Multifaceted Aspects of MCR-mediated Colistin Resistance: Fitness, Virulence, Environmental Reservoirs and Genomic Insights

By

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Summary

To date, eight different *mcr*-variants have been identified encoding phosphoethanolamine transferases contributing to phosphoethanolamine addition to lipid A and thereby conferring colistin resistance. This novel mechanism of plasmid-mediated colistin resistance provides an efficient way in distributing colistin resistance. This thesis aims to further our understanding of the multifaceted aspects of *mcr*-like genes using detailed molecular analysis including whole-genome sequencing (WGS) for 17 *mcr*-positive *Klebsiella pneumoniae* (MCRPKP) and 219 *mcr*-positive *Escherichia coli* (MCRPEC) from Thailand and Vietnam.

Fitness is an important factor to predict the spread and development of antibiotic resistance. In chapter 3, the data show that over-producing MCR-1 in *E. coli* imposes a great fitness loss, observed by slow growth rates, impaired membrane integrity and decreased competitiveness. The fitness burden conferred by *mcr-3* seems to be more moderate and *mcr-3*-positive plasmids are more stable and competitive, than that of *mcr-1*-carrying plasmids.

In addition, I provided evidence that blowflies serve as reservoirs of *mcr*-genes and 48 *mcr-1*-positive strains recovered from blowflies have been characterized. In particular, the clonal relationship among 17 MCRPKP strains obtained from blowflies was observed by indistinguishable PFGE patterns and an identical phylogenetic tree. The virulence potential of this MCRPKP clone was measured in the *Galleria mellonella* model.

Furthermore, 219 MCRPEC isolates were characterized using WGS and bioinformatics analysis. WGS analysis shows that MCRPEC isolates are highly diverse, with distinct phylogenetic groups and various accessory genes including heavy-metal/antibiotic resistance determinants, virulence factors and toxin-antitoxin systems. The genetic context analysis on the origin and spread of gene *mcr-1* and *mcr-3*, provides important knowledge of understanding on the movement and dissemination of *mcr*-like genes bacterial population.

Journal Publications and Oral Presentations

Publications resulting from data presented in this thesis

Qiu E Yang, Siham Rajab Agouri, Jonathan Mark Tyrrell, Timothy R. Walsh: *Heavy metal resistance genes are associated with bla_{NDM-1} and $bla_{CTX-M-15}$ -Enterobacteriaceae*. ***Antimicrobial Agents and Chemotherapy*** 03/2018; DOI:10.1128/AAC.02642-17

Qiu E Yang, Mei Li, Owen B. Spiller, Diego O. Andrey, Philip Hinchliffe, Hui Li, Craig MacLean, Pannika Niumsup, Lydia Powell, Manon Pritchard, Andrei Papkou, Yingbo Shen, Edward Portal, Kirsty Sands, James Spencer, Uttapoln Tansawai, David Thomas, Shaolin Wang, Yang Wang, Jianzhong Shen, Timothy R. Walsh: *Balancing mcr-1 expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms*. ***Nature Communications*** 12/2017; 8(1). DOI:10.1038/s41467-017-02149-0

Qiu E Yang, Timothy R Walsh: *Toxin-antitoxin systems and their role in disseminating and maintaining antimicrobial resistance*. ***FEMS microbiology reviews*** 03/2017; 41(3). DOI:10.1093/femsre/fux006

Philip Hinchliffe, **Qiu E. Yang**, Edward Portal, Tom Young, Hui Li, Catherine L. Tooke, Maria J. Carvalho, Neil G. Paterson, Jürgen Brem, Pannika R. Niumsup, Uttapoln Tansawai, Lei Lei, Mei Li, Zhangqi Shen, Yang Wang, Christopher J. Schofield, Adrian J Mulholland, Jianzhong Shen, Natalie Fey, Timothy R. Walsh, James Spencer: *Insights into the Mechanistic Basis of Plasmid-Mediated Colistin Resistance from Crystal Structures of the Catalytic Domain of MCR-1*. ***Scientific Reports*** 01/2017; 7:39392. DOI:10.1038/srep39392

Manuscript in preparation

Qiu E Yang, Uttapoln Tansawai, Diego O. Andrey, Shaolin Wang, Yang Wang, Kirsty Sands, Anong Kiddee, Kanit Assawatheptawee, Nophawan Bunchu, Brekhna Hassan, Timothy Rutland Walsh, Pannika R. Niumsup. *Blow flies service as reservoirs facilitating the spread of mcr-1-horboring Enterobacteriaceae in Thailand*. (Under review, from Chapter 5)

Qiu E Yang, Craig Maclean, Andrei Papkou, Manon Prichard, David Thomas, Diego O. Andrey, Kirsty Sands, Timothy Rutland Walsh. *Compensatory mutations ameliorate mcr-3-conferring fitness costs in Escherichia coli.* (From Chapter 4)

Qiu E Yang, Kirsty Sands, Timothy Rutland Walsh et al., *High prevalence of co-existed mcr-1- and mcr-3- plasmids in multidrug-resistant Escherichia coli from human in Vietnam.* (From Chapter 4)

Oral Presentation at major international conferences

1. Antibiotic tolerance induced by a toxin-antitoxin system (*hipBA*) from a bla_{NDM}-positive plasmid from *Acinetobacter baumannii*. Mini-Oral presentation at 27th ECCMID, Vienna, April 2017.

2. Colistin resistance caused by *mcr-1* and *mcr-3* genes is associated with decreased virulence in *Klebsiella pneumoniae*, Oral presentation at 28th ECCMID, Madrid, April 2018, O0099.

3. Amelioration of the fitness costs of *mcr-3*-mediated resistance due to compensatory mutations in *Escherichia coli*. Oral presentation at 28th ECCMID, Madrid, April 2018, O0100.

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List of Abbreviation

Abbreviation	Full name
AMR	Antimicrobial resistance
bla	β-lactamase gene
NDM	New Delhi metallo-β-lactamase
MCR	Mobile colistin resistance
KPC	*Klebsiella pneumoniae* carbapenemase
HGT	Horizontal gene transfer
Inc	Incompatibility (referring to plasmid typing)
MDR	Multi-drug resistant
XDR	Extensively-drug resistant
PDR	Pan-drug resistant
GNB	Gram-negative bacteria
MCRPE	*mcr*-positive Enterobacteriaceae
MCRPEC	*mcr*-positive *Escherichia coli*
MCRPKP	*mcr*-positive *Klebsiella pneumoniae*
HLCRMs	High-level colistin resistance mutants
CRE	Carbapenem-resistant Enterobacteriaceae
pmr	Polymyxin resistance
phoP/phoQ	Phosphorylated, *pho*
LptA	LPS PEA transferase A
L-Ara4N	4-amino-4-deoxy-L-arabinose
PEtN	phosphoethanolamine
AMPs	Antimicrobial polypeptides
LPS	Lipopolysaccharides
TAs	Toxin-antitoxin systems
Tn(s)	Transposon(s)
T4SS	Type IV secretion system
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
qPCR	Quantitative PCR

Abbreviation	Full name
MLST	Multilocus sequence typing
SNP(s)	Single nucleotide polymorphism(s)
WGS	Whole genome sequencing
Ct	Cycle threshold value(s)
TE	Tris-EDTA (buffer)
TBE	Tris-borate-EDTA (buffer)
LB	Luria Bertani (agar/ broth)
MH	Mueller Hinton (agar)
CFU	Colony-form units
MIC	Minimum inhibitory concentration

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Chapter 1

General Introduction

1. 1 The end of antibiotics against Gram-negative bacteria?

As the discovered antibiotic in 1928 by Sir Alexander Fleming (FLEMING, 1944), penicillin has been described as a wonder drug which transformed medical treatment in 1941 and saved millions of lives during World War II. Penicillin's discovery was exemplary of many pioneering researchers, and the period between 1950s and 1970s was considered as 'the golden era of antibiotic discovery'. By the 1970s many of history's endemic and almost incurable human infections including tuberculosis, cholera and malaria, were no longer fatal. Unfortunately, even before the first medical use of penicillin in 1941, penicillin resistance had been reported in 1940 (Ventola, 2015). But at that time most doctors remained optimistic and hopeful for the success of this new drug, which had defeated diseases such as syphilis and cholera, so the first emergence of possible penicillin resistance was overlooked. The conclusion from an early report for acquired penicillin resistance in 1952 was (Rollo *et al.*, 1952): '*Syphilis has now been treated with arsenicals for about 40 years without any indications of an increased incidence of arsenic-resistance infections, and this work gives grounds for the hoping that the widespread use of penicillin will equally not result in an increasing incidence of infections resistance to penicillin'*. Antibiotic treatments have two conflicting consequences: in the short-term, antibiotics can effectively block bacterial growth and cure bacterial infections; but in the long-term, the use of antibiotics promotes the evolution of resistance. History has shown that shortly after the introduction of a new antibiotic, it is usually followed by the emergence and development of resistance, and resistance has been seen to almost all current available antibiotics (**Fig.1.1)**. Today, there is

considerable concern with the mounting prevalence of pan-drug resistant Gram-negative Bacteria (GNB) in clinical and environmental settings, in particular Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Worse still, the discovery and development of novel antibiotics have declined considerably in the last two decades (Livermore, 2004, Brown & Wright, 2016). The U.S. CDC has estimated that each year at least 2 million people suffer from infectious illnesses and 23,000 deaths are directly linked to antibiotic-resistant bacteria (https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf#page=6).

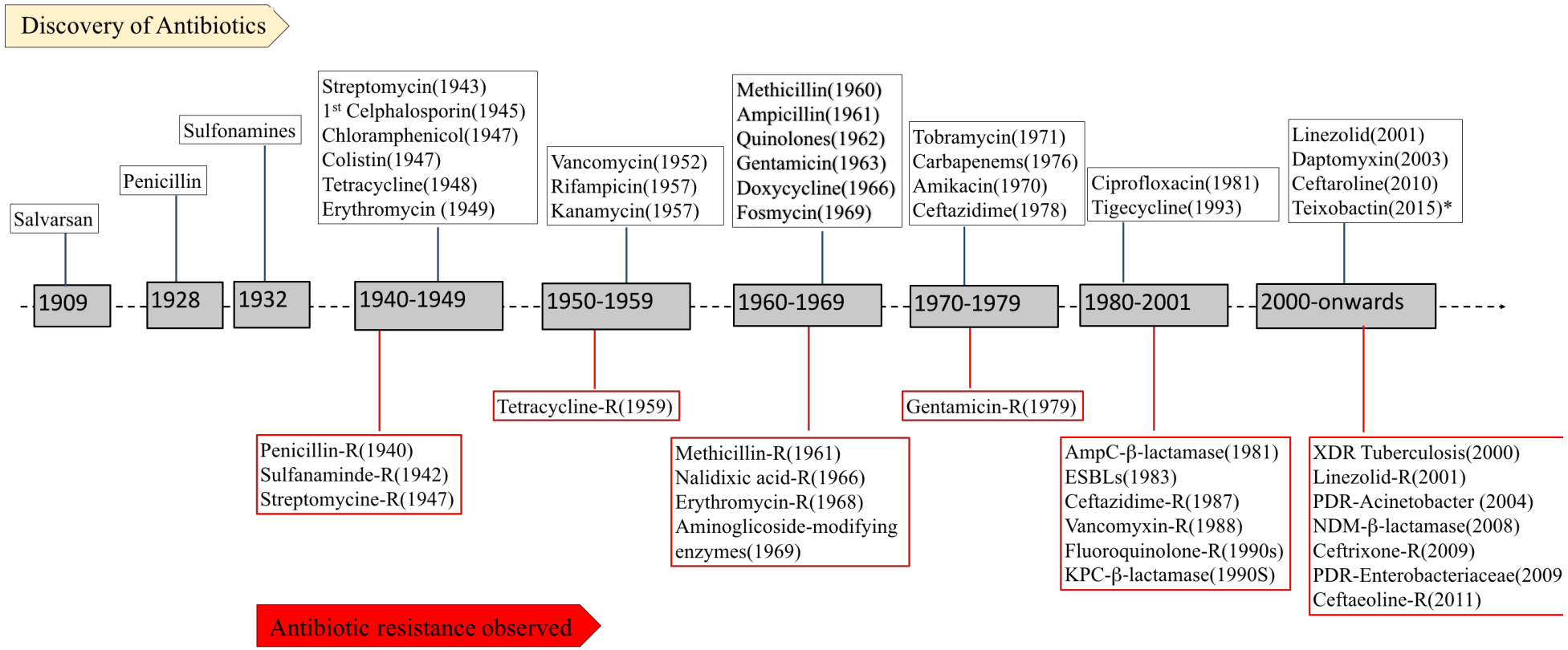


Fig.1.1 key dates in the history of antibiotic discovery and subsequent resistance development. -R: resistance; XDR: extended-drug resistance; PDR: Pan-drug resistance. *Teixobactin is not in clinical use yet. (updated from (Bbosa *et al.*, 2014, Ventola, 2015)

Currently, the main treatment options for Gram-negative infection are β-lactams, which consist of three subclasses, namely, narrow-spectrum (penicillins and early generations of cephalosporins), extended-spectrum (cephalosporins) and broad-spectrum (carbapenems) (**Table 1.1**). However, bacteria have rapidly developed several strategies to counter the effects of β-lactams, one of the most effective mechanisms is the production of β-lactamases, enzymes that hydrolyse β-lactams. High levels of extended-spectrum β-lactamases (ESBLs), which can inactivate the majority of β-lactams including celphalosporins but not carbapenems, have been reported globally (Bonnet, 2004, Arcilla *et al.*, 2017). One of the most prevalent ESBLs, CTX-M-15, is associated with the widespread pathogenic *E. coli* clone, ST131. With the occurrence of ESBLs, carbapenems were one of a few remaining β-lactam agents left for the treatment of Gram-negative infections. However, after the first report of the *K. pneumoniae* carbapenemase (KPC), several novel carbapenemases have been subsequently found in GNB, including OXA-types and the NDM-β-lactamases (Walsh *et al.*, 2005, Queenan & Bush, 2007, Doi & Paterson, 2015). Carbapenemase-producing GNB thus can inactivate virtually all β-lactams including carbapenems, regarded as the last resort against Gram-negative infections. Considerably high resistance rates (>50%) to carbapenems have now been reported in *K. pneumoniae* in some European countries (Canton *et al.*, 2012). In 2013, the U.S. CDC classified carbapenem-resistant *Enterobacteriaceae* (CRE) as first-line 'urgently treated' (https://www.cdc.gov/narms/resources/threats.html), and high burdens of CRE strains have also been reported globally (Munoz-Price *et al.*, 2013, Nordmann, 2014). Successful spread of β-lactam-resistance in the hospitals and communities, have forced clinicians to explore combination therapies. Fluoroquinolones, aminoglycosides and polymixins are often used in combination with β-lactams for the treatment of serious Gram-negative infections. Frequent exposure to mixed antibiotics, ultimately pave the way for

bacteria to develop more complex mechanisms of resistance conferring protection against more than one group of antibiotics. We now are facing more complex and chronic situations of multi-drug resistant GNB (MDR-GNB), for instance, resistance genes encoding β-lactamases often co-exist with other antibiotic resistance genes conferring resistance to quinolones, aminoglycoside or tetracyclines (Crofts *et al.*, 2017). According to latest NARS-Net surveillance data in 2017 (https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/report), the high prevalence of MDR phenotypes (resistance to fluoroquinolones, aminoglycosides and carbapenems) in *Acinetobacter* species, has been frequently reported in southern and south-eastern Europe (EARS-Net, 2017), which makes therapeutic options for *Acinetobacter* infections even more limited. Moreover, over one-third of *K. pneumoniae* were resistant to more than one of the antibiotic classes and combined resistance to multiple antibiotics were common, according to 2017 EARS-Net surveillance. There is increasing evidence that MDR (and increasing reports of pan-drug resistance [PDR]) in GNB, have spread globally (Crofts *et al.*, 2017, Logan & Weinstein, 2017, Navon-Venezia *et al.*, 2017), such that if we do not act promptly to preserve the activity of current available antibiotics and focus on the development of new antimicrobial agents, we will soon return to an equivalent of the 'pre-antibiotic era'.

The situation of antibiotic resistance in animals is equally worrisome. Since the first industrial production of low-dose tetracycline to enhance chicken growth and boost poultry profits in 1948, the process of routinely feeding antibiotics to food-producing animals has been implemented in the majority of countries (Popescu, 2018). All major species of food animals, including fish, sheep, cattle and pigs, derive some benefit from the use of antibiotics as prophylaxis, chemotherapy and/or growth promotion. In the United States, food-producing animals consume approximately 70% of the nation's annual antibiotic consumption, and a significant fraction (62%) are medically important antimicrobials including tetracycline,

aminoglycosides, cephalosporins and fluoroquinolones (FDA, 2016). A recent study has estimated that the global consumption of antibiotics in livestock will rise by 67% by 2030, from approx. 63,151 tons in 2010 to approx. 105,596 tons, and nearly double in Brazil, China, Russia, India and South Africa (Van Boeckel *et al.*, 2015). The intensive use of antibiotics, especially in low- or middle-income countries, where most of those counties have no standard guidelines on antibiotic use in animal production, potentially increase selective pressure on bacteria and aid the emergence of antibiotic resistances (Founou *et al.*, 2016). There is a proven relationship between the consumption of antibiotics and the prevalence of antibiotic-resistance in commensal *E. coli* from pigs, cattle and poultry. It is therefore not surprising that the high prevalence of MDR pathogens has been identified from food products, posing a significant public health threat through food consumption (Founou *et al.*, 2016). There are numerous transmission pathway for MDR bacteria from animals to human. For instance, the resistance genes can be transferred horizontally between commensal species like *E. coli* and Enterococci, and then to pathogens, aided and abetted by exposure through food or contact with animals. Direct spread of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) to people exposed to pigs, cattle or poultry have been well studied (Graveland *et al.*, 2011, Alba *et al.*, 2015, Goerge *et al.*, 2017). The occurrence of ESBL-producing *E. coli* with MDR phenotypes from animals and food products is also increasing (Ewers *et al.*, 2012, Vitas *et al.*, 2018). The potential effects of antibiotic use in animals on human health has attracted public attention and resulted in those antibiotics used for growth promotion in animals, being banned in some countries (Walsh & Wu, 2016). In 1985, Sweden was the first country to regulate a ban on the use of antibiotic as growth promoters in food animal production (Wierup, 2001, Maron *et al.*, 2013). It was followed by Norway (1995) and Denmark (1998-1999) with the withdrawal of antimicrobials used as routine preventative use in food-producing animals (Grave *et al.*, 2006). In 2006, the European

Union declared a ban on the use of all antibiotic growth promoters in food animals; however, this did not cover antibiotics used for metaphylaxis treatment in large flocks, and they are still in use in non-EU countries. In 2013, the Indian government changed antibiotic regulation laws aiming to end the sale of over-the-counter antibiotics (Kakkar *et al.*, 2017); however due to lack of regulatory scrutiny in India, their increasing sales of unapproved antibiotics made this initiative soon nullified (https://theconversation.com/the-indian-government-is-not-doing-enough-to-tackle-the-sale-of-unapproved-antibiotics-85432). There are no geographic boundaries to impede the global spread of AMR-humans move and therefore so do bacteria. If preventive and containment efforts can be made locally, nationally and regionally, and apply the One Health approach to ensure food safety and security, we will be in a better position to combat infectious diseases and potentially curb the spread of AMR (Founou *et al.*, 2016).

Table 1.1 Currently available antibiotics for the treatment of GNB infections

Antibiotic classes	Some important antibiotics	Infections and drug-resistant pathogens	Major resistance mechanisms and comments
narrow-spectrum β-lactams: penicillis and early generation cephalosporins	penicillin, cephalothin, cefazolin, cephaprilin, cephalexin	In the absence of resistance, these drugs are still active against a wide range of bacterial pathogens. But resistance is widespread, it is not recommended for serious infections	The production of β-lactamases: such as penicillinase
extended-spectrum β-lactams: celphalosporins	ceftriaxone, ampicillin, cefepime, ceftazidime	for pneumonia bloodstream infections and UTI[#]	ESBLs, mainly CTX-M-types, they can hydrolyze most of β-lactams expect carbapenems.
broad-spectrum β-lactams: carbapenems	meropenem, imipenem, ertapenem, doripenem (considered as the last-line antibiotic against serious infections)	for ESBLs-producing Enterobacteriaceae, pneumonia, bloodstream infections and UTI.	A variety of potent carbapenemases (such as NDM, OXA, KPC) that are effective against all β-lactams.
β-lactamase inhibitors	clavulanic acid, sulbactam, tazobactam	combined with other β-lactams such as ampicilin, these drugs are important for serious infections caused by GNB that have β-lactamases with limited activities	potent β-lactamases including ESBLs and carbapenemases
Aminoglycosides	amikacin, gentamicin, tobramycin	for pneumonia, bloodstream infections and UTI.	16S rRNA methylases that confer a high level of resistance to all aminoglycosides, often co-produce with ESBLs and carbapenemases(Doi & Arakawa, 2007)
Fluoroquinolones	ciprofloxacin, levofloxacin, moxifloxacin	for pneumonia bloodstream infections and UTI	target-site mutations like *gyrA*, efflux pumps, *qnr* genes (Jacoby, 2005, Redgrave *et al.*, 2014)
Tetracyclines and Glycyclines	Tigecycline[+], (*tetracyclines serve as secon-or third line therapy)	for CRE, CRA	resistance to tigecycline has been identified but it is still rare.
Polymyxins	colistin[+]	for CRE, CRA, CRP	plasmid-borne *mcr*-genes, chromosomal *pmrAB* and *phoPQ* systems

Footnotes: * tetracyclines are not fist-line treatment options, but increasing resistance to other classes, it is considered as a therapeutic option). [+] tigecycline and colistin are last-line antibiotics for the treatment of serious infections caused by CRE (Carbapenem-Resistance Enterobacteriaceae), CRA (Carbapenem-Resistance *A. baumannii*) and CRP (Carbapenem-Resistance *P. aeruginosa*). [#] UTI: urinary tract infection. This detail was updated from (Peleg & Hooper, 2010) and U.S. CDC 2013 report (https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf#page=6).

1.2 Re-introduction of the old antibiotic colistin for multidrug resistant Gram-negative bacterial infections

As seen in **Table 1.1**, Gram-negative pathogens (*A. baumannii*, *P. aeruginosa*, and Enterobacteriaceae) that cause the majority of hospital infections, have evolved a variety of strategies to escape the action of antibiotics. The increasing gulf between the escalation of carbapenem-resistance and the shortage of novel antibiotic entering clinical trials has aroused renewed interest in older antimicrobial agents to be redeployed in the battle against MDR bacteria (Li *et al.*, 2006). One of these old antibiotics are polymyxins, which were first obtained from the soil bacterium *Paenibacillus polymyxa* (previously *Bacillus polymyxa*) in 1947 (Ainsworth *et al.*, 1947, Benedict & Langlykke, 1947, Stansly *et al.*, 1947). Polymyxins are a group of antimicrobial polypeptides (AMPs), they are bactericidal and mainly active against GNB (Storm *et al.*, 1977). Polymyxins include five different chemical compounds (polymyxin A, B, C, D and E), each consisting of a cyclic heptapeptide, a linear tripeptide and a long hydrophobic tail which are integrated into the bacterial membrane inflicting damage (**Fig.1.2**). There are only one amino acid difference at position 6' between polymyxin B and polymyxin E (colistin), where D-Phe (D-phenylalanine) in polymyxin B is replaced by D-Leu (D-leucine) in colistin, as shown in **Fig.1.2** (Falagas et al., 2005). Colistin was first discovered from soil bacterium *Aerobacillus polyaerogenes* in 1949 (Koyama, 1950), and it was not correctly classified as belonging to the polymyxin group until 1963 (Wilkinson,

1963). Colistin was comprised mainly of colistin A and B, varying slightly by different attachment of fatty acids ('R2'), where colistin A was MOA and colistin B was IOA (Suzuki *et al.*, 1965) (**Fig.1.2**). Therefore, the chemical structure of polymyxins are a mixture of lipophilic, such as cationic L-Dab (α,γ-diaminobutyric acid) residues and hydrophobic groups (N-terminal fatty acid chain and D-Leu/Phe^{6}-L-Leu^{7} segment), and this amphipathic structure is essential for antibacterial activity (Velkov *et al.*, 2010, Yu *et al.*, 2015).

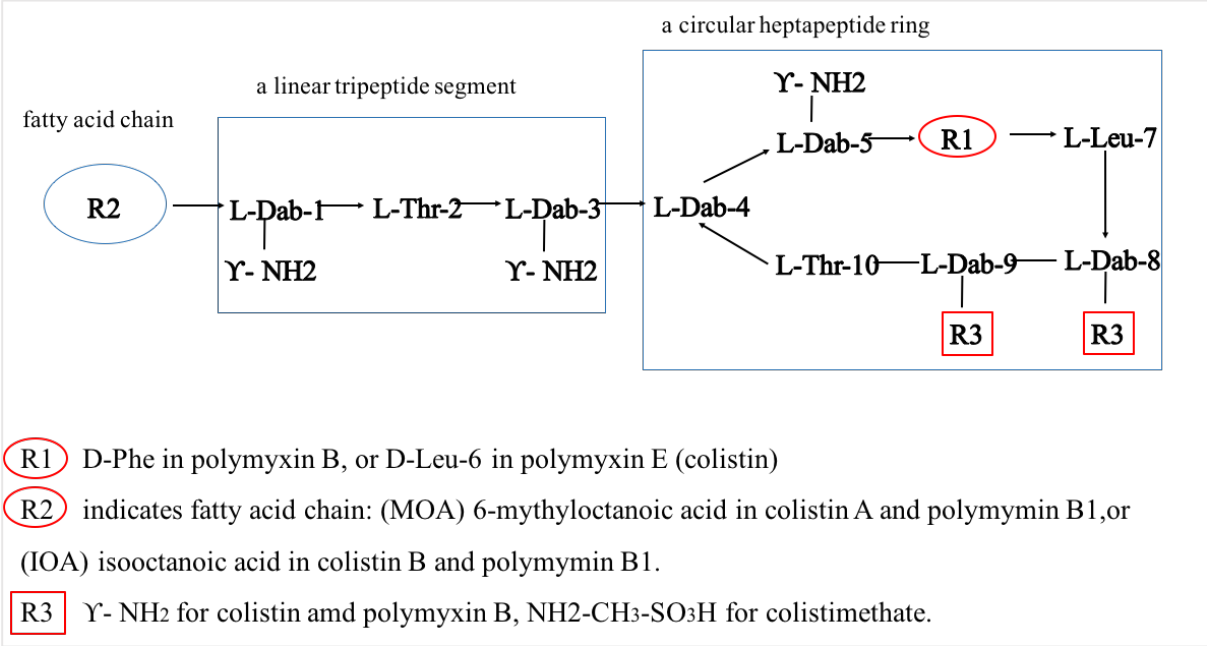


Fig.1.2 Chemical structure of colistin and polymyxin. Dab:α,γ-diaminobutyric acid; Phe: phenylalanine; Leu: leucine; Thr: threonine. Roman numbers mean the position of amino acid residues in polymyxins.

1.2.1 Antibacterial Mechanism of polymyxins

It is well-established that the broad-spectrum bactericidal activity of polymyxins is mediated by altering GNB membrane structure/topology. A prerequisite for antibiotic activity against GNB is to overcome the outer membrane permeability barrier and reach the inner membrane, which is the primary target of polymyxins. Lipopolysaccharides (LPS) is the

major component of the outer membrane in GNB and consists of a lipid and a polysaccharide composed O-antigen, an outer core and inner core joined by a covalent bond. LPS contributes greatly to the structural integrity of the bacteria, and protecting the other cell structures from harmful substances (Ruiz *et al.*, 2009). The lipid component of LPS, lipid A, which is responsible for the toxic stimulus in sepsis, consists of two glucosamine-base disaccharides attached to six fatty acid chains **(Fig.1.3**). The lipid A disaccharide contains two phosphoryl groups at the 1' and 4'-position, both phosphates can be further modified by the addition of groups inducing ethanolamine, glucose, mannose or 4-amino-4-deoxy-L-arabinose (Erridge *et al.*, 2002, Needham & Trent, 2013). It has been proposed that lipid A can increase the negative charge of the cell membrane and help stabilize the overall membrane topology. It is generally believed that the antibacterial activity of polymyxins depends on electrostatic attraction between cationic residue of polymyxin and the anionic phosphate head-groups of lipid A in the outer membrane of GNB, resulting in disarrangement of the bacterial cell membrane (Newton, 1956, Falagas *et al.*, 2005). A classic model of polymyxin B binding to lipid A, which is based on the three-dimensional nuclear magnetic resonance (NMR) structure of polymyxin in complex with lipid A (Pristovsek & Kidric, 1999, Velkov *et al.*, 2010) is: the positively-charged Dab residues closely interact with the negatively-charged 1'- and 4'-phosphate groups of lipid A, reducing the net-negative charge of lipid A. The hydrophobic leucine residues and tail of polymyxin interact with the fatty acid tails of lipid A. This initial electrostatic interaction between polymyxin and to LPS molecules in the outer membrane of the GNB leads to the displacement of the divalent cations, magnesium (Mg^{+2}) and calcium (Ca^{+2}), which normally act as a bridge to stabilize the LPS molecules. (Storm *et al.*, 1977, Yu *et al.*, 2015). As a result, this insertion of hydrophobic residues [($_{D}$-Phe^{6} -$_{L}$-Leu^{7} (polymyxin) or $_{D}$-Leu^{6} -$_{L}$-Leu^{7} (colistin)) and the fatty acyl chain acts to disrupt and destabilize the monolayer of outer-membrane, and eventually the

permeability of the cell wall to polymyxins is increased, that ultimately causes cell death (**Fig.1.3**).

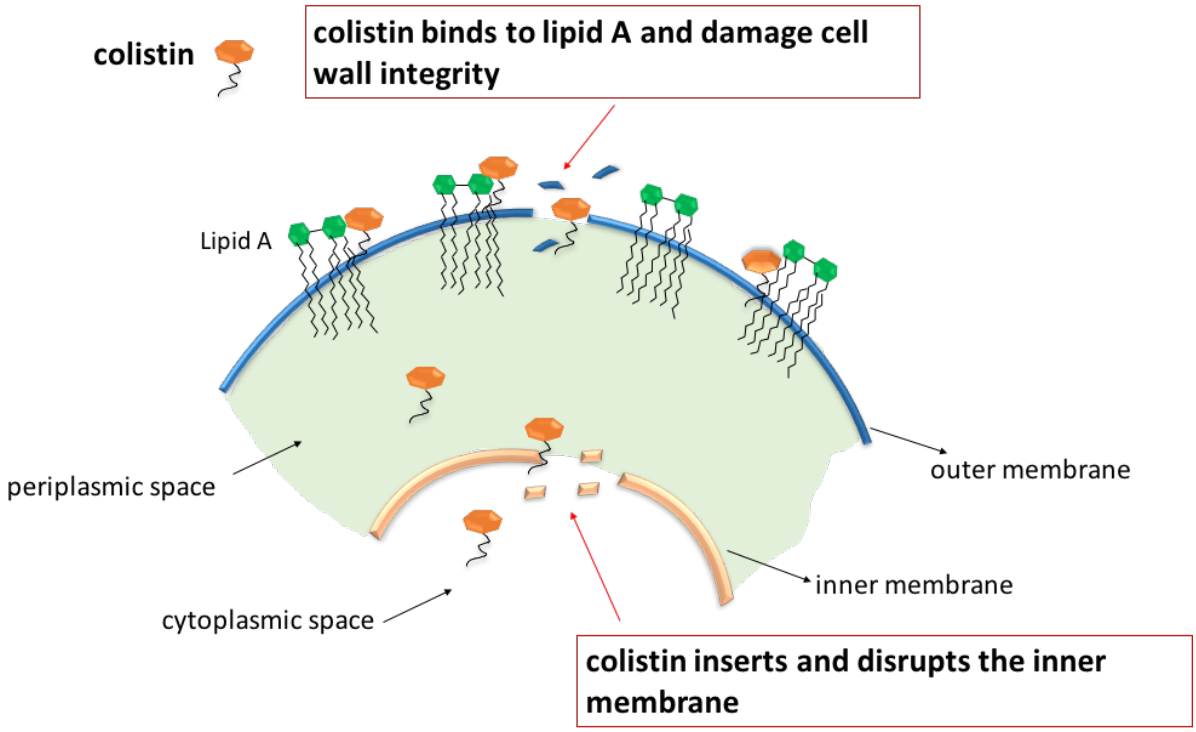


Fig. 1.3. A schematic of putative antimicrobial action of colistin on the outer membrane of Gram-negative bacteria (modified from (Velkov *et al.*, 2010). The activity of colistin on bacterial cells are usually divided into three steps: firstly, disruption of outer-membrane through colistin-lipid A interaction; self-promoted uptake into inner membrane and finally lysis of cell through disruption of the stability of bacterial inner membrane.

1.2.2 Antimicrobial activity of polymyxins

Due to the high toxicity of polymyxin A, C and D, only polymyxin B and E (colistin) have been used in clinical practice. Colistin has excellent antibacterial activity against most GNB, including *E.coli*, *Acinetobacter* species, *P. aeruginosa*, *Salmonella* species, *Enterobacter* species, *Citrobacter* species and *Shigella* species, with minimum inhibitory concentrations less than 2 mg/l (Storm *et al.*, 1977, Falagas *et al.*, 2005). Generally, polymyxins are less active against Gram-positive bacteria though there are polymyxin sensitive strains including

Staphylococcus, *Bacillus* and *Corynebacterium* with MIC less than 2 mg/l (Storm *et al.*, 1977). In Edgar's report, colistin was described as a successful antibiotic to cure infections caused by *Pseudomonas*, which is resistant to almost all chemotherapeutic agents, but most of the organisms remain susceptible to colistin (Edgar & Dickinson, 1962). It has been demonstrated that colistin possesses the same level of bactericidal activity as polymyxin B, but is less toxic than polymyxins, which is commonly associated with serious adverse effects (Hall, 1960, Reed *et al.*, 2001). Subsequently, this led to the development and clinical availability of colisin in Japan and Europe during the 1950s. Soon after, significant colistin-associated renal and neurological toxicity was also reported and the intravenous use of colistin and polymyxin B were gradually abandoned in the early 1980s (Yu *et al.*, 2015).

Until the last decade, colistin has been shelved for over 30 years in most countries and replaced by the less toxic aminoglycosides and beta-lactams. However, outbreaks of MDR superbugs, such as NDM-1-producing bacteria that are resistant to most classes of antimicrobial agents (Yong *et al.*, 2009, Kumarasamy *et al.*, 2010), has led to the reintroduction of colistin, in particular, as a valuable clinical drug. Furthermore, the reassessments of the efficacy and safety of colistin in the treatment of serious infections suggests that colistin is less nephrotoxic than previously believed, and revised dosing regimens can minimize its toxicity (Reed *et al.*, 2001, Li *et al.*, 2006, Falagas *et al.*, 2010, Honore *et al.*, 2013). According to Matthew et al study in 2010, 258 patients with MDR bacterial infections received intravenous colistin therapy for at least 72 hours with no adverse effects (including nephrotoxicity) observed (Falagas *et al.*, 2010). More importantly, due to its significant activity against MDR Gram-negative pathogens, including *A. baumannii*, *P. aeruginosa* and carbapenem-resistant *K. pneumoniae*, colistin has been revived as a 'salvaged' antibiotic employed against MDR bacterial infections (**Table 1.1**) (Linden et al., 2003, Falagas et al., 2010, Biswas et al., 2012).

Another encouraging outcome of colstin's reemergence is its synergistic effects when conbined with other antibiotics . The synergistic effect of colistin with rifampicin has also been reported *in vitro* against carbapenem-resistant *A.baumannii* (Song et al., 2007), the amount of colistin-rifapicin combination to achieve bactericidal activity was 4-8 fold lower when compared monotherapy with colistin. However, the efficacy to the combination therapy is still poorly undestood because of the sparisity of clinical outcome data. With the emergence of increasing colistin resistance, more clinical studies for colistin combination regimens is warranted. The recent results show that several antibiotics including clarithromycin, in combination with colistin, display stronger antimicrobial effecacy than the mono-treatment, suggesting a viable therapeutic alternative for highly antibiotic resistant GNB (MacNair *et al.*, 2018).

1.3 The acquired resistance mechanisms of polymyxins

Since the target of antimicrobial polypeptides (AMPs) is the bacterial membrane, which is a fundamental structure and critical to bacterial survival, it has been long thought that the acquisition of resistance by significantly altering the outer-membrane is improbable (Zasloff, 2002). However, there are many reports showing that lipid A can undergo extensive re-modelling that allow bacteria to adapt to their rapidly changing and often hostile environment. For instance, modification of lipid A by adding a phosphate group, results in a change in the outer-membrane permeability and can promote bacterial resistance to AMPs (Erridge *et al.*, 2002). The diversity of lipid A modification has been identified previously and has provided a deep insights into the interaction between lipid A modification, AMPs and bacterial resistance.

The World Health Organization (WHO) has classified polymyxins as 'critically important'

antibiotics for human medicine to treat serious infections caused by MDR Enterobacteriaceae (http://www.who.int/foodsafety/publications/antimicrobials-third/en/). Resistance to colistin is a great challenge to global health as it means virtually no remaining antibiotics are available to fight against colistin-resistant 'superbugs'. Unfortunately, the reintroduction of polymyxins, colistin in particular, for last-line therapy will provide some selective pressure and potentially facilitate the outbreak of 'superbugs' and increase their global numbers (Moffatt *et al.*, 2010). However, certain bacterial species are known to have natural or intrinsic resistance to colistin, such as *Neisseria spp.*, *Moraxella catarrhalis*, *Helicobacter pylori, Proteus mirabilis, Serratia marcescens*, *Morganella morganii*, *Chromobacterium* and *Brucella species*, with MICs greater than 128 mg/l (Velkov *et al.*, 2010, Biswas *et al.*, 2012). Polymyxin resistance is most often mediated by a variety of LPS modifications, including changing the length of O-Antigen, lipidA modifications with phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N), and deacylation (Gunn, 2001, Olaitan *et al.*, 2014). Polymyxins are positively charged peptides, which are proposed to kill bacteria by compromising the integrity of the bacterial outer-membrane. The LPS modifications, which alters the net negative charge of the outer-membrane, will reduce the avidity between the AMPs and their target resulting in polymyxin resistance (Olaitan *et al.*, 2014) (**Fig.1.4**). Other strategies, such as overexpression of outer-membrane protein OprH or the use of efflux pumps, have also evolved in GNB to protect themselves from AMPs.

1.3.1 Chromosomal-dependent colistin resistance

1.3.1.1 The two-component systems govern LPS with PEtN/L-Ara4N additions in Enterobacteriaceae

Because the outer membrane is the initial site of interaction with colistin, the most efficient colistin resistance mechanisms are those directly connected to LPS modification as they

protect the peptide from entering the cell by decreasing colistin affinity. The most common LPS modifications are the cationic substitution of the phosphate groups by L-Ara4N and PEtN: the resulting positively charged LPS reduces the binding of colistin, leading to resistance. LPS modifications are induced by the two-component regulators, *phoP/phoQ* (phosphorylated, *pho*) and *pmrA-pmrB* (polymyxin resistance, *pmr*), which can be found in a wide variety of bacterial species, including *E. coli*, *Salmonella Typhimurium*, *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and *Citrobacter rodenium* (Chen & Groisman, 2013). As described below, it is now recognized that specific mutations in both *pmrAB* and *phoPQ* systems contribute to constitutively activated *pmrA* and *phoP*, resulting in the upregulation of *arnBCADTEF* and *pmrCAB,* and a high level of LPS modification with PEtA and Ara4N, mediating polymyxin resistance (Olaitan *et al.*, 2014, Poirel *et al.*, 2017)(**Fig. 1.4**).

(i) *pmrA-pmrB* system: The two-component system *pmrA-pmrB*, first identified in *S. Typhimurim*, consists of the response regulator PmrA and its cognate sensor PmrB. The mechanism of polymyxin resistance mediated by the *pmrA-pmrB* system, is to modify the LPS core and lipid A region with L-Ara4N and PEtN addition (Gunn *et al.*, 1998, Gunn, 2001, Zhou *et al.*, 2001, Lee *et al.*, 2004). Two loci *arnBCADTEF* (also known as *pmrHFIJKLM*) and *pmrE* (previously identified as *pagA* or *ugd*, encoding a UDP-glucose dehydrogenase) (Gunn *et al.*, 1998), are responsible for the biosynthesis and transportation of L-Ara4N to the lipid A, and deletion of these genes can cause 60 plus-fold decrease in resistance to polymyxin (Gunn, 2001, Falagas *et al.*, 2010). The activation of this system is triggered by high concentrations of polymyxins, Fe^{3+} or low pH, which are sensed by PmrB. In response to high Fe^{3+}, PmrB autophosphorylates by binding Fe^{3+} in an iron-binding motif and then activates the expression of *pmrA*, a regulator of *arn* operon. Once activated, phosphorylated PmrA triggers the activation of the *arnBCADTEF*, resulting in the addition of L-Ara4N to the 1'- or 4'-phosphate group to lipid A (Mitrophanov *et al.*, 2008). This modification decreases

the net charge from about -1.5 to 0. The *pmrAB* system can also enhance the addition of PEtN to lipid A by activating the *pmrCAB* operon (**Fig.1.4**), which consists of three proteins: 1). PEtN phospotransferase, PmrC (often referred as EptA), 2). A regulator, PmrA; and 3). A sensor kinase, PmrB. The addition of cationic PEtN decreases the net charge from -1.5 to -1 (Nikaido, 2003). Because of the nature of charge alteration, the efficiency of L-Ara4N addition probably is higher than that of PEtN addition (Nikaido, 2003, Olaitan *et al.*, 2014).

In addition, *pmrA*-regulated genes including *eptB*, *eptC* (Salazar *et al.*, 2017), *pmrR* (Nowicki et al., 2014) and *lpxR* (which deacylates lipid A)(Reynolds *et al.*, 2006, Olaitan *et al.*, 2014), have been identified as modifying the structure of LPS and thus altering the levels of bacterial resistance to AMPs (**Fig.1.4**). For example, EptB and EptC belong to the PEA transferase family identified in *E. coli*, EptB catalyzes the addition of PEtN to the kdo (Olaitan *et al.*, 2014), while EptC promotes resistance to polymyxin by decoration of PEtN of the LPS inner core (Salazar *et al.*, 2017). A *pmrA*-activated membrane peptide, PmrR, has been reported to inhibit the activity of LpxT (Kato *et al.*, 2012), a kinase responsible for the transfer of a phosphate group (Und-PP, undecaprenyl pyrophosphate) to the 1'-phosphate group of lipid A (Touze *et al.*, 2008). The inhibition of LpxT leads to regeneration of the undecaprenyl phosphate (Und-P) which forms a phosphorylated lipid A, thereby decreasing the negative charge of bacterial surface and ultimately reducing the affinity to polymyxins (Herrera *et al.*, 2010, Kato *et al.*, 2012).

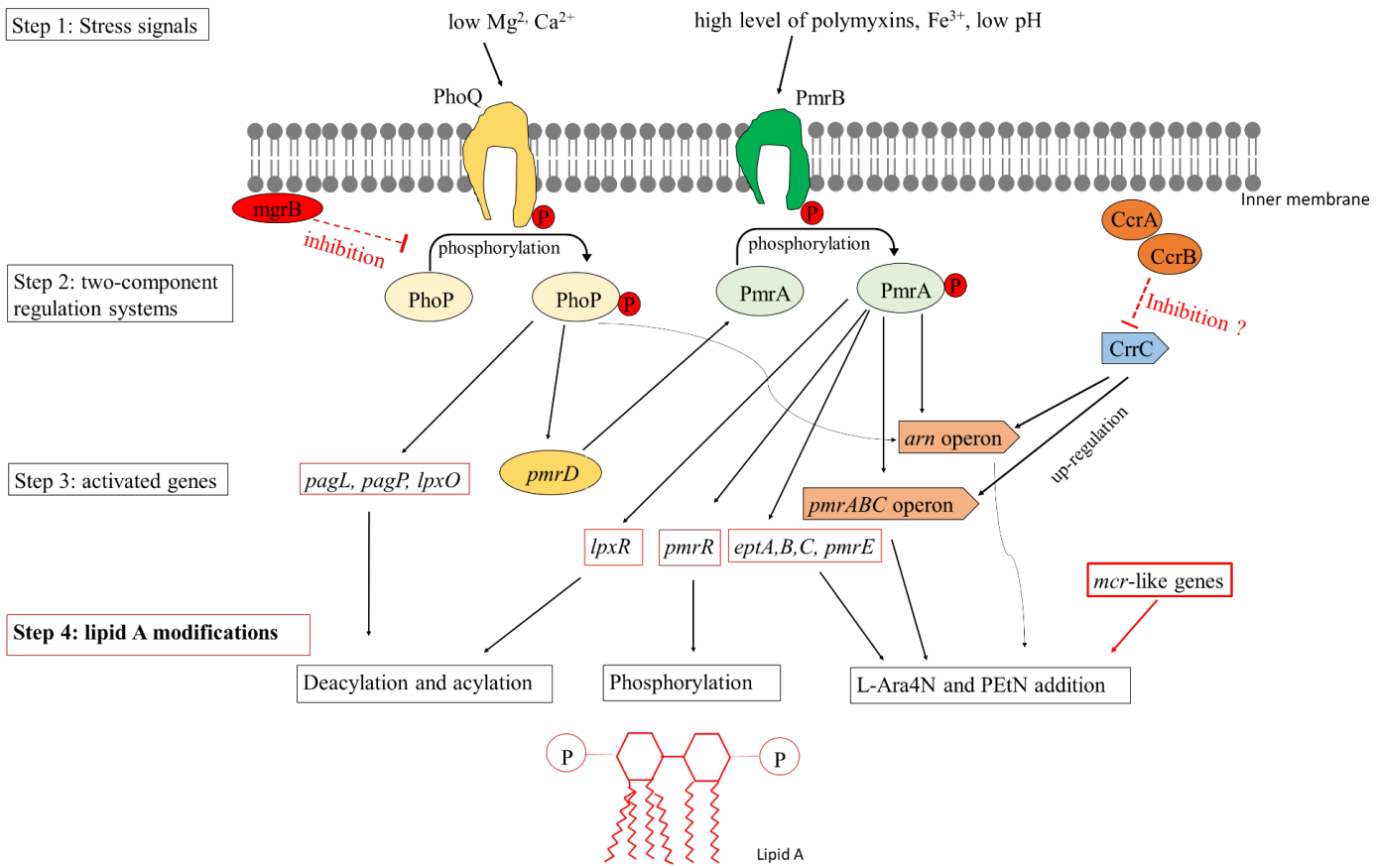


Fig. 1.4 Diagram of the regulations of three putative two-component systems, *phoPQ*, *pmrAB* and *crrAB*, responsible for polymyxin resistance in Gram-negative bacteria (updated from (Chen & Groisman, 2013, Olaitan *et al.*, 2014)).

Fig.1.4 (legend continued) Environmental stress signals such as high concentrations of polymyxins and low Mg^{2+} can trigger the phosphorylation of the sensor PmrB and PhoQ, respectively. The former can activate its cognate PmrA, once induced, several lipopolysaccharide-modifying genes including *arn* operon and *pmrABC* operon are activated by phosphorylated *pmrA* (see main text). The activation of the latter *phoQ-phoP* system can trigger *arn* operon directly in some *K. pneumoniae* or indirectly via the activation of PmrD, which in turn activates *pmrA*. In addition, the activated PhoP in *Salmonellae* can trigger other gene such as *pagL*, *pagP*, and *lpxO*, which can further modify lipid A through the removal of a 3-hydroxymistate, the addition of palmitate and 2-hydroxymyristate, respectively (Miller & Mekalanos, 1990). The *mgrB* exerts negative feedback on the *phoQ-phoP* system. Therefore, mutations in *mgrB* lead to constitutive induction of the *phoQ-phoP* system. Finally, the third two-component system, CrrAB, consists of a regulator CrrA and its activator CrrB. It has been proposed that the *crrB* can repress the expression of *crrC*, acting as a connector between *crrAB* system and *arn* operon. The mutations in *crrB* lead to the over-expression of *crrC,* which in turn up-regulates both *arn* and *pmrABC* operons and eventually modifications of lipid A. Black arrows indicate positive regulation of LPS-modifying genes. In addition, the mechanism of *mcr*-like genes is also added in this figure, MCR proteins catalyse the transfer of PEtN to the phosphate group of lipid A (Liu *et al.*, 2015).

(ii) *phoP-phoQ* system: In the PhoP-PhoQ system, PhoQ is a sensor kinase that phosphorylates its cognate regulator, PhoP. The activation of PhoQ is initiated during growth in limited Mg^{2+}, and an array of genes such as *pmrD* and *pmrAB*-regulated genes encode gens that are involved in modifying the structure of LPS (Nikaido, 2003). Upon activation, the phosphorylated PhoP directly activates the *arnBCADTEF* operon by binding its promoter, or indirectly via activating expression of PmrD protein (Mitrophanov *et al.*, 2008). PhoP-activated *pmrD* gene acts as a connector by creating regulatory links between the two-component systems: one is downstream of the *phoP-pmrQ* system, and the other is upstream of the *pmrA-pmrB* system. PmrD stabilizes the phosphorylated state of PmrA by binding phosphorylated PmrA at the N-terminal domain, preventing PmrA de-phosphorylation by its cognate sensor PmrB (Gunn, 2001, Kato & Groisman, 2004, Falagas *et al.*, 2010). As a result,

phosphorylated PmrA promotes the transcription of the *arn* operon that mediates the synthesis and addition of L-AraN4 to lipid A.

Additionally, the PhoP-PhoQ system is the major mechanism for polymyxin resistance in Salmonellae (Ernst *et al.*, 2001), the expression of *phoP* can simultaneously change the activation of more than 40 genes, termed PhoP-activated genes (*pag*) or PhoP-repressed genes (*prg*), respectively (Miller & Mekalanos, 1990, Guo *et al.*, 1997). The *pagL* gene encoding a lipid A 3-o-deacylase (Trent *et al.*, 2001), has been found to promote resistance to polymyxin B in Salmonellae via catalyzing 3-o-deacylation of lipid precursors (Guo *et al.*, 1997, Kawasaki *et al.*, 2007). Another *pag* gene, *pagP*, has been showed to increase lipid A acylation, leading to AMPs resistance in Salmonellae. This observation has been further confirmed by mutations in *pagP*, resulting in increased outer membrane permeability in response to several AMPs in Salmonellae (Guo *et al.*, 1998, Ernst *et al.*, 2001). Taken together, these results further suggest the important role of PhoP-activation in LPS modifications and polymyxin resistance.

(iii)The regulation of *phoP-phoQ* system: *mgrB* gene, which encodes a 47 amino acid peptide and plays an important role in regulating the transcription of the PhoP-PhoQ system (Lippa & Goulian, 2009). MgrB is a membrane protein with its N-terminus in the cytoplasm and C-terminus in the periplasm, which interacts directly with the periplasmic sensor domain of PhoQ. *mgrB* acts as a mediator in the negative feedback loop of the PhoP-PhoQ circuit that is prepressed by *mgrB* overexpression, and PhoQ inhibits PhoP phosphorylation (Lippa & Goulian, 2009). Inactivation or down-regulation of MgrB has been reported to be associated with colistin resistance in *K. pneumoniae* (Cannatelli *et al.*, 2013, Cannatelli *et al.*, 2014, Poirel *et al.*, 2015). *mgrB* alterations, can occur either by mutations resulting in amino acid substitutions (Olaitan *et al.*, 2014, Cheng *et al.*, 2015, Poirel *et al.*, 2017), importation of

insertion sequences (Cannatelli *et al.*, 2014, Cannatelli *et al.*, 2015, Jayol *et al.*, 2015, Poirel *et al.*, 2015), or insertion or deletion of small nucleotide sequences in the *mrgB* gene (Giani *et al.*, 2015). Such inactivation of *mgrB* facilitates the up-regulation of the *phoP-phoQ* operon, subsequently activating the *arnBCADTEF* operon and leading to the LPS modification with L-Ara4N responsible for colistin resistance. *mgrB*-mediated colistin resistant bacteria had fully restored susceptibility to colistin, once acquired a functional *mgrB* gene (**Fig.1.4**) (Poirel *et al.*, 2015). *mgrB* mutations appear to be the most common colistin resistance mechanism found in *Klebsiella*, and this *mgr*-mediated colistin mechanism so far has not been found in other bacteria (Poirel *et al.*, 2017).

(iv) *crrAB* system: A third two-component system, *crrAB* (Colistin Resistance Regulation, *crr*), has been reported to be associated with colistin resistance in *K. pneumoniae.* The *crrAB* system is present in most *K. pneumoniae*, but not in *E. coli* (Wright *et al.*, 2015, Jayol *et al.*, 2017). The CrrAB system consists of a histidine kinase CrrB and an adjacent response regulator CrrA. Six specific amino acid substitutions (Q10L, Y31H, N141I, P51S, L94M and S195N) in CrrB have shown to be responsible for mediating high-level colistin resistance by inducing the expression of *crrC*, which acts as a connector between *arnBCADTEF* operon and the *crrAB* system (Wright *et al.*, 2015, Cheng *et al.*, 2016). Inactivation of the *ccrB* gene elevates the expression of connector gene *ccrC*, followed by the up-regulation of *arnBCADTEF* operon and *pmrC*. This leads to the production of L-Ara4N and PEtN, consequently, mediating an increased colistin resistance.

1.3.1.2 Other species-specific colistin resistance mechanisms

(i) The loss of LPS: The disruption of LPS biosynthesis is known to play a significant role in colistin resistant *A. baumannii*. Mutations or insertional inactivation of the first essential genes for lipid A biosynthesis, *lpxA*, *lpxC*, or *lpxD*, leads to loss of the ability to produce lipid

A (Moffatt *et al.*, 2010, Moffatt *et al.*, 2011). Because the initial target of colistin is lipid A, LPS deficient *A. baumannii* results in loss of colistin binding ability to its bacterial outer membrane, and consequently yields high-level colistin resistance (MIC>128 mg/l). *pmrAB* mutation is another colistin mechanism found in *A. baumannii* (Arroyo et al., 2011). Both these colistin resistance mechanisms confer a noticeable biological cost observed by striking reduction of bacterial fitness and virulence in murine infection models (Beceiro *et al.*, 2014, Wand *et al.*, 2015). Unsurprisingly, the fitness burden of *lpx* mutant strains are significantly higher than that of *pmrB* mutant strains.

(ii) Efflux pumps: Colistin was primarily used to treat lung infections caused by *P. aeruginosa* in patients afflicted with cystic fibrosis (Littlewood *et al.*, 1985, Valerius *et al.*, 1991). The efflux pumps, *mexAB-oprM* are involved in developing tolerance to colistin in *P. aeruginosa* (Pamp *et al.*, 2008). *P. aeruginosa mexAB-oprM* knock-out mutants have restored colistin sensitivity and its complementary strain exhibited a significant increase in colistin tolerance. (Breidenstein *et al.*, 2011).

(iii) Production of the capsule polysaccharide (CPS) in *K .pneumoniae*: *K .pneumoniae* is able to release anionic CPSs, which is a crucial pathogenic factor for *K. pneumoniae*, and a variety of chemical compositions of CPSs have been identified in *K .pneumoniae (Cortes et al., 2002)*. It has been shown that CPS is associated with LPS by an ionic interaction (Fresno *et al.*, 2006). In Llobet's study in 2008, purified CPS from *K .pneumoniae* increased the bacteria survival and MIC of polymyxin B for an unencapsulated *K. pneumoniae* mutant. It has been speculated that CPS would act as a protective shield on the bacterial surface by trapping cationic polymyxins, resulting in reducing the amount of free polymyxins reaching the bacterial outer membrane and ultimately mediating resistance to polymyxins (Campos *et al.*, 2004, Llobet *et al.*, 2008).

(iv) Role of porins: OmpD/NmpC, which belong to a member of the trimeric β-barrel outer membrane general porin family, has been shown to contribute to polymyxin B resistance in *S. enterica* (Pilonieta *et al.*, 2009). In this study, the authors data showed that the gene, *ydeI*, which encodes an oligosaccharide/oligonucleotide binding-fold (OB-fold) periplasmic protein has an interaction with OmpD/NmpC and thus mediates colistin resistance. This suggests that the OB-fold protein, YedI, can protect bacteria from cationic polymyxins in connection with the general porin, OmpD. In addition, a second OB-folding protein, YgiM, and the porin, OmpF, also have been confirmed to confer to resistance to polymyxin B.

1.3.2 Plasmid-mediated colistin resistance

In November 2015, the first discovery of a mobile colistin resistance gene (*mcr-1*) was reported in food animals, food and humans from China (Liu *et al.*, 2015), shortly after, *mcr-1* was identified in more than 40 countries across five continents (**Fig.1.5**). The discovery of *mcr-1* attracted worldwide attention, due to it further limiting treatment options in humans. A recent study indicated that the emergence of *mcr-1* is likely to be linked with antibiotic exposure, suggesting that the reduction of colistin use in food-producing animals would, potentially, be an effective way to minimize the further emergence and spread of colistin resistant *E. coli* (Wang *et al.*, 2017). In order to control colistin consumption in agriculture, the Chinese government has banned colistin as an animal food additive and switched colistin to human therapy in 2017 (Walsh & Wu, 2016). Subsequently, the European Medicine Agency re-evaluated their advice on the use of colistin products in animals in the European Union, and aims to reduce the use in animal by 65% in next few years (EMA, 2016).

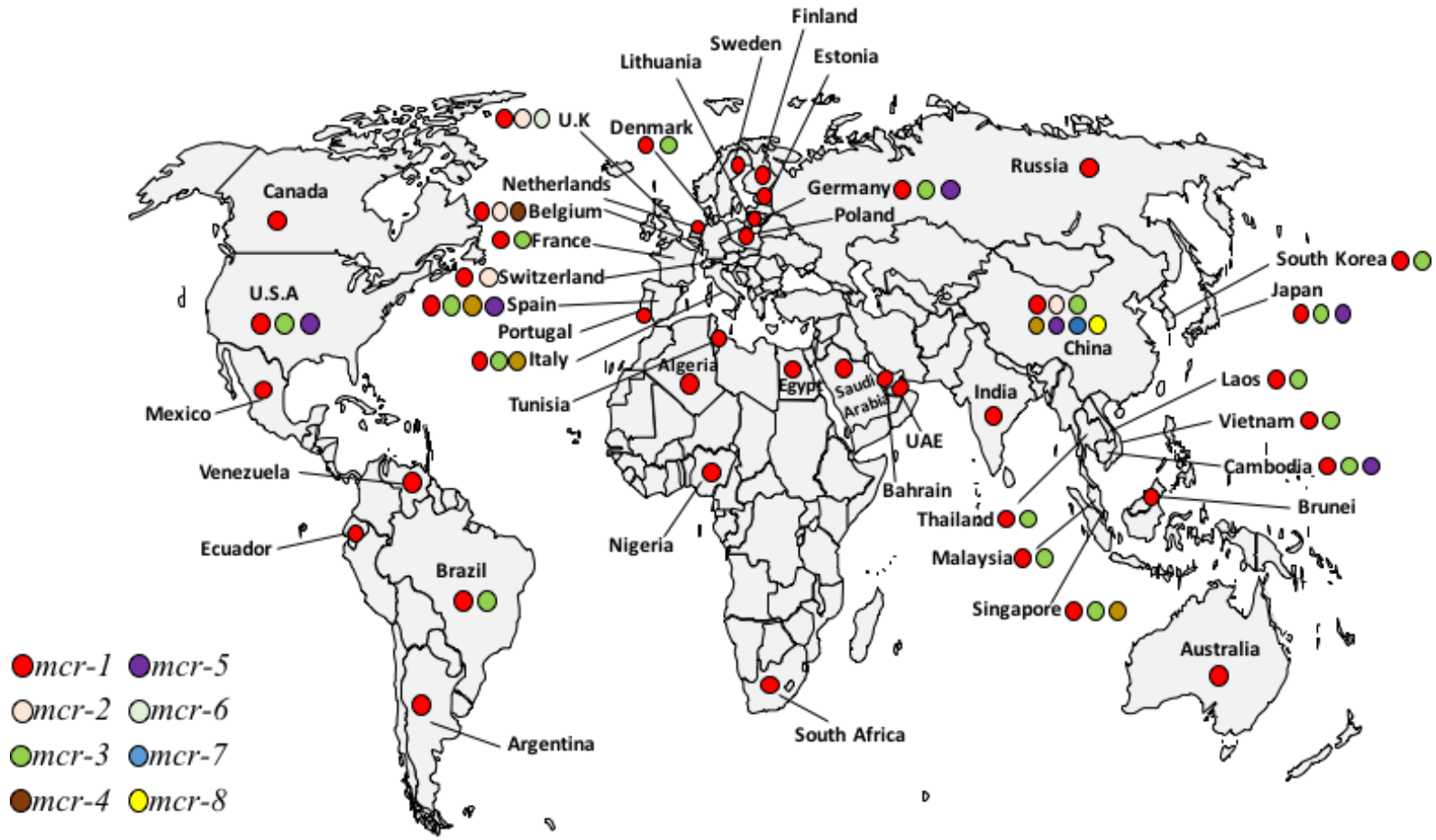


Fig.1.5 Global distribution of *mcr*-like genes (*mcr-1*, *-2*, *-3*, *-4*, *-5*, *-6*, *-7* and *mcr-8*). This map shows the countries (n=43) that have reported plasmid-mediated colistin resistance conferred by *mcr*- genes, updated from (Giamarellou, 2016, Al-Tawfiq *et al.*, 2017, Ellem *et al.*, 2017, Yin *et al.*, 2017, AbuOun *et al.*, 2018, Alba *et al.*, 2018, Eichhorn *et al.*, 2018, Garcia *et al.*, 2018, Wang *et al.*, 2018, Wang *et al.*, 2018, Wise *et al.*, 2018, Yamaguchi *et al.*, 2018, Yang *et al.*, 2018).

A retrospective study indicated that the *mcr-1* gene was circulating in the 1980s, when colistin was firstly introduced in veterinary medicine as a food additive and curative treatment in food-producing animals in China (Shen *et al.*, 2016). *mcr-1* has been identified both in strains obtained from livestock and clinical human patients, but the detection rates of *mcr-1* in livestock is markedly higher than that in human patients (Gao *et al.*, 2016, Health, 2016, Quan *et al.*, 2017). Worryingly, *mcr-1* (and other variants) have been shown to coexist with other MDR genes, including genes encoding NDM-1 (Wang *et al.*, 2017) and ESBLs (Kluytmans, 2017), highlighting the possibility that pan-drug resistance is now emerging. Following the detection of *mcr-1*, a number of distantly related *mcr*-variants, *mcr-2* (Xavier et al., 2016), *mcr-3* (Yin et al., 2017), *mcr-4* (Carattoli *et al.*, 2017) and *mcr-5* (Borowiak *et al.*, 2017) genes were detected and added to the list of mobile PEA transferases conferring colistin resistance in Enterobacteriaceae. The amino acid sequence identity among these MRC-variants are distinct from each other (33.71%-44%), except for MCR-1 and MCR-2, which show an 81.23% amino acid sequence identity. Nevertheless five residues (E246, T285, H395, D465 and H478) that are hypothesized to be critical for substrate binding and MCR-mediated colistin resistance, are conserved in all MCR-variants (Borowiak et al., 2017, Coates et al., 2017, Hinchliffe et al., 2017). It is believed that *Moraxella* species has acted as a potential reservoir of *mcr-1* and *mcr-2* (Gao *et al.*, 2016, Kieffer *et al.*, 2017), while *mcr-3* and *mcr-4* align closely to PEA transferases originating from *Aeromonas* species (Yin *et al.*, 2017) and *Shewanella frigidimarina* (Carattoli et al., 2017), respectively. While the new *mcr-5,* first discovered in *Salmonella enterica,* shows high similarity to PEA transferases from *Proteobacteria* and *Pigmentiphaga* species (Borowiak *et al.*, 2017). Most recently, three new MCR homologs, MCR-6 (AbuOun *et al.*, 2017), MCR-7 (Yang *et al.*, 2018) and MCR-8 (Wang *et al.*, 2018), were detected on the chromosome of *Moraxella pluranimalium*, in an

IncI2 plasmid recovered from *K. pneumoniae*, and an IncFII plasmid from *K. pneumoniae*, respectively.

1.3.2.1 Global spread of mcr-like genes

Colistin resistance serves as an interface between human and animal health, because colistin has been used in both human and veterinary medicine for more than 50 years, although their usage in human patients had been limited due to its nephrotoxicity and neurotoxicity (Falagas *et al.*, 2010). Whereas, colistin is widely used in veterinary medicine (particularly in China and SE Asia), especially for therapeutic treatment of gastrointestinal infections caused by GNB in pig and poultry populations. Polymyxins, mainly colistin, were the 5th highest group of antimicrobials sold for agriculture use in Europe in 2013, and the level of colistin usage ranged from below 1 mg/PCU such as in Denmark and the UK, up to 20-25mg/PCU in Italy and Spain (Health, 2016). As noted by Liu and colleagues, China was one of the world's highest users of colistin in agriculture, and eight out of top ten largest colistin producers are Chinese, the remaining two colistin producers are Indian and Danish. Until recently, China produces 71.1% of the world's colistin exporting 28.7% including to Europe. The heavy use of colistin in food-producing animals in Asia have resulted in a selective pressure in the farming sector and environment, and ultimately an outbreak of *mcr-1* positive *E.coli* (MCRPEC). Unlike chromosomal-mediated colistin resistance, *mcr* genes are located on plasmids, which are efficient vehicles for the dissemination of antibiotic resistance and virulence genes among Enterobacteriaceae. The initial description of *mcr-1*-bearing plasmids had the IncI2 exclusion determinant, subsequently, genetic and sequencing revealed that plasmid-borne *mcr*-genes have been observed in at least 14 different plasmid backbones possessing different replicon incompatibilities, including IncFII, IncX4, IncX3, IncX1-X2, IncHI2, IncHI1 and IncP plasmids (Matamoros *et al.*, 2017, Wang *et al.*, 2017, Sun *et al.*,

2018). It has been estimated that *mcr-1*-harbouring plasmids is highly transferrable with an *in vitro* transfer frequency as high as 10^{-1}-10^{-4}. A continuously increasing trend of *mcr-1* gene positive strains isolated from chicken samples has been found in China, with positive rates rising from 5.2% in 2009 to 30% in 2014 (Shen *et al.*, 2016). Besides China, a high prevalence of *mcr-1* has been circulated in Vietnam, where nine MCRPEC were identified from 24 (37.5%) $bla_{\text{CTX-M}}$ containing *E.coli* isolated from food animals (Malhotra-Kumar *et al.*, 2016). In France, the dissemination of *mcr-1* in commensal *E. coli* isolated from livestock were detected, with a prevalence rate ranging from 0.5% found in pig samples to 5.9% from turkeys. However, it is difficult to estimate the exact prevalence of *mcr-1* genes, as *mcr-1* screening from most of the current studies are from colistin resistant isolates, and the prevalence of *mcr-1* has almost certainly been underestimated. Interestingly, from the study by Thanh et al., an inactive form of *mcr-1* gene blocked by a 22-bp tandem repeat has been identified in the human pathogen *Shigella sonnei* under antibiotic-free selection (Thanh et al., 2016). However, upon colistin exposure, the *mcr-1* gene could restore its activity via deletion of one copy of the 22-bp repeat, resulting a high-level colistin resistance (32 mg/l). This inactivated form of MCR-1 may explain the reason that some isolates are positive by PCR but not phenotypically resistant to colistin.

Unlike the worldwide spread of *mcr-1*-producing Enterobacteriaceae in food animal samples (Malhotra-Kumar *et al.*, 2016, Schrauwen *et al.*, 2017, Wang *et al.*, 2017), the prevalence of other *mcr-like genes*, *mcr-2*, *-3*, *-4*, *-5*, *-6*, *-7* and *mcr-8*, is sporadic and low (**Fig. 1.5**). *mcr-2* has been firstly identified in *E.coli* (Xavier *et al.*, 2016) and Salmonella isolates (Garcia-Graells *et al.*, 2018) from European countries, but recently, it has been found in human vaginal swabs in from China (Zhang *et al.*, 2018). In Xavier and colleagues study, the prevalence of *mcr-2* in porcine colistin resistant *E.coli* (11/53) in Belgium was higher than that of *mcr-1* (7/53) (Xavier *et al.*, 2016). In Belgium, *mcr-2* has also been found in

Salmonella isolated from food at retailers but its occurrence is very low (1/105) (Garcia-Graells *et al.*, 2017). Variants (*mcr-2.2)* with 87.9% nucleotide identity with *mcr-2* were detected in *Moraxella* species isolated from healthy pigs at slaughter in the UK. The downstream genetic context of *mcr-2.2-pap2* was conserved with *eptA* and *mcr-1*-like genes in *Moraxella* species, further suggesting that Moraxella species may act as a reservoir of *mcr-1* and *mcr-2* genes (AbuOun *et al.*, 2017, Poirel *et al.*, 2017). This phenomenon is consistent with a new *mcr*-variant, *mcr-4*, which was first identified in *Salmonella enterica* and *E. coli* obtained from pigs in Europe (Carattoli *et al.*, 2017). Surprisingly, *mcr-3* and *mcr-5* have been reported in *E. coli* obtained from pigs in Japan, with detection rates of 8.3% (10/120) and 28.3% (34/120), respectively (Fukuda *et al.*, 2017). In this study, it stated that the co-occurrence of *mcr-1* and *mcr-5* has an addictive effect with higher level of colistin resistance (MIC>16 mg/l), compared to strains carrying single *mcr*-genes (MIC 2mg/l or 4mg/l). The prevalence of *mcr-3* gene has been determined in Asia, including China, (Yin *et al.*, 2017), Japan (Fukuda *et al.*, 2017) and Thailand (Wise *et al.*, 2018); Europe including Denmark (Roer *et al.*, 2017), Spain (Hernandez *et al.*, 2017) and France (Perrin-Guyomard *et al.*, 2016). *mcr-4* (Carattoli *et al.*, 2017) and *mcr-5* (Borowiak et al., 2017) are first found in *E.coli* and Salmonella isolates from Europe, respectively, and further detected in Asia, including Thailand (Wise *et al.*, 2018), Singapore (Teo *et al.*, 2018) and Japan (Fukuda *et al.*, 2017) (**Fig.1.5**).

In contrast with a much higher prevalence of *mcr-1* detected in animal samples, especially in Asia, it has been shown that *mcr-1* has so far only been found sporadically in humans with low detection rate (<1%) in both Asia and Europe (EMA, 2016). For instance, in a four-year cross-sectional study in Spain, the overall prevalence of colistin-resistant clinical isolates was as low as 0.67% (91/13579), and the *mcr-1* gene was only identified in one *E.coli,* strain and no *mcr-2* was detected (Prim et al., 2017). The overall prevalence of *mcr*-producing (*mcr-1*

and *mcr-2*) Enterobacteriaceae strains from human UTIs in Switzerland was also very low (0.05%) (Liassine *et al.*, 2016). It might be because of the limited use of colistin for the treatment of community-acquired infections therefore limiting the selective pressure for the emergence of MCR-1-producing bacteria. Although the detection rate of *mcr-1* in humans is still rare when compared to the higher abundance of *mcr-1* gene in animals, the finding of *mcr-1* gene in Enterobacteriaceae from patients with severe infections, highlights the risk of increasing co-resistance in pathogens causing difficult-to-treat infections in hospitals. It has been hypothesized that *mcr-1* originated from animals, due to their extensive use of colistin on the animals, and subsequently transferred to humans (Al-Tawfiq *et al.*, 2017, Wang *et al.*, 2018). The major host of *mcr-1* gene, *E. coli*, is a part of the normal human intestinal microbiome, but also a common cause of urinary tract and bloodstream infections world-wide. It has been reported that the *mcr-1* gene was detected in *E. coli* from human bloodstream infections and human gut microbiota (Hasman *et al.*, 2015). In Wang's report, 76/91 (83.5%) *mcr-1* positive isolates were *E.coli*, followed by *K. pneumoniae* (13/91, 14.3%), indicating that *E. coli* is responsible for the substantial horizontal dissemination of *mcr-1*. A variety of sequence types (ST) including the most virulent, ST131 clone, were found among MCRPEC (Wang *et al.*, 2017). ST131 is a worldwide pandemic clone, which potentially harbors a broad range of virulence and resistance genes, and the link between *mcr-1* gene and ST131 clone is concerning. Moreover, the *mcr-1* gene has also been detected in ESBL- and carbapenemase-producing *E. coli* isolated from human wound infections (Falgenhauer *et al.*, 2016). These XDR/PDR pathogens are of special concern, as very few antibiotics are left to treat life-threatening infections. Apart from *mcr-1*, *mcr-3* gene also has been found in clinical strains (Litrup *et al.*, 2017, Liu *et al.*, 2017), and chromosomal-linked *mcr-5* has been found in a *P. aeruginosa* strain from a patient from United States (Snesrud *et al.*, 2018). However, *mcr-2*, *-4*, *-6*, *-7* and *mcr-8*, have not been reported in patients. Most recently, a high prevalence of

mcr-1 gene ranging from 3.7% to 32.7%, was determined from human samples from 30 provinces in China (Shen *et al.*, 2018). In Shen et al. report, *mcr-1* is already disseminating in the community, which posts a great concern for the usage of colistin in clinical treatment, In addition, the high abundance of *mcr-1* gene in some provinces are closely associated with higher aquaculture industries, suggesting that the complex interface between human, environment and food-producing animals (Shen *et al.*, 2018) (**Fig.1.6**).

Taken together, the prevalence of *mcr*-like genes in both animal and human samples are varied; the *mcr-1* gene is most prevalent, followed by *mcr-3*. It is speculated that the origin of *mcr-1* gene is from the animal world, supporting by the following evidence: i) in contrast with only some scattered reports of *mcr-1*-producing isolates obtained from humans, a much higher prevalence of *mcr-1* gene has been identified worldwide from animals and animal products including pigs and piglets, chickens and chicken meat, cattle and turkeys. ii) polymyxins have been widely used in veterinary medicine as growth promoter/food additive or disease treatment. Retrospective data shows that *mcr-1* increased dramatically in China, in 2008/9 coinciding with the increase in colistin usage at exactly the same time (Shen *et al.*, 2016). iii) The genetic features of *mcr-1* also support the hypothesis of animal origin for *mcr-1*. For instance, *mcr-1* is often associated with the insert region *ISAPI1*, which was identified in a common animal pathogen, *Pasteurella multocida*. As the use of colistin in human infections is increasing, the spread of *mcr*-like genes may be largely underestimated, suggesting that prospective epidemiological surveys are needed, and the association between various 'hot spots' of MCR-producing microbiotas need to be further explored (**Fig.1.6**).

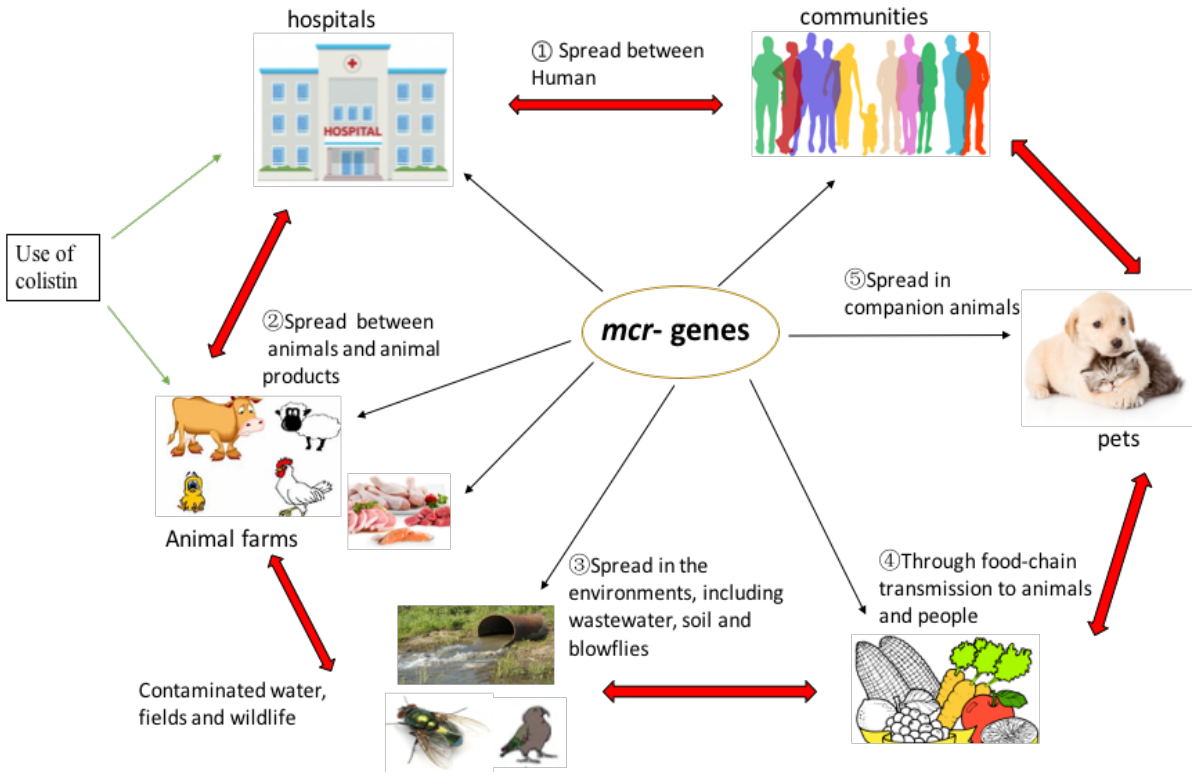


Fig.1.6 Schematic representation of complexity of the transmission pathways of *mcr*-like genes between human, animals, food and the environment. *mcr*-like genes have been detected in all sections shown in this figure, including human (Liassine *et al.*, 2016, Zhang *et al.*, 2018), animals (Fukuda *et al.*, 2017) (Shen *et al.*, 2016), wastewater (Lekunberri *et al.*, 2017), soil (Zheng *et al.*, 2017), blowflies (Zhang *et al.*, 2018), vegetables (Luo *et al.*, 2017) and meat products (Nishino *et al.*, 2017, Yi *et al.*, 2017). Light green arrows show major selective pressure of the emergence of *mcr* genes. The various reservoirs of *mcr*- genes are indicated by black arrows (updated from (Rhouma *et al.*, 2016)).

1.3.2.2 Mechanism of mcr-1 mediated colistin resistance

Generally, the mechanism for colistin resistance is associated with the decrease in membrane affinity for polymyxins by the modification of LPS, as described above. Plasmid-borne *mcr-1* represent a novel transferable mechanism for colistin resistance. MCR-1 acts as a membrane-anchored enzyme that catalyses the transfer of phosphoethanolamine to the head group of lipid A, ultimately resulting in the reduced polymyxin affinity of the bacterial outer membrane (Liu *et al.*, 2015, Gao *et al.*, 2016, Hinchliffe *et al.*, 2017). Therefore, the colistin

resistance mechanism mediated by the plasmid-borne *mcr-1* is similar to the chromosome-encoded two-component systems (*pmrAB* and *phoPQ*) (Gunn *et al.*, 1998, Olaitan *et al.*, 2014). Structural analysis of MCR-1 shows that it is closely related to LptA (LPS PEA transferase A, also referred as EptA), which is intrinsically responsible for polymyxin resistance in *Neisseria* via addition of PEA to 1' and 4' head groups of lipid A (**Fig.1.5**) (Wanty *et al.*, 2013, Liu *et al.*, 2015). Despite the fact that plasmid-borne *mcr-1* and chromosomal-encoded *lptA* share very low DNA sequence identity, phylogenetic analysis suggests that both *mcr-1* and *lptA* are highly homologous to the PEtN lipid A transferase family, and fall into two different subclades (Gao *et al.*, 2016). Both mechanisms of colistin resistance mediated by *mcr-1* and *lptA* are associated with the modifications of lipid A that decrease colistin affinity for LPS, but their regulation systems seem to be different: the expression of *lpxA* is influenced by *pmrAB* (**Fig.1.4**), whereas the pathway or regulation of plasmid-borne *mcr-1* is not yet fully understood. In comparison with chromosomal resistance genes mediating vertical distribution of colistin resistance, the plasmid-borne *mcr* seems to be a more effective mechanism for colistin resistance to mobilise horizontally. Most intriguingly, just within the space of a few months after its first discovery, *mcr-1* had been reported around the world testimony to the strength of whole genome sequencing and publicly available sequence databases, even if those same databases did not originally recognise it as a PEA transferase demonstrating the frailty of DNA databases (Skov & Monnet, 2016).

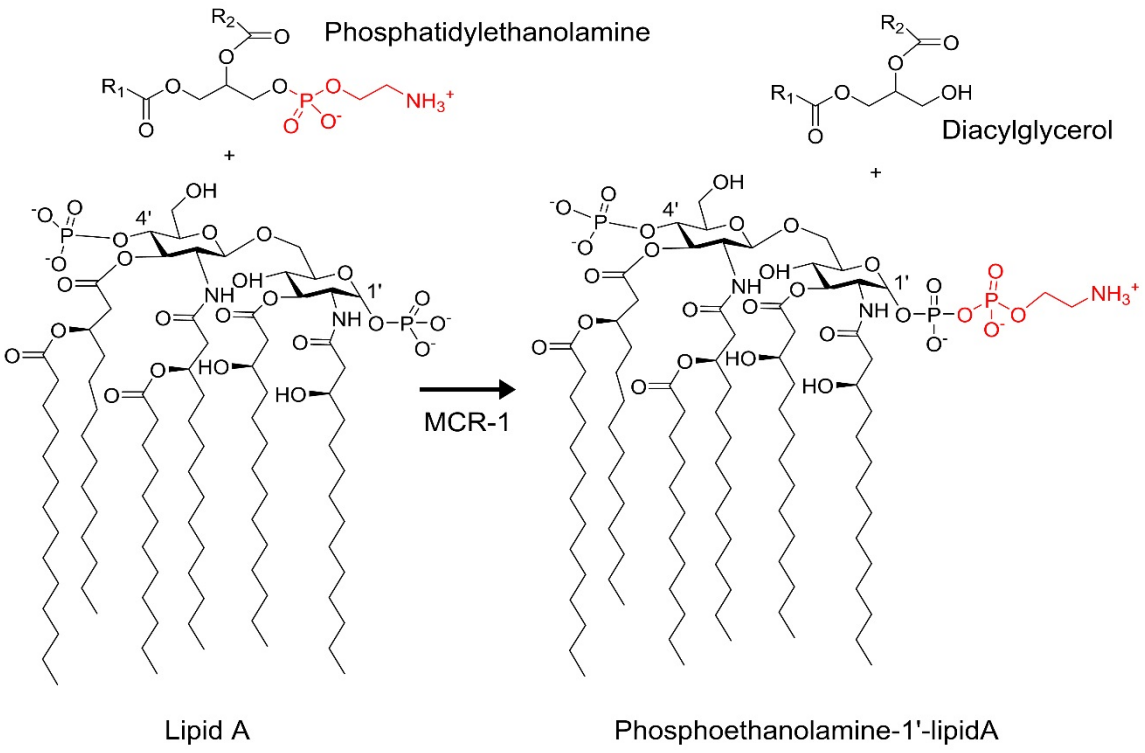


Fig.1.7. Phosphoethanolimine transfer to lipid A by MCR-1 mediating colistin resistance**.** MCR-1 catalyses transfer of phosphoethanolamine from a phosphatidylethanolamine donor substrate onto lipid A with 1'or 4'-phosphate position.

1.3.2.3 Structural mechanism of lipid A PEA transferases conferring colistin resistance

Plasmid-encoded MCR-1 protein is an integral membrane protein, consisting of a trans-membrane domain anchoring the enzyme to the periplasmic space of a soluble catalytic domain. It has been shown that removal of the transmembrane domain from MCR-1 protein can restore colistin susceptibility in recombinant *E .coli*, suggesting that MCR-1 transmembrane domain anchoring of its soluble domain is required for MCR-1 catalytic activity (Gao *et al.*, 2016). So far, the structures of the soluble domain of three different colistin-resistance associated PEA transferases: MCR-1 from *E .coli* (Hinchliffe et al., 2017), EptA (also labelled as LptA) from *Neisseria meningitidis* (Wanty *et al.*, 2013) and EptC from *Campylobacter jejuni* (Fage et al., 2014), have been determined. The crystal structures of

MCR-1 have been further examined following substitutions of five putative conserved sites in the catalytic domain of MCR-1 protein (E246, T285, H395, D465, and H466) that are essential for its function and all were found to induce significant reductions in the MIC of colistin (Gao *et al.*, 2016, Hinchliffe *et al.*, 2017). These results indicate that both transmembrane and soluble domains are critical for catalytic activity of MCR-1. The former would be required to enable the MCR-1 enzyme to correctly localize in the outer membrane and orientate the binding of substrates (Wanty *et al.*, 2013); the latter contains the catalytic nucleophile (T285) essential for PEA transfer (Gao *et al.*, 2016, Hinchliffe *et al.*, 2017). The structure of the MCR-1 catalytic domain reveals that the MCR-1 enzyme is a zinc metalloproteinase with an alkaline phosphatase/sulphatase-fold containing three disulphide bonds. Zinc ions are present in the active site and associated with T285, which is a phosphorylated form of the putative nucleophile. Zinc deprivation can restore colistin susceptibility in MCR-1-producing *E. coli* demonstrating that the zinc is critical to MCR-1 activity (Hinchliffe *et al.*, 2017).

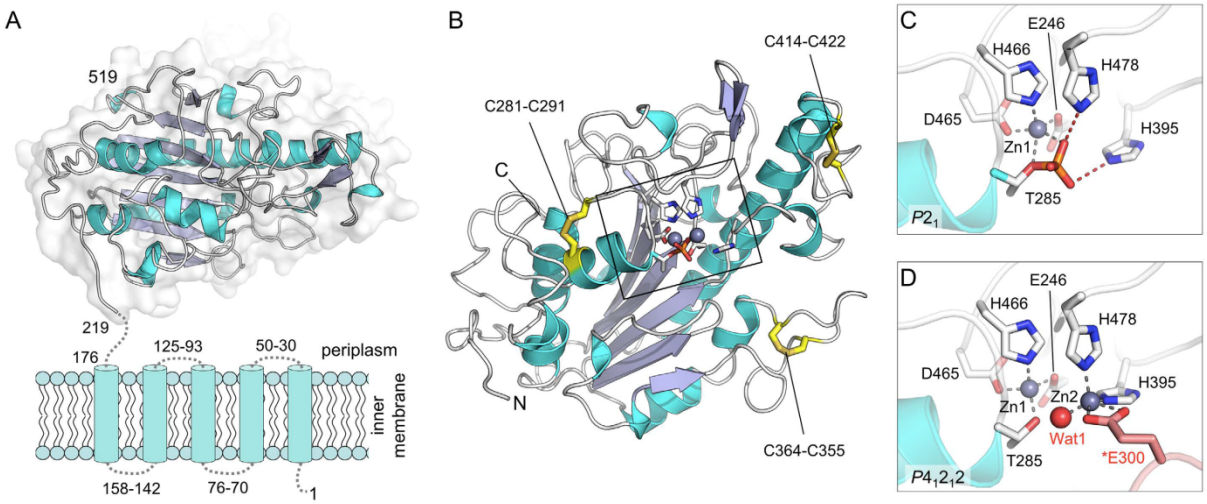


Fig.1.8 Structure of MCR-1 catalytic domain. (A) putative organisation of MCR-1 showing five predicted membrane-spanning α-helices and the soluble periplasmic domain (residues 219–541)

crystallised here. (B) Overall fold of MCR-1 catalytic domain. Intramolecular disulphide bonds are labeled and metal centre (active site) is boxed. (C) Active site of P21 crystal form showing single bound zinc ion (grey sphere) and phosphorylation of Thr285. (D) Active site of P41212 crystal form showing dinuclear zinc centre and coordination of Zn^2 by residue Glu300 (pink) from an adjacent molecule in the crystal lattice (cited from (Hinchliffe *et al.*, 2017).

It has been proposed that the crystal structure of a full-length PEA transferase may facilitate our understanding of the lipid structure binding and catalysis. Very recently, structure of a full-length LptA protein has been determined (Anandan *et al.*, 2017). This provides a model of its homologous PEA transferases such as MCR-1, and insights into the structural mechanism of lipid A modification by PEA addition. LptA structure also reveals that the helical transmembrane domain and periplasmic facing soluble domain are linked by a bridging helix that embeds in the outer membrane interacting with the phospholipid head group. Five transmembrane helices (TMH1-TM5) forming an aromatic belt, are predicted to be parallel to one another and span the inner membrane (**Fig.1.6**). There are a number of tryptophan, tyrosine and histidine residues observed in the transmembrane domain that may facilitate the stabilization and orientation of the protein in the bacterial membrane bilayer **Fig.1.6D**. TMH5 is connected to the soluble domain by an extended periplasmic loop followed by a helix structure. The full-length structure confirms that there is a ligand bond between the two domains, this kind of structural arrangement can efficiently sequester the catalytic site (Anandan *et al.*, 2017). The substrate binding pocket involves a soluble domain and two helices, PH2 and PH2', which are linked with TMH3 and TMH4 on the membrane domain. In a recent study, two substrate-binding pockets were demonstrated in the MCR-1 catalytic reaction, and addition of the PEA analog, ethanolamine, can restore bacterial susceptibility of polymyxin (Wei *et al.*, 2017). These data suggests the crucial role of PEA- and lipid A-binding pockets in the action of PEA transferases mediating colistin resistance.

However, except the crystal structure of soluble domain of MCR-1(Hinchliffe *et al.*, 2017) and MCR-2 (Coates *et al.*, 2017), full-length MCR protein are still under explored.

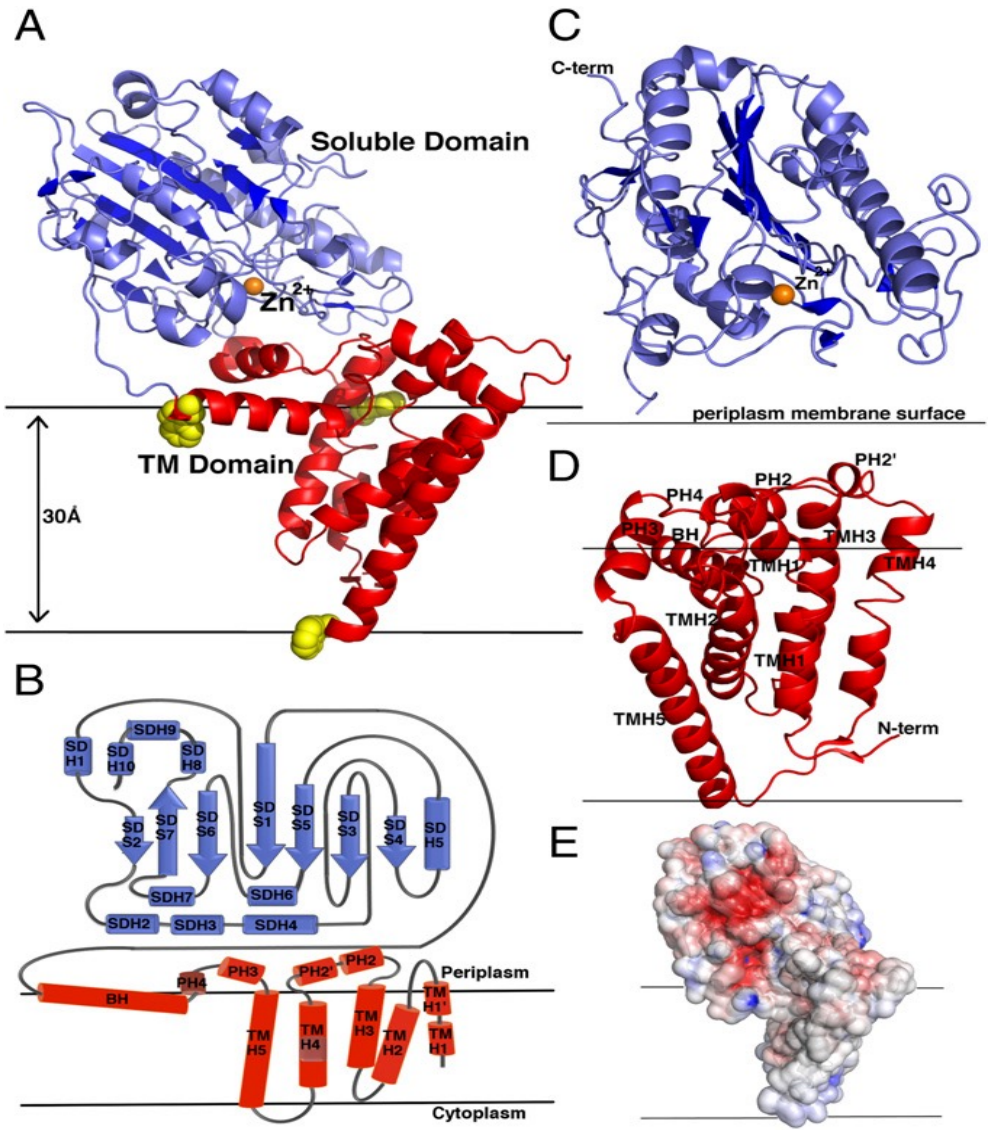


Fig.1.9 Molecular structure of full-length EptA from *Neisseria meningitidis*. In **A**, red and blue ribbons are represented the amino-terminal TM domain and the carboxylterminal soluble domain, respectively. The yellow spheres are indicated as the three TM domain tryptophan residues (Trp126, Trp148, and Trp207). In **B**, the putative orientation of the EptA protein relative to the periplasmic and cytoplasmic membrane is presented. **C** and **D** indicate the secondary structure of the soluble domain and TM domain with the helical numbering labeled, repectively. (**E**) Electrostatic surface representation of EptA, calculated using APBS. The bound Zn^{2+} ion in A and C is shown as an orange sphere (cited from (Anandan *et al.*, 2017))

1.4 Fitness costs of antibiotic resistance and compensatory evolution

The actions of antibiotics interfere with bacterial normal physiological processes including cell wall synthesis, DNA replication, RNA transcription and protein synthesis. It is not therefore surprising that resistant mutants usually cause the perturbation of cellular activities. Any resistance mechanism seems to be an 'equilibrium double-edge sword': on one hand, it protects bacterium from antibiotics by altering antibiotic targets; but, on the other hand, it can potentially confer a biological cost, associated with diminished bacterial growth, increased survival and transmission inside or outside a host (Andersson & Levin, 1999, Andersson, 2006) . It has been proposed that the high fitness cost of fosfomycin resistance may account for the low prevalence of *E. coli* clinical isolates from UTIs (Pourbaix *et al.*, 2017). Both experimental and theoretical data suggest that the biological cost of resistance is a major determinant of resistance development and potentially reversibility. It has been proposed that the fitness-cost plays a significant role in limiting the emergence of antibiotic resistance. Ergo: the higher the fitness-cost, the slower the rate of resistance development, which would lead to a decreased proportion of resistant bacteria and perhaps even a disappearance from the population (Hughes & Andersson, 2015). Therefore, understanding bacterial fitness-cost is important to predict the development of MDR/XDR in different clinical settings, as well as to evaluate intervention to reduce resistance (Andersson, 2006).

1.4.1 The impact of trade-offs and constraints on adaptive evolution

The fitness cost of antibiotic resistance can be compensated by either reversion (which is rare) or compensatory mutations. It has been long thought that because the deleterious fitness burden caused by antibiotic resistance, resistant bacteria may reverse back to antibiotic sensitivity or be out-competed by their ancestral susceptible bacteria. This theory raises the hope that the frequency of resistant bacteria will decrease through natural selection, and the

current problem caused by MDR/XDR bacteria would simply be solved in a reasonable amount of time (Andersson & Levin, 1999). However, there is increasing evidence suggesting that resistance mutations such as those found in bacteria isolated from patients, have evolved to ameliorate the bacterial fitness-cost imposed on them by antibiotic resistance. As a result, compensated-resistant bacteria would stabilize in the population, becoming less reversible to their ancestral susceptible phenotypes. Furthermore, in a computer simulation model, it has been suggested that mutation rates for intermediate-fitness compensatory mechanisms are higher than those for high-fitness revertants, probably because of larger mutational targets (Levin *et al.*, 2000, Sommer *et al.*, 2017). As shown by theoretical modelling, bacteria carrying antibiotic resistance genes, which engender deleterious effects on bacterial fitness, subsequently evolved, by mutation with a moderate fitness burden, or even no fitness-cost in the absence of antibiotics, rather than reversion to complete antibiotic sensitivity (Bjorkman *et al.*, 1998, Rozen *et al.*, 2007, Pacheco *et al.*, 2017). Compensatory mutations in *Pseudomonas* spp. carrying a MDR plasmid, RP4, has been found to increase plasmid permissiveness, because the fitness cost turned into a benefit after 600 generations (Andersson & Hughes, 2010). Another example of adaptive compensations has been examined by Sousa J et al. (de Sousa *et al.*, 2017), who stated that double-resistant bacteria (an *E. coli* resistant to both streptomycin and rifapicin) compensate faster than those of single-resistant strains, more importantly, compensated mutations that only found in double resistance strains have strongly beneficial effects, observed by increased fitness.

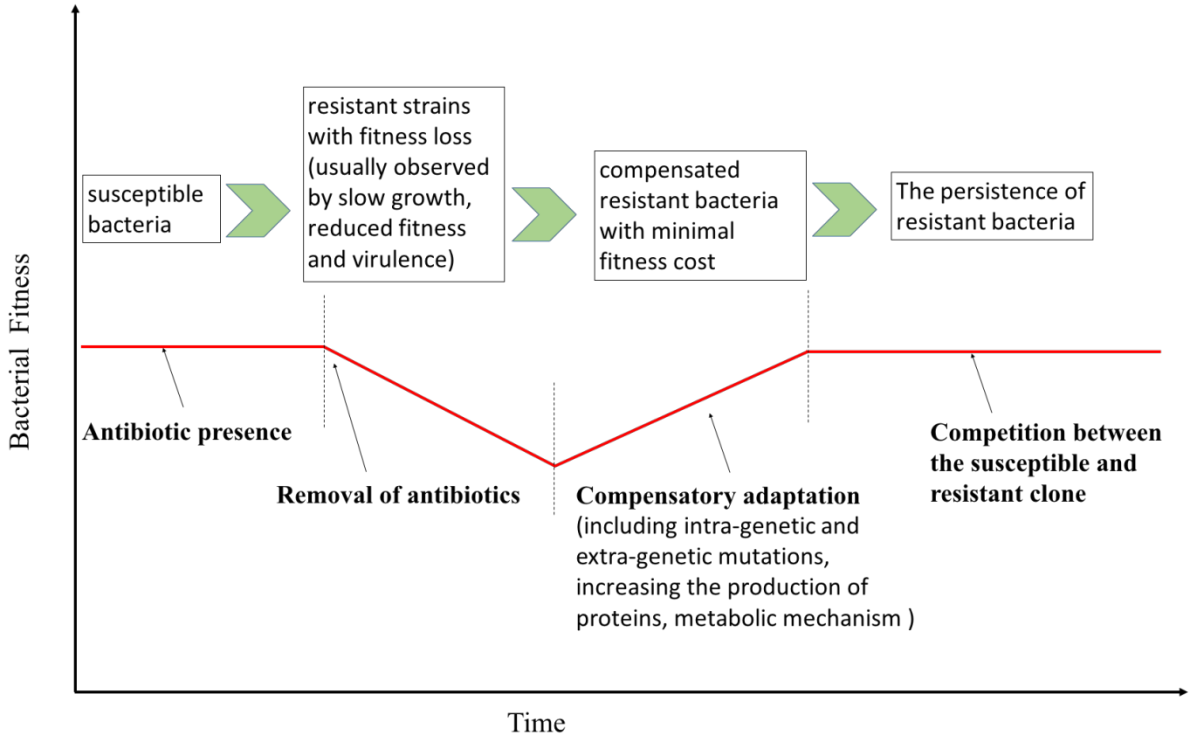


Fig.1.10 the development and evolution of fitness burdens imposed by antibiotic resistances (updated from (Hughes & Andersson, 2015))

1.4.2 Fitness cost of antibiotic resistance conferred by plasmids carrying AMR genes

In many cases, plasmids can carry beneficial genes like AMR genes to the host bacteria by enabling them to persist in hostile environments, e.g. protection against potentially lethal antibiotics. However, because plasmids are an extra-chromosomal genetic element presented in host cells, an additional metabolic burden would be imposed by intragenomic conflict between chromosomal genes and plasmids, which are prone to elimination from bacterial genomes in the absence of selective (antibiotic) pressure (Zielenkiewicz & Ceglowski, 2001, Yang & Walsh, 2017). Therefore, the acquisition of plasmids generally causes a fitness cost, associated with the replication and maintenance of the plasmids themselves within the host. In spite of this fitness loss, it can be alleviated over time through compensatory changes, the initial fitness burden associated with plasmid-bearing is the main constraint on the horizontal transferability and maintenance of these genetic elements in the host bacterium (San Millan

& MacLean, 2017). Generally, there are pleiotropic fitness effects of plasmids in their every life cycle in the host. For example, during the first phase of plasmid entry into a new host via conjugation, plasmid DNA transiently becomes single-stranded, which is the trigger for the bacterial SOS response. Conjugation-triggered SOS responses mediate the inhibition of cell division, which would cause bacterial slow growth and fitness loss, in a recent study, the loss of the conjugation region in the IncN antibiotic resistance plasmid pKP33, reduced the fitness cost in *E. coli* (Porse et al., 2016). It has been proposed that the plasmid-encoded replication initiation proteins, which control the plasmid copy number, are responsible for a fitness loss. Initiation of plasmid replication play a crucial role in the maintenance of plasmids, such that they must keep producing enough copies to allow their distribution to all daughter cells after cell division. Thus, more DNA polymerases and helicases are needed to process plasmid replication from the bacterial host. Over-producing plasmid replication proteins therefore would cause energetic exhaustion of the host leading to disruption of the efficiency of cellular DNA replication machinery, inducing stress responses and ultimately inhibiting cell division (Wegrzyn & Wegrzyn, 2002). This deleterious effect has been observed in bacteria carrying multi-copy plasmid pNUK73 in *P. aeruginosa* (San Millan *et al.*, 2014, San Millan *et al.*, 2015) and pQBR103 in *E. coli* (Harrison et al., 2015). However, there is a conflict between plasmid-bearing fitness-cost and the widespread occurrence of plasmids in environmental or clinical bacteria, suggesting that some mechanisms contributing to plasmid stabilization and/or ameliorating the fitness-costs, would may explain this plasmid paradox. Genes involved in the genetically expensive conjugative process are tightly self-regulated. It has been found that the plasmid compensatory evolution repeatedly targeted to the a global regulatory system, *gacA*/*gacS*, and its mutations may ameliorate the cost of plasmid carriage by relieving the translational demand imposed by the plasmids (Harrison *et al.*, 2015, Harrison *et al.*, 2016). In the Dahlberg and Chao study, in spite of initial fitness cost,

plasmids R4 and RP4, were persistent and the fitness-cost of the plasmids was reduced by compensatory evolutions through changes in both plasmids and bacteria (Dahlberg & Chao, 2003). Moreover, evolved plasmids no longer imposed a cost on their host when transferred to a plasmid-free ancestral *E. coli* (Dahlberg & Chao, 2003, Dionisio et al., 2005), indicating that even with the reduction of antibiotic use, plasmid-mediated AMR can still persist in bacterial populations (Lopatkin *et al.*, 2017).

1.4.3 Assessing horizontal evolution of resistance under the perspective of bacterial fitness

On the basic knowledge we have today on the evolution of resistance genes (here we mainly focus on plasmids-mediated resistance, which is a particularly common genetic route to emerge antibiotic resistance in clinical relevant bacteria), the risk assessments of the acquisition of gene that confers antibiotic resistance would be of great importance in predicting future emergence and development of AMR and therefore guiding therapeutic choices. Several parameters that affect HGT (horizontal genetic transfer) need to be considered, including genetic characteristics of plasmids (such as some plasmids have broad host range and can transfer across species, whereas others have a much narrower host range) (van Hoek *et al.*, 2011); the frequency of HGT between relevant species, the fitness effects on the host following the acquiring of resistant plasmids, and gene expression that would mediate high-level antibiotic resistance to clinical pathogen (**Fig.1.11**) (Sommer *et al.*, 2017). All of these parameters can be experimentally determined to provide a rational assessment of the risk of the evolution, transmission and maintenance of resistance (**Fig.1.11**).

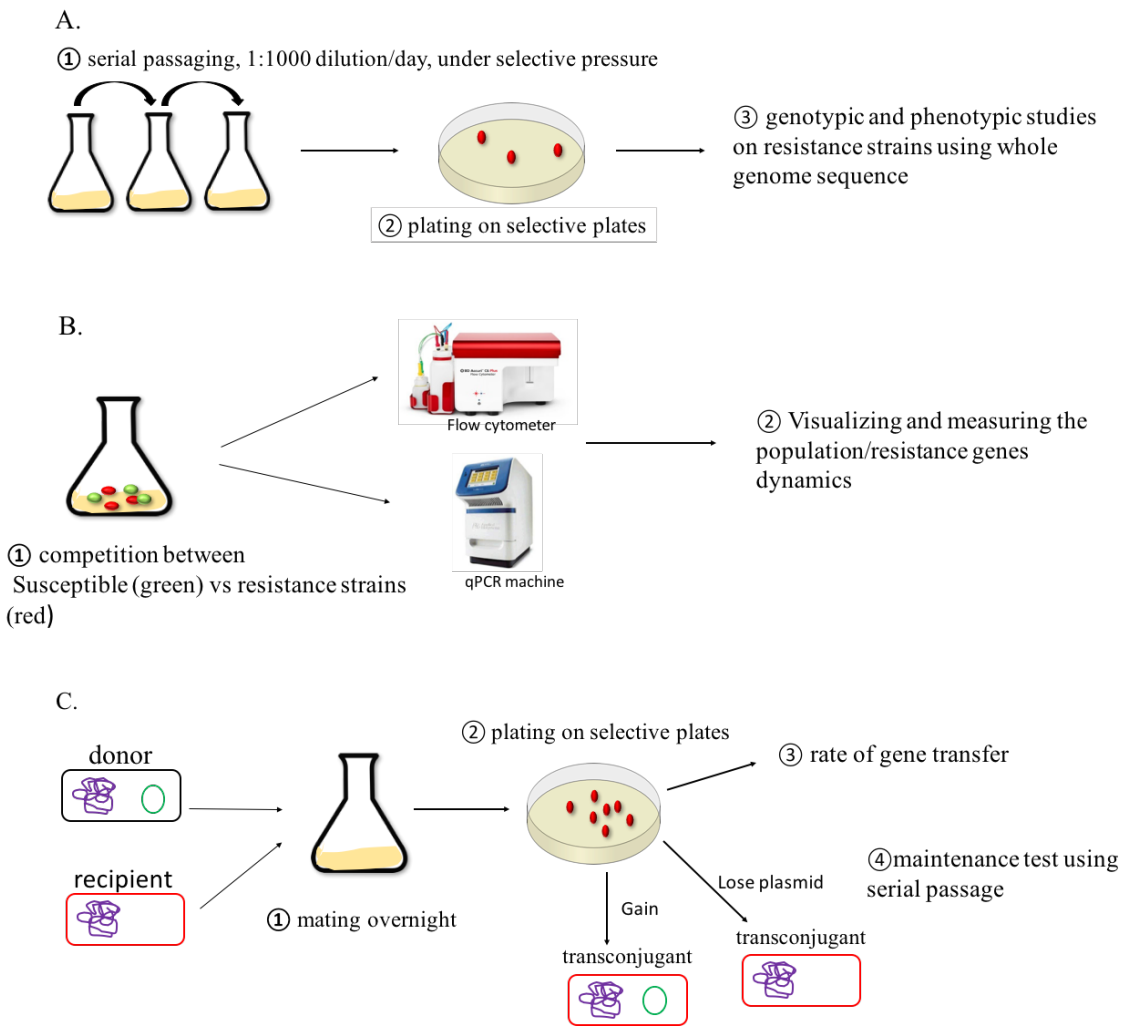


Fig.1.11 Methods summary for the experimental assessments of the risk of bacterial resistance

A. generating resistant bacteria in vitro: antibiotic resistant bacteria can be evolved by challenging with an increasing concentrations of antibiotics in agar plates. Any viable colony is further studied by its phenotypic and genotypic characterizations.

B. magnitude of bacterial fitness: bacterial fitness can be measured by its growth rate in pure culture over time or by competitive fitness against the wild-type ancestral strains. The fitness differences between susceptible and resistant strains can be achieved by measuring their exponential growth rates. It is possible to discriminate differences of ~5% per generation (Andersson & Hughes, 2010). In a competition model (*in vitro* or *in vivo*), the susceptible and resistant strains are initially mixed at 1:1 ratio and then are serial passaged through growth cycles, followed by measuring the changes in the ratio of both populations. It would be more sensible to discriminate fitness differences of 0.1% per generation, by using florescent-activated cell sorting with fitness-neutral fluorescent markers (Andersson & Hughes, 2010). Additionally, combination with epidemiological studies, fitness burden

conferred by antibiotic resistance can provide insights into real-life fitness over longer time periods in clinical and nature environments and prediction of dynamics and development of the resistance (Rossolini *et al.*, 2010, Hughes & Andersson, 2015). Furthermore, compensatory adaptation also can be observed by laboratory experiments. Briefly, resistant bacteria are cultured in the fresh medium without antibiotics and then serial passaged every day. The changes in fitness can be detected quantitatively by measuring growth rate or competitive fitness as described above (B), and qualitatively by a change in colony size and resistance phenotype when bacteria are plated on selective agar plates (MacLean *et al.*, 2010). Furthermore, any intragenetic/extragenetic changes responsible for compensated fitness can be confirmed by whole genomic sequence and targeted sequencing (MacLean *et al.*, 2010, de Sousa *et al.*, 2017).

C. The game of gains and loses. The rate of gene transfer and the stability of the acquired resistance gene in the pathogen (the possibility of being lost?), can be measured by conjugation and serial passaging with or without selective pressure.

Taken together, the following is the summary of factors influencing bacterial fitness cost.

(i) Fitness cost can be compensated by different trajectories. There are substantial findings to suggest that the fitness-cost can be compensated without loss of resistance, by second-site mutations during the evolution of bacterial resistance in an experimental host or laboratory medium (Hall *et al.*, 2010, Harrison *et al.*, 2015). The differences in compensatory mutations and fitness cost were observed in the bacteria evolving through serial passages in a murine model and in a laboratory medium (Bjorkman *et al.*, 2000). These data indicate that different growth conditions would affect the spectra of compensatory mutations. Bjorkman and colleagues provided an elegant example to show that the fitness-cost of resistance is compensated by different mutations under different growth conditions (Bjorkman *et al.*, 2000). In a *S. Typhimurium* strain with streptomycin resistance (SmR) caused by mutations in *rpsL* gene, the bacterial growth rate and the opportunity for compensated mutations to arise and evolve were similar in LB and the murine model. However, different spectra of compensated mutations were identified under both growth conditions: in LB-selective

conditions, all SmR strains contained extragenic mutations in either *rpsD* or *rpsE*, whereas all murine-selected mutants preferentially contained one specific intragenic compensatory mutation. The specific types of compensatory mutations observed in both LB and mice suggests that different evolutionary pathways are responsible for reducing the cost of AMR within and outside a host.

(ii) The genetic interactions (epistasis) can affect fitness cost. Since mutations that confer multiple AMR are known to impair bacterial fitness, the genetic interactions (epistasis) between resistance mutations may provide additional insight as to how bacteria cope with deleterious effects mediated by AMR (MacLean *et al.*, 2010, Chou *et al.*, 2011). Here, epistatic interactions describe how the bacterial fitness effects of a mutation depend on second mutations, usually in the core genome. Theoretically, if the epistasis is synergistic (when mutations are cumulative), then the cost of multiple AMR would be high and eventually resistant microbes will be eliminated in the absence of antibiotics (Khan *et al.*, 2011). Unfortunately, antagonistic epistasis (when mutations compensate each other's fitness-cost), which would reduce fitness loss by interaction with resistance mutations, have been identified in MDR bacteria (Trindade *et al.*, 2009, MacLean *et al.*, 2010). In Trindade's study, the majority of antagonistic epistasis has been identified in *E. coli* associated with the *rpsL*, *rpoB* and *gyrA* alleles, which confer resistance to streptomycin, rifampicin and nalidixic acid, respectively (Trindade *et al.*, 2009). This specific genetic combination therefore alleviates the costs of double resistance, allowing the accumulation of resistant bacteria in the population. Another example of an epistasis effect on fitness is shown by second mutations in the genes, *par* and *gyr*, responsible for resistance to fluoroquinolones in *Streptococcus pneumoniae* (Rozen *et al.*, 2007). Generally, single mutation in both *par* and *gyr* gene, cause low level resistance to fluoroquinolones in *S. pneumoniae*, associated with a fitness burden. Interestingly, with the addition of second mutation in *par* or *gyr* gene, a higher level of

fluoroquinolone resistance was observed without any fitness loss and even increasing the fitness (Rozen *et al.*, 2007). Furthermore, the genetic linkage between resistance mutations are important to the fitness cost associated with its acquisition. One example is the fitness cost imposed by SmR in *P. aeruginosa*, which seems to depend on environmental and epistatic effects (Ward *et al.*, 2009). In the absence of antibiotics, the cost of SmR mutants is greater in a rifampicin sensitive (RifS) *P. aeruginosa*, than those in a rifampicin resistance genetic background (RifR). In the presence of rifampicin, SmR mutants have no detectable costs in some RifR background, while massive fitness cost detected in others (Ward *et al.*, 2009). Taken together, the patterns of epistasis may differ from within- and between-gene networks during bacterial adaptations.

(iii) Some mutations are cost free by metabolic pathways. One largely accepted paradigm is that the acquisition of resistance is generally associated with a fitness cost, but there is always an exception. It has been reported that fitness costs depend on the mutation involved, with some of them occurring with no cost. For instance, the mutations in the β-subunit of the rifampicin target enzyme, RNA polymerase, contributes to rifampicin resistance in *Staphylococcus aureus*, with no measurable cost (O'Neill *et al.*, 2006). It has been determined that overexpression of efflux pumps MexAB-OprM, MexCD-OprJ, or MexXY, responsible for AMR in *P. aeruginosa*, did not cause any fitness loss. In turn, metabolic changes including an increase in anaerobic nitrate respiratory chain when cells are under full aerobic environment, may serve as a feasible strategy to cope with the cost associated with the acquisition of AMR (Pacheco *et al.*, 2017).

(iv) The cost of resistance to the same antibiotic can be different by alternative mechanisms. There are two main mechanisms of colistin resistance in *A. baumannii*, the PEtN modification of LPS regulated by a two-component system *pmrAB* (Mu *et al.*, 2016), and the complete loss of LPS due to the mutations of LPS synthetic genes *lpxACD* (Moffatt *et*

al., 2010). Both mechanisms are associated with a noticeable fitness loss evaluated by the growth rates and competitive experiments *in vitro* (Beceiro *et al.*, 2011, López-Rojas *et al.*, 2011) and *in vivo* (Lopez-Rojas *et al.*, 2013), with LPS deficient conferring a higher fitness burden (Beceiro *et al.*, 2014, Mu *et al.*, 2016). It has been shown that the *lpx* mutant can be selected in one single passage on Mueller-Hinton agar supplemented with 10 mg/l of colistin sulfate, and the mutants usually confer high-level of colistin resistance (MICs>128 mg/l) (Beceiro *et al.*, 2014). On the contrary, colistin-resistance strains with *pmrAB*-mutationts were generated from multiple passages with increasing concentrations of colistin and give rise to lower levels of colistin resistance. This may explain the higher fitness cost mediated by *lpx* mutations, with significant genetic and LPS structural changes in a single step. Moreover, *pmrAB*-mutants did not alter the susceptibility to other antibiotics, while the LPS-deficient mutations affect the sensitivity to other antibiotics including cefepime and azithromycin (Beceiro *et al.*, 2014). These laboratory observations that LPS-deficient *A. baumannii* with the higher fitness burden and more susceptible to other antimicrobials, also mirrors the literature - that a higher number of *pmrAB*-mediated colistin resistance *A. baumannii* have been reported than that of *lpx*-mutants.

Taken together, and as noted above, the acquisition of AMR generally imposes a fitness burden. Concurrently, in order to stabilize the resistant population in unfavorable environments, bacteria evolve to compensate the pleiotropic fitness effects mediated by antibiotic resistance. Unsurprisingly, some compensatory adaptations increase the fitness-cost in bacteria that can even out-compete their wide-type sensitive phenotypes, resulting in the predominance and persistence of MDR/XDR even in the antibiotic-free environments (Bjorkman *et al.*, 1998, Hall *et al.*, 2010). The knowledge of AMR mediated fitness burden and its complex interplay between bacterial genetics and both natural and antibiotic-rich

ecology, provides some insights to predict and combat the development of AMR in the pathogenic bacteria.

1.5 The main objectives of my study

To date, eight *mcr*- variants have been identified as PEA transferases, contributing to PEtN addition to lipid A and thereby conferring colistin resistance. This novel mechanism of plasmid-mediated colistin resistance provides an efficient way in distribution and evolution of colistin resistance. The worldwide emergence and epidemiology of *mcr*-mediated colistin resistance has been actively studied, a deeper understanding of how these transferable *mcr*-genes evolve and disseminate successfully among animals, the environment and human, is lacking. Furthermore, the biological and clinical role of *mcr-1*-mediated colistin resistance is still poorly understood. Thus, there are two major aims in this study:

Firstly, I seek to define the mechanism(s) of *mcr-1*-mediated colistin resistance in *E. coli* and the effects of *mcr-1* expression on the overall fitness, including morphological changes, cell viability, competitiveness and the virulence impact in an *in vivo* model. Ultimately the objectives of such work are to provide mechanistic insights into *mcr*-conferring biological costs, and evidence to debate necessary actions that might prevent the spread of colistin resistance pathogens.

Secondly, to characterize *mcr*-positive strains, including the identification of new *mcr* variants, their genetic characteristics by using whole genome sequencing, and their potential reservoirs by screening samples from various sources (food-producing animals, water, human, flies, pets and meat). This will help to expand the knowledge regarding the diversity of genetic contexts associated with *mcr* genes mainly found in *E. coli* and *K. penumonae* strains from Thailand and Vietnam, in where large amount of colistin has been consumed in food-producing animals.

Chapter 2

Materials and Methods

2.1 Bacterial strains studied and materials used in this study

Ready-made reagents supplied directly from the manufacturers are given in the main text of this chapter. Recipes for reagents made up locally are provided in **Appendix I**.

In this study, a total of 236 *mcr*-positive Enterobacteracae have been characterized. These isolates are contributed from our collaborators from Thailand and Vietnam and all details of these strains used in this study are described in **Appendix II Table 2.1**. The following are the brief methods of collecting samples from blowflies: blowflies were trapped at different areas in Northern Thailand, 100 blowflies from each area: local market in urban community, rural area and suburb of the city Phitsanulok. These locations are approximately 10 kilometers apart. The flies were individually pulverized in enriched peptone water for 30 min and then aliquots of the resultant suspensions (100 µl) were plated on Eosin-Methylene-Blue (EMB)-agar plates supplemented with 2 mg/l colistin and incubated at 37^{o}C overnight. One to three representative colonies with different species from each plates were purified and subsequently identified the *mcr-1* gene by PCR. The *mcr-1*-positive bacteria were sub-cultured in liquid nutrient broth for 18 h before DNA extraction for species identification and whole-genome sequence.

2.2 Plasmid constructs and cloning

All constructed plasmids in this study are described below. All *E. coli* strains were grown at 37 ^{o}C in LB medium unless indicated differently. The antibiotics used for selections were used at the following concentrations: Ampicillin 100 mg/l, Kanamycin 50 mg/l, Tetracycline

20 mg/l, and Chloramphenicol 25 mg/l. several strategies have been used to construct the target plasmids (the details described below).

2.2.1 *mcr-1*-plasmid construct

The *mcr-1* containing fragment was excised from plasmid pUC-19-*mcr-1*(Liu *et al.*, 2015) by double digestion with *EcoR*I and *Xba*I and T4-ligated into pSU18 and pBAD vector, respectively. The resultant constructs, pSU18-*mcr-1* and pBAD-*mcr-1*, were transformed into *E. coli* TOP10 (Invitrogen, UK). The plasmids were purified and their integrity verified by PCR and DNA sequencing.

The generation of *mcr-1*-mutation: An MCR-1 non-active enzyme was created by a single substitution (E246A). Its encoding gene was excised from *mcr-1*-pUC19 (E246A) (Hinchliffe *et al.*, 2017) and sub-cloned into plasmid pBAD-HisA to generate E246A/pBAD. To test the effect of the MCR-1 soluble domain on bacterial growth, the gene encoding the MCR1 soluble domain (lacking the five predicted transmembrane helices; codons 219–541) was cloned into pBAD-HisA vector with forward and reverse primers (*mcr-1*-F soluble and *mcr-1*-R soluble, **Table 2.1**). As a negative control, a ß-lactamase gene, $bla_{\text{TEM-1b}}$, an 861bp fragment were generated by PCR with forward and reverse primers (**Table 2.1**) and then inserted into pBAD-HisA vector using the restriction sites *EcoR*I and *Kpn*I. All resultant plasmids were transformed into *E. coli* TOP10 cell (Invitrogen, UK), purified and its integrity confirmed by PCR, double restriction digestion and DNA sequencing.

2.2.2 *mcr-3.5* plasmid construct

The *mcr-3.5* coding region with its own promoter (201 bp) and downstream sequence (36 bp) was PCR amplified from strains PN218 using primers *mcr-3.5*-F and *mcr-3.5*-R (**Table 2.2**). The fragment was purified by gel purification kit (Qiagen, Germany), digested with Pst1 and EcoRI enzymes (NEB, UK), and cloned into pUC19 and pBAD vectors. The resulting

plasmids were confirmed by PCR using the primers (*mcr-3.1*-F and *mcr-3.1*-R, in **Table 2.2**) and the following cycling conditions: 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s, followed by 1 cycle of 72°C for 7 min. The presumptive 542-bp PCR product of *mcr-3* was sent for sequencing (Eurofins Genomics, Germany).

Table 2.1 primers of gene *mcr-1* and *mcr-3* for construction, screening and qPCR

primer name	Sequence (5'->3')	length	Tm (°C)	application	reference
mcr-1-F	GCTACTGATCACCACGCTGT	958bp	60	PCR screening	(Yang *et al.*, 2017)
mcr-1-R	TGGCAGCGACAAAGTCATCT				
mcr-3.1-F	TTGGCACTGTATTTTGCATTT	542bp	50	PCR screening	(Yin *et al.*, 2017)
mcr-3.1-R	TTAACGAAATTGGCTGGAACA				
mcr-3.5-F-*pstI*	AAAACTGCAGATGTTACAATGTGGGAGTATCAG	1863bp	60	plasmid construct	This study
mcr-3.5-R-*EcoRI*	CGGAATTCCAGATGATTGGGGGCCTGA				
mcr-1 soluble-F	GGGGTACCACCATTTATCACGCCAAAGACG	969	60	PCR (plasmid construct)	This study (Yang *et al.*, 2017)
mcr-1 soluble-R	CGGAATTCGCGGATGAATGCGGTGCG				
bla_{TEM-1b}-F	GGGGTACCATGAGTATTCAACATTTTCGTGTCG	861	60	PCR (plasmid construct)	This study (Yang *et al.*, 2017)
bla_{TEM-1b}-R	CGGAATTCTTACCAATGCTTAATCAGTGAGGC				
16S-qF	CATTGA CGTTACCCGCAGAA	100bp	60	qPCR	(Spano *et al.*, 2005)
16S-qR	CGCTTTACGCCCAGTAATTCC				
16S-probe	(FAM)CGTGCCAGCAGCCGCGGTA-(TAMRA)				
rpoB Ec F3	TCCTTTCTATCCAGCTTGACTCGT	~200bp	60	qPCR	Chapter3 (Yang *et al.*, 2017)
rpoB Ec R3	CGCAGTTTAACGCGCAGCGG				
rpoB Ec probe	(HEX)ACGTCAGCTACCGCCTTGGCGAACCGGTGT-(BHQ1)				
mcr-3-qF	CGTGTTCCTATGCAGGTGTG	~150bp	60	qPCR	Chapter 4
mcr-3-qR	CGAGTATCAGCGGCTTTCTG				
mcr-3-probe	(FAM)TGCAAACACGCCATATCAACGCCT-(BHQ1)				
mcr-1-qF	TGGCGTTCAGCAGTCATTAT	~120bp	60	qPCR	Chapter 4 (Yang *et al.*, 2017)
mcr-1-qR	AGCTTACCCACCGAGTAGAT				
mcr-1-probe	(FAM)AGTTTCTTTCGCGTGCATAAGCCG-(BHQ1)				

2.2.4 Generation of site-directed point mutations in *mcr-3*-encoding sequence region

A total of six targeted mutations were generated at 1626bp mcr-3.5 sequence using the Q5® Site-Directed mutagenesis kit (NEB, UK). Primers were designed by NEBaseChanger (http://nebasechanger.neb.com/), as specified in **Table 2.2**. In brief, PCR amplicons were acquired using specific primers and a master mix containing Q5 hot start High-Fidelity DNA polymerase (Invitrogen, UK) (reaction conditions as see in **Table 2.3** and **2.4**), follows by incubated with kinase-ligase-DphI (KLD) enzyme mix. This step is designed for the circulation of the PCR product and removal of the template. Then the ligated plasmid DNA was transferred into chemically competent TOP10 cells (ThermoFisher, UK). The mutations were confirmed by plasmid extraction and sequencing.

Table 2.2 primers for point mutations in *mcr-3*-encoding sequencing region

Plasmid template	amino acid substitution (nucleotide mismatch)	primers	Resultant mutants
mcr-3.5/pBAD	M23V+A457E+T488I	See **Table 2.1**	*mcr-3.5*
mcr-3.1/pBAD	M23V(a67g)_forward	TTTTGCATTTaTGCTGAACTG	*mcr-3* (E457A+ I488T)
	_reverse	TACAGTGCCAAAAAGAAC	
mcr-3.5/pBAD	A457E (c1370a) _forward	TCACTGGGAGcATTAGGGCTTTAC	*mcr-3* (M23V+ T488I)
	_reverse	TTCACCATGATCGGAGAC	
mcr-3.5/pBAD	I488T(c1463t) _forward	CCTGGATTTAcCAAAGAGAAAGGC	*mcr-3*(V23M+ E457A)
	_reverse	TGACATCCACACCTGCAT	
mcr-3 (M23V+ T488I)	I488T(c1463t)	Primers see above	*mcr-3* (M23V)
mcr-3 (M23V+ A457E	M23V(a67g)	Primers see above	*mcr-3* (A457E)
mcr-3 (A457E+ T488I)	A457E (c1370a)	Primers see above	*mcr-3* (T488I)

* *mcr-3.1*/pBAD plasmid was provided by our collaborators in China. mismatched nucleotides are indicated in red

Table 2.3 Components of PCR conditions for the generation of *mcr-3*-positive PCR products with targeted point mutations

	Component	Final Concentration	Volume pipetted (µl)
Reaction Conditions	H_20	-	9.0
	Q5 2X Master Mix	1x	12.5
	Forward Primer	0.5µM	1.25
	Reverse Primer	0.5µM	1.25
	Template DNA	1-25ng	1.0
Template DNA	Plasmid size (bp) (same as expected PCR product size)	4100bp of pBAD backbone, plus 1826bp (with additional 200bp of promoter sequence of *mcr-3* gene)	
	Purify Method	plasmid miniprep	

Table 2.4 PCR conditions for the generation of *mcr-3*-encoding sequencing region with targeted point mutations

	Cycle	Temperature (°C)	Time (seconds)
Reaction Conditions	Initial Denaturation	98	30
	Denaturation	98	10
	Annealing	58-61*	30
	Extension	72	180 (30seconds/kb)
	Number of cycles:	25	
	Final Extension	72	120
Template DNA:	Hold	4	

*annealing temperatures are 60°C for these different primers shown in Table 2.3.

2.2.5 Construction of promoter-*gfp* fusions

The vector pGLOW-TOPO (Invitrogen, UK) was used to measure the transcription activities of *mcr* genes (*mcr-1* and *mcr-3*). The features of pGLOW-TOPO vector include a TOPO cloning site, pUC-original high copy number replication, ampicillin resistance gene and a green fluorescent protein (GFP) allowing to monitor transcriptional changes in real time. The PCR reverse primers were designed including a ribosomal binding site (-AGGA-),

an initiation codon (ATG) in frame with the GFP initiation codon and additional 8-12 nucleotides between the ribosomal binding site and the initiation codon, according to the manufacturer's instructions. primers were listed in **Table 2.5**. Promoter regions were amplified by PCR using Supermix high fidelity (Thermo Fisher Scientific, UK), and confirmed by agarose gel electrophoresis. The amplified PCR products were ligated with the pGLOW-TOPO vector at room temperature for 10-15 min and transferred into chemical competent *E.coli* TOP10 cell (Invirtogen, UK), according to the manufacturer's instructions. The orientation of the inserted promoter was confirmed by PCR and sequencing using the upstream GFP primer. Overnight cultures were used to detect i*n-vitro* GFP Fluorescence by flow cytometer (Becton Dickenson, Biosciences, UK).

Table 2.5 primers used for promoter-*gfp* constructs

primer name	Sequence (5'-3')	length	Tm (°C)
P_mcr-1-F	GCCCACATATTTGCGGGCTTAA	500bp	60
P_mcr-1-R*	TCATAGATCCTTTC**TCCT**+GAGAAACTAC TCAAAAAATAAACGG		
P_*mcr-3*-F	TACTGGTCGGAGATATGGGT	300bp	60
P_mcr-3-R*	TCATAGATCCTTTC**TCCT**+ ACTTACTCCATTAATAGTCCAAC		

*Underlined sequence are the additional nucleotide and a reverse RBS site –TCCT- (in bold), followed by promoter sequence.

2.3 plasmid DNA extraction

Plasmid DNA was extracted from 5 ml bacterial cultures, grown to stationary phase in LB, using the QIAprep Spin Miniprep Kits (Qiagen, Germany), according to the manufacturer's specifications, and eluted in 30-50 µl sterile water.

2.4 Polymerase Chain Reaction (PCR)

Amplification of DNA, for cloning or mutagenesis, was carried out using the Thermocycler high Fidelity PCR system (ThermoFisher, UK), according to the manufacturer's specifications. A total volume of 12 µl in a reaction contained 1xPCR master mix, 0.1 µM forward primer and 0.1 µM reverse primer and 1 µl DNA template (sequence of primers used in this study are list in **Table 2.1, -2.2** and **-2.6**). Thermocycler parameters varied depending on the amplicon size and GC content. A typical PCR cycle was: denaturation at 95 ˚C for 5 min and then 30 cycles of denaturation at 95 °C for 30 sec, annealing at 50 ˚C for 30 sec, extension at 72 ˚C for 30s-60s per kb of amplicon, final extension at 72˚C for 10 min. PCR products were separated via electrophoresis through 1.5% agarose dissolved in 1xTris-borate EDTA buffer (TBE) gels and visualised with Ethidium Bromide Stain (Invitrogen, UK) at a dilution of 1:10 000, using a Geneflash UV transilluminator (Syngene, Bangalore, IND). Fragment size was determined using SmartLadder (Eurogentec, UK) as a marker.

After gel electroporation, DNA can be purified using gel purification kit (Qiagen, Germany), according to the manufacturer's specifications. The purified DNA can be further used for Sanger sequence (Eurofins, Germany) and DNA-probing (see 2.17.3).

2.5 Reverse-Transcription quantitative PCR (RT-qPCR)

To optimize the expression of *mcr-1* gene, I tried a series concentration of L-arabinose over a 10,000-fold range (0.00002% to 0.2%). Each transformant or control was inoculated in 2 ml of LB medium containing 100 µg/ml ampicillin with a single recombinant *E. coli* colony for overnight at 37°C with shaking (225 r.p.m.). The next day, 10 µl of overnight culture were added unto fresh 10 ml of LB containing 100 µg/ml ampicillin. When OD_{492} reached to approximate 0.4 (the cells normally reach in their mid-log phase), 10-fold serial

dilutions of 20% (w/v) L-arabinose (e.g., 2%, 0.2%, 0.02%, and 0.002%) were added to the five 10 ml cultures as follows, to induce the expression of *mcr-1* gene and then grow at 37°C with shaking for 6-8 hours.

After for 6-8h incubation, the bacterial pellets were collected and total RNA was extracted using the RNeasy Plus kit with on column DNase digestion (Qiagen, Germany), according to manufacturers' protocol. RNA was quantified by measurement at OD_{260}/OD_{280} using a Nanodrop (Thermo Scientific, UK), followed by cDNA synthesis with DNA integrated genomic DNA removal using QuantiTect Reverse Transcription kit (Qiagen, Germany), according to manufacturers' protocol. Quantitative PCR amplification was performed on the 150-200 ng of cDNA cDNA using SsoAdvanced universal probes supermix (Bio-Rad, Hemel Hempstead, UK) on the Bio-Rad CFX384 (Corbett Research, Sydney, AUS) with the necessary primer pairs and the control primers specific to the *rpo*B housekeeping gene (Table 2.1). A typical reaction mix contained 10 µl of SsoAdvanced universal probes supermix, 0.5 mM of each primer, 0.2 mM of each probe and 100-300 ng of cDNA in a total volume of 20 µl in triplicate. The absence of carry-over genomic DNA was verified for every experiment by comparative qPCR (or standard PCR) in the absence of reverse transcriptase. Typical parameters for the PCR amplification were as follows: 2 min denaturing at 95 ˚C, 40 cycles of 10 sec denaturing at 95 ˚C and 30 sec annealing at 60 ˚C. Analysis was based on comparative quantitation of a treated sample and the control mRNA levels were normalized to the control gene, rpoB. Relative expression results were obtained by the ΔΔCT analysis method using mean CT value. For details of used primers and probes, see **Table 2.1**.

2.6 *mcr-1* gene copy number measured by qPCR

DNA extraction as stated above. For qPCR determination of *mcr-1* copy numbers per cell, 0.1 ng of total genomic DNA was used as template with primers *mcr-1*-qF, *mcr-1*-qR and

mcr-1 probe and 16S primers and probe (**Table 2.1**). In parallel standard curves for *mcr-1* and 16s were obtained using as template serial dilutions of *mcr-1*-carring DNA extracted from pSU18-*mcr-1* strain (Hinchliffe *et al.*, 2017) (4.3 pg of DNA corresponding to 10^6 copies, calculated through the website: http://cels.uri.edu/gsc/cndna.html) and *E. coli* TOP10 (Invitrogen, UK) total genomic DNA (5 ng corresponding to 10^6 cells)(Bontron *et al.*, 2016), respectively.

2.7 *E. coli* multi-locus sequencing typing (MLST)

The *E.coli* MLST scheme used in this study was available at Warwick Medical School (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The following seven housekeeping gene were used in this scheme: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate synthetase) and *recA* (ATP/GTP binding motif). The primers sequences are listed in **Table 2.6**. The reaction condition were an initial denaturation step at 94^{o}C for 2 min, followed by 35 cycles of the following conditions: denaturation at 94^{o}C for 2 min, annealing at 58 ^{o}C (for *gyrB*, *mdh* and *recA*) or 54 ^{o}C (for *adk*, *fumC*, *idc* and *purA*) for 1 min and extension at 72 ^{o}C for 1 min, with the final extension step at 72 ^{o}C for 10 min. The size of PCR products were confirmed by agarose gel electrophoresis, followed by gel purification using gel purification kit (Qiagen, Germany), according to the manufacturer's instructions. The seven housekeeping genes were confirmed by sequencing (Eurofins genomics, Germany). The allelic profile was summarized by the assignation of an ST via the online database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/).

Table 2.6 The list of primers used for MLST typing

primers	primer's sequence (5'-3')	Length	Tm
adk-F	ATTCTGCTTGGCGCTCCGGG	583 bp	54° C
adk-R	CCGTCAACTTTCGCGTATTT		
fumC-F	TCACAGGTCGCCAGCGCTTC	806 bp	54° C
fumC-R	GTACGCAGCGAAAAAGATTC		
gyrB-F	TCGGCGACACGGATGACGGC	911 bp	60° C
gyrB-R	ATCAGGCCTTCACGCGCATC		
icd-F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878 bp	54° C
icd-R	GGACGCAGCAGGATCTGTT		
mdh-F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932 bp	60° C
mdh-R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT		
purA-F	CGCGCTGATGAAAGAGATGA	816bp	54
purA-R	CATACGGTAAGCCACGCAGA		
recA-F	CGCATTCGCTTTACCCTGACC	780 bp	58° C
recA-R	TCGTCGAAATCTACGGACCGGA		

2.8 Antimicrobial susceptibility testing

2.8.1 Agar Dilution Method

Minimal inhibitory concentrations (MICs) of antibiotics were performed by the agar dilution method, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST). Briefly, organisms were inoculated on agar plates and adjusted the turbidity of bacterial suspensions reached the McFarland 0.5 Standard (approximately $1.5x10^8$ CFU/ml) for the tests. Mueller-Hinton (Becton Dickinson, USA) agar plates supplemented with different concentration of colistin in a range of 0.125 -128 mg/L. The strain *E. coli* (ATCC 25922) used for quality control purposes. Isolates were classified as either susceptible or resistant according to the interpretative standards recommended by the EUCAST clinical breakpoints.

2.8.2 Broth Microdilution Method

The colistin susceptibility of tested *E.coli* isolates was assessed by broth microdilution according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Briefly, overnight cultures were resuspended to an optical density corresponding to a 0.5 McFarland standard (approximately $1.5x10^8$ CFU/ml). Briefly, overnight cultures were diluted in sterile saline to an optical density corresponding to a 0.5 McFarland standard (approximately $1.5x10^8$ CFU/ml), and then the standardised cell suspensions were aliquoted to 96-well microtiter plates containing cation-adjusted Mueller-Hinton medium (Becton Dickinson, USA) at colistin concentrations from 0.25 to 128 mg/L. Plates were incubated overnight at 37°C and optical density read on a microplate reader (EZ Read 400, BIOCHROM, UK.) at 492nm.

2.9 The transferring procedures of *mcr*-bearing plasmids

2.9.1 Heat shock transformation of Chemical competent bacteria

One vial of 50 µl TOP10 cells (ThermoFisher Scientific, UK) was thawed on ice for each transformation. 100 ng of each constructed plasmids/ligation reaction were added directly into the vial of competent cells and mixed by tapping gently. The remaining ligation mixture(s) can be stored at -20°C. Then, the mixture was incubated for 30 minutes on ice, followed by heat shock in the 42°C water bath for 45 s and placed them on ice immediately for 2-3 minutes. 950 µl of pre-warmed LB broth were then added to each vial and incubated at 37°C for 1 hour at 200 r.p.m. in a shaking incubator. 100 µl cultures from each transformation vial are spread on selective agar plates and incubated at 37°C overnight. The transformants were confirmed by plasmid isolation, PCR and sequencing.

2.9.2 Electroporation

(i) Preparation of electrocompetent cells. Overnight cultures were diluted (1:50) in 5 ml fresh LB broth and incubated at 37°C (220 r.p.m) until the OD600 of cultures are reached to 0.5-0.7. Bacterial cell cultures were kept on ice for 20 minutes, followed by centrifugation at 5000 r.p.m/min, 4°C for 15 min. The pellets were then re-suspended in 1ml chilled 10% glycerol and re-centrifuged for 15 min at 4°C. After another twice washes in chilled 10% glycerol, supernatants were discarded and the pellets were suspended in the residual glycerol (about 100 µl). The electrocompetent cells are ready to use for electroporation.

(ii) Electroporation programme. 0.2 cm electroporation cuvettes (Bio-Rad, UK) must be kept on ice for at least 30min before use. 50-100 ng of plasmid DNA were added into 50 µl of electrocompetent cells as described above and then the DNA/cells mixture were transferred into a chilled cuvette without introducing bubbles. The following conditions were used for electroporation (Bio-Rad MicroPulser, France): EC3/EC2 (3.0/2.5kv) programme was used for *E. coli*, the typical time constant is ~5.5 milliseconds. Immediately 950 µl of LB broth were added to the cuvette and mixed well, followed by incubation at 37°C for 1 hour, shaking vigorously at 220 r.p.m. 100 µl of aliquot were plated onto a pre-warmed selective plate and incubated overnight at 37°C.

2.9.3 Conjugation experiments

To investigate the transferability of *mcr*-like-carrying plasmids, we performed conjugation assays with sodium-azide resistance *E.coli* J53 as the recipient strains. Briefly, overnight cultures of MCR-producing donors and the recipient strain were 1:2 or 1:4 mixed and incubated in 37^{o}C without shaking for 16-20 h. After incubation, we subsequently ten-fold serial diluted the mixed culture in sterile saline and aliquoted 100 µl of diluted culture onto selective agar plates containing with 2-4 mg/l colistin and 150-200 mg/l Sodium Azide. The *mcr*-positive transconjugants were confirmed by PCR and transfer frequency was calculated

by the number of transconjugants per recipient (equations see below). Plasmid analysis were done by whole genome sequence as described below.

$$CFU/ml = \frac{(Number\ of\ colonies)}{dillution\ factors * (volume\ of\ aliquot)}$$

$$conjugation\ frequency = \frac{\text{CFU of transconjugants}}{\text{CFU of recipients}}$$

2.10 Selection for high-level colistin resistant mutants (HLCRMs)

HLCRMs were generated from seven wild-type *mcr-1* positive strains through 14-day serial passaging with increasing concentrations of colistin (Alfa Aesar, USA). Briefly, overnight cultures were resuspended to an optical density (OD) corresponding to approximately 1.5x10^8 CFU/ml, followed by challenging with different concentrations of colistin (from 0.125 to 256 mg/L) in a 96-well microtiter plates containing cation-adjusted Mueller-Hinton medium (Becton Dickinson, USA). The next day, cultures in the last well that yielded visible bacterial growth were mixed with the first clear well (normally registered as the MIC) and challenged with colistin as described above. This passage lasts for 14 days and cultures at 0, 3, 7 11 and 14 days were retained and stored at -80^{o}C for further analysis.

2.11 Stability of high-level colistin resistance in HLCRMs

To detect whether the colistin resistance in HLCRMs is stable i.e. reversible or not, serial passages of HLCRMs were performed in colistin-free medium. Overnight cultures of HLCRMs were diluted (1:500) into fresh LB broth without colistin and incubated with vigorous shaking (220 r.p.m.) for 18h. To measure the proportion of colistin resistance bacterial population during reversion, overnight cultures were serial diluted, then plated on

chromogenic agar plates with or without colistin. Various concentrations of colistin (8, 16, 32, 64, 128, 256 mg/l) were depending on the level of colistin resistance mediated by the HLCRM. The CFU/ml of colistin resistance cells were counted after 18-22 h incubation at 37^{o}C.

2.12 Morphological analysis by transmission electron microscopy (TEM)

Overnight cultures were diluted into 50 ml of fresh media supplemented with 100 mg/L ampicillin for *mcr-1*/pBAD. For *mcr-1*/pBAD strain, 0.2% (w/v) of L-arabinose was added to induce the overexpression of *mcr-1*. After 8h incubation, samples were fixed by addition of glutaraldehyde to the broth to a final concentration of 1%. Bacteria were harvested by collection onto 0.45 mm pore filters, gently scraped off and dispersed in 4% low melting point agarose at 50^{o}C. Preparations were allowed to gel at room temperature and cut into 1 mm cubes. Cubes were post-fixed for 2 hours in 2% uranyl acetate, washed for 3 x 20 minutes in reverse osmosis purified water and dehydrated through graded propan-2-ol (50%, 70%, 90% for 10 minutes each, 100% for 2 x 15 minutes), infiltrated with LR White acrylic resin (London Resin Company, Aldermaston, U. K.) (50% in propan-2-ol 30 for minutes, neat resin for 4 x 20 minutes) placed in size 0 gelatine capsules with fresh resin and heat polymerised overnight at 50^{o}C. Thin (80 nm) sections were cut on an Ultracut E.ultramicrotome with a glass knife and collected onto 300 mesh copper grids, stained with lead citrate and examined in a Philips CM12 (FEI U. K. Ltd. UK) TEM at 80kV. Digital images were captured with a Megaview III digital camera and AnalySIS (Soft Imaging System GmbH, Germany).

2.13 Methods used for *in-vitro* competition assays

2.13.1 Bacterial competitiveness measured by flow cytometry

In-vitro competition experiments were used to measure the relative fitness of the *mcr-1/*pBAD HLCRMs, *E. coli* TOP10 (*mcr-1*/pBAD) and *E. coli* TOP10 (pBAD only) and *E. coli* TOP10 (**Table 2.8**). These strains were competed against a GFP-labelled *E. coli* DH5-α carrying plasmid pHT315-pAphA3'-gfp for constitutive expression (Daou *et al.*, 2009). All competitions were carried out in M9 medium (SIGMA-ALDRICH, UK) (**Table 2.9**) with six biological replicates per strain/condition, as previously described with some modifications(San Millan *et al.*, 2016). For *E. coli* TOP10 (*mcr-1*/pBAD), *mcr-1* expression was induced by adding different concentrations of L-arabinose (0, 0.0002, 0.002, 0.02 and 0.2%, w/v).

Table 2.7 Strains and plasmids used in this assay

plasmids	Strains list				
mcr-1/pBAD plasmid*(w/v)	*mcr-1* 0.0002%	*mcr-1* 0.002%	*mcr-1* 0.02%	*mcr-1* 0.2%	*mcr-1* 0%
mcr-1/pBAD mutants	*mcr-1* D0	*mcr-1* D3	*mcr-1* D4	*mcr-1* D8	*mcr-1* D14
Control strains	TOP10	pBAD	pHT315-GFP DH5-α		

* indicated the concentration of L-arabinose used to induce *mcr-1* expression

Table 2.8 preparation of M9 medium

Components*	Volume (ml)
Sterile distilled water	492
0.5M Magnesium (0.22uM filter)	2
Total volume	500

*Normally, M9 salts contain sterile distilled water, magnesium and glucose. But glucose in this experiment has been removed, because it may inhibit inserted gene GFP expression.

(i) Preparation for initial mixture. The bacteria were cultured overnight in LB broth (SIGMA ALDRICH, UK), supplemented with the appropriate antibiotics (2 mg/l of colistin for HLCRMs, or 100 mg/l of ampicillin for *E. coli* TOP10 (*mcr-1*/pBAD), *E. coli* TOP10 (pBAD) and the GFP-labelled *E. coli* DH5-α. The overnight cultures were diluted 1:400 in M9 broth and mixed at 1:1 ratio with GFP-labelled cells. Before starting the competitions, the exact initial proportion of fluorescent/non-fluorescent cells was estimated using flow cytometry (for details see below). Briefly, overnight cultures were firstly diluted into M9 salts (1:200) and then their cell density (events/µl) were measured Accuri C6 flow cytometer (Becton Dickenson, Biosciences, UK). If the events/µl is higher than 3000, need to re-dilute samples to 1000-3000 events/µl. This measurement is more accurate than that using microplate reader. In order to get 1:1 mixture of the control and samples, a rough guideline to follow for *E.coli* as below: (for instance, the highest density was considered as template, 500 µl as a volume of the control)

Volume (µl) of sample = [events/µl (template)*500 µl]/ events/µl (sample)

Finally, if the actual ratio was close to 1:1, we started the competition by diluting the initial mixtures into LB broth (1:2) and shifting the mixtures to a shaking incubator (MAXQ 8000, Therom Scientific, UK) in 37°C, 225 r.p.m. Otherwise, the preparation procedure was repeated.

As with all experiments, good controls are essential. Performing a competition between a tested strain and a GFP-tagged cheater strain, a pure culture of each as controls are needed. This is because the "fluorescence intensities" of tagged and untagged strains have a small region of overlap (see **Fig.2.1**). The pure cultures must be present so that mixtures can be corrected for this overlap and yield more accurate results.

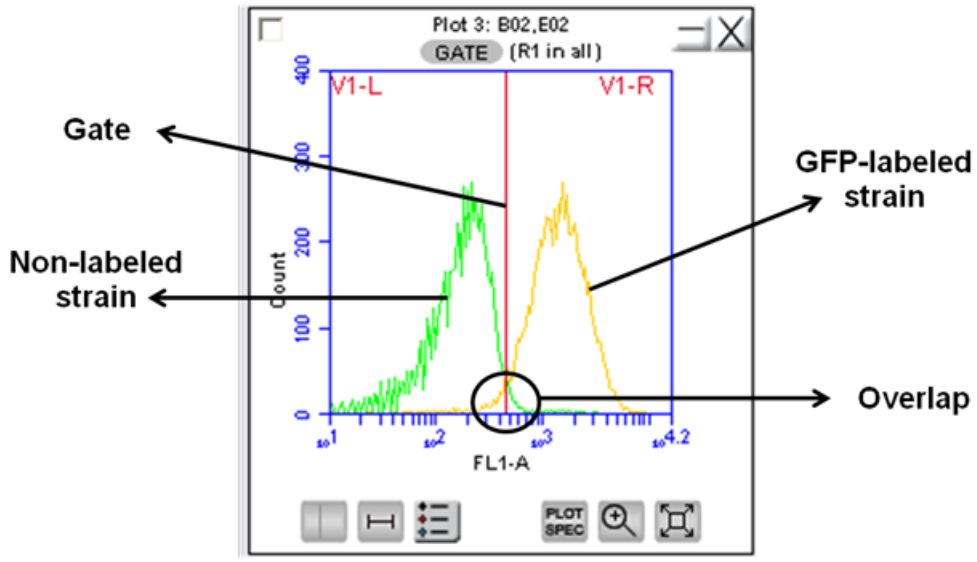


Fig. 2.1 Controls used in a competition experiment between a GFP-labelled strain and a non-labelled strain (tested strain). There is a small region of overlap which can be corrected for mathematically.

(ii) Data collection. After 22h, the competed bacteria were diluted 1:400 in Nunclon^{TM} Delta Surface 96-well plates (Therom Scientific, UK) with M9 medium and analyzed on a flow cytometer to estimate the resulting proportion of labelled versus unlabelled cells. Flow cytometer was performed on an Accuri C6 (Becton Dickenson, Biosciences, UK). The cell densities were adjusted to give approximately 1000-3000 events per 1uL. During data acquisition, a lower cut off was set at 10 000 for FSC-H and at 8000 for SSC-H. For each competition, we ensured that the GFP-labelled strain can be well separated from non-fluorescent strains by comparing non-mixed controls (overlap is usually less than 2% of the cells). Relative fitness was calculated using formula:

$$Relative\_fitness = \frac{\log_2(\frac{p_1}{p_0/n_{dilution}})}{\log_2(\frac{1-p_1}{(1-p_0)/n_{dilution}})}$$

where p_0 is an initial proportion of an unlabelled stain, and p_1 is a final proportion of an unlabelled stain after competition. The $n_{dilution}$ is the factor, which reflects a fold difference in

cell density at the beginning and at the end of the competition. For HLCRMs, we expressed the fitness of daughter strains relative to their parental strains (i.e $f_{daughter}/f_{parental}$) and followed the procedure of error propagation to account for the uncertainty of the two estimates:

$$SE = \sqrt{\left(\frac{SD_{daughter}}{\bar{f}_{daughter}}\right)^2 + \left(\frac{SD_{parental}}{\bar{f}_{parental}}\right)^2}$$

where $\bar{f}$ and SD are a mean estimate and its standard deviation for each corresponding strain based on 6 replicates. Similarly, the relative fitness of the HLCRMs at different L-arabinose concentrations was represented as the relative fitness at no induction (no L-arabinose). The differences in fitness were tested using non-parametric Mann-Whitney test, and the p-values were adjusted by Bonferroni method.

2.14.2 Bacterial competitiveness measured by qPCR

2.14.2.1 mcr-carrying plasmids competition in E.coli J53 model

To examine the stability and competitive ability of *mcr-1*- and *mcr-3*- carrying plasmids, the qPCR was used to measure changes in the abundance of *mcr* genes over 14-day passage. Two representative *mcr-1* (PN23, IncX4) and mcr-3(F203, IncF) plasmids were transferred into the same recipient genetic background (*E.coli* J53) by conjugation. Each competition was initiated with 1:1 mixture (equal CFU/ml) of *mcr-1* and *mcr-3.5* plasmid bearing bacteria in 5ml tubes containing 3 ml of culture medium. Competitions were maintained for at least 80 generations of growth by serially transferring 1% of the overnight mixtures to fresh medium with or without colistin (2 mg/l) for 14 days. The negative controls, *mcr-1* or *mcr-3*-plasmid monocultures, were also processed in the same manner. In total, the experiment have the following six treatments (biological triplicates were performed):

(a) control mono-cultures

(1) *mcr-1* transconjugant (PN23), no colistin

(2) *mcr-1* transconjugant (PN23), 2 mg/l colistin

(3) *mcr-3* transconjugant (F203), no colistin

(4) *mcr-3* transconjugant (F203), 2 mg/l colistin

(b) mixed cultures

(5) Mix of *mcr-1* transconjugant (PN23) and *mcr-3* transconjugant (F203), no colistin

(6) Mix of *mcr-1* transconjugant (PN23) and *mcr-3* transconjugant (F203), 2 mg/l colistin

gDNA was extracted from overnight culture (2 ml) using a QIAcube automated machine (Qiagen, Germany) at the following time points: day 0, 1, 3, 5, 8, 11, 14. The concentrations of gDNA were measured by a Qubit (ThermoFisher Scientific, UK). This is followed by measuring the dynamics of the *mcr-1* and *mcr-3* genes by qPCR (ABI StepOnePlus). 1 ng of gDNA was used as template with specific primers and probes (**Table 2.1**). A housekeeping gene *rpoB* was used as internal control using primers *rpoB-qF*, *rpoB-qR* and *rpoB* probe. Biological triplicates were performed and the ΔΔCt method were used to compare the relative abundance between *mcr-1* and *mcr-3* genes in the mixture:

$$[1]\Delta Ct\ (mcr\ genes) = Ct(\textit{mcr-3 gene})\text{-Ct}\ (\textit{mcr-1 gene})$$

$$[2]\Delta Ct\ (ropB\ genes) = Ct(\textit{mcr-3 sample})\text{-Ct}\ (\textit{mcr-1 sample})$$

$$[3]\Delta\Delta Ct = \Delta Ct(target\ gene) - \Delta Ct(reference\ gene)$$

Where Ct is the threshold cycle, target genes are *mcr-1* and *mcr-3*, *rpoB* is the control to normalize the target gene expression. Target is mcr-1 and reference is mcr-3 gene. The fold change of genes were considered statistically enriched if $p<0.01$ using linear regression analysis. If there was no amplification, detection limit Ct 40 was used. The relative fitness of mcr-3 and mcr-1 plasmid were then estimated by the regression coefficient of the log value of

the ratio of mcr-3 to mcr-1 plasmid plotted against time (generation of competition). The bacterial generation time is calculated by the below formula:

$$\text{Generation Time} = \log(2)\ (dilution\ factor\ on\ bacterial\ passasing)$$

(One condition for this formula is bacteria need to grow to saturation condition in everyday passaging. 1000-fold dilution I have used in this passaging, which means that 10 bacterial generations was achieved per day).

2.14.2.2 *mcr*-carrying Plasmids competition in a clinical strain model

Co-existence of *mcr-1* and *mcr-3* plasmids were found in MCRPE collection in this study, and they are often located in different plasmids, which have been confirmed by conjugation and S1-PFGE. I have selected three of them as my wild-type competition model and examined the stability and competitiveness of both *mcr-1*- and *mcr-3*-carrying plasmids in the presence/lackness of colistin, the qPCR was used to measure changes in plasmid abundance over 14-day passage as above describe with slightly differences. Briefly, each competition was initiated with equal optical density in 5ml tubes containing 3 ml of culture medium. Competitions were maintained for at least 100 generations of growth by serially 1:1000 dilution of the overnight mixtures to fresh medium with (4 mg/l) or without colistin for 14-day serial passaging. Biological triplicates were performed.

2.15 LPS isolation and macrophage stimulation

2.15.1 LPS extraction

LPS was extracted using LPS extraction kit LPS was extracted from induced *E.coli* TOP10 *mcr-1*/pBAD strain and control *E.coli* TOP10 pBAD strain, using LPS Extraction Kit (iNtRON Biotechnology, UK), according to the manufacturers' instructions. Briefly, the

bacteria were cultured overnight in 5 ml of LB broth, supplemented with 100 mg/L of ampicillin, and for *mcr-1*/pBAD strain, *mcr-1* gene was induced by 0.2% arabinose for approximately 12h. In order to eliminate contamination of protein, treatment with protease K (20 mg/ml, AppliChem, Germany) was performed prior to the extraction steps. Bacterial cultures were spun down at 13,000r.p.m at room temperature. Supernatants were removed and resuspended pelleted cells by adding 1 ml of Lysis Buffer, followed by adding 200 ml of chloroform and mixing well. The mixtures were incubated at room temperature for 5min. The purpose of adding the chloroform is to separate the phenol layer from aqueous layer and eventually isolate RNA and genomic DNA/protein. Then, 400 ml of supernatant were transferred to new 1.5 ml tube after centrifuged at 13,000r.p.m for 10 min at 4℃. In order to purify LPS from other extract of cell, such as protein, nucleic acids and lipids, 800 ml of purification buffer was added into supernatants and incubated for 10-20 min at -20℃. After centrifuging the solution at 13,000r.p.m for 15 min at 4℃, 1 ml of 70% EtOH was added to wash the LPS pellet to remove impurities such as salts. The upper layer was discarded after centrifuging the mixtures for 3 min at 13,000r.p.m at 4℃. Final purified LPS pellet was dried at room temperature and dissolved in 30-50 ml of 10mM Tris-HCl buffer (pH 8.0) by boiling it for 2 min. LPS was stored at -20℃ for further experiments.

2.15.2 The quantity of LPS samples

To eliminate protein contamination, treatment with protease K was performed after the extraction steps. The protease K was added to the cell mixture and incubated at 56^{o}C for one hour. The potency of LPS samples was determined by Limulus Amebocyte Lysate (LAL chromogenic Endotoxin Quantitation kit, ThermoFisher, UK) to measure the LPS concentration, according to the manufacturers' instructions.

2.15.3 Propagation of THP-1 cell lines

THP-1 cell lines are monocyte-like cell line derived from leukemia from a one year old boy (American Type Culture Collection, ATCC TIB-202™). The cells are harvested with the complete medium (R.P.M. I 1640 medium supplemented with 10% FBS, 2 mM L-Glutamine, 1 mM sodium pyruvate, 100 mg/l penicillin and 100 mg/l streptomycin, in order to inhibit bacterial contamination) in a humidified cell culture incubator with 5% CO_2 at 37°C. Cell media can be changed when cell concentration reaches around $8x10^5$ cells/mL by adding 1ml cells into 9ml of fresh media. THP-1 monocytes were differentiated into macrophage cells by stimulation with phorbol myristate acetate (PMA). In brief, cells were centrifuged and resuspended in fresh media to a concentration of $2x10^5$ cells/ml. PMA (10 ng/ml) was added to diluted cells, and then aliquoted into 24-well plate (ThermoFisher, UK) by adding 1 ml of cell suspension per well. The differentiation of THP-1 monocytes were reached after 48 hours of incubation. Differentiated cells were adherent to the bottom of plate observed by 10X objective microscope, while undifferentiated cells were removed by discarding the cell media.

2.15.4 LPS stimulation

To prepare the 24-well assay plate, 1 mL of macrophage cell cultures was first added to every well followed by adding serial concentrations of LPS (4.5, 0.45, 0.045, 0.0045, 0.00045 ng/mL) to the appropriate wells in duplicate/triplicate. Macrophage cell cultures without LPS stimulation served as the negative controls. The wells are thoroughly mixed and incubated at 37 °C for 24h. The samples were collected at 4, 6, 8 and 24h and stored at -20°C. The production of macrophage-derived cytokines (IL-6 and TNF-α) were analyzed using DuoSet® ELISA kit (R&D systems, UK), and cytokine concentrations were calculated according to the manufacturers' instructions.

2.16 *Galleria mellonella* pathogenicity model

In vivo pathogenicity of seven wild type MCRPEC isolates and their corresponding mutants were evaluated using *G .mellonella* infection model. The wax moth *G.mellonella* in larval stage (Live Foods UK Ltd, http://www.livefood.co.uk) were stored in dark and used within three days from shipment. Prior to inoculation into larvae, bacterial pellets were washed with sterile saline and then diluted to an appropriate cell density (OD_{600} is approximately 0.6). Using a 50 µl Hamilton Syringe, 10 µl aliquots of serially diluted bacterial suspension (from 10^3 to 10^7 CFU/ml) were injected into the hemocoel of each larvae, through the rear left pro-leg (Peleg *et al.*, 2009). A group of ten larvae were randomly chosen to inject for each level of inoculation in triplicate. Followed by injection, larvae were incubated at 37ºC, and the survival of larvae was monitored daily for three days. Death was denoted when larvae no longer responded to touch. Results were analyzed by Kaplan-Meier survival curves (GraphPad Prism statistics software). For all experiments, three control groups were used: ten larvae in first groups were injected with 10 µl sterile saline, the second group included larvae that received empty stabbed to test if killing caused by physical trauma, and the larvae in third group with no injection. In all case, no dead larvae were observed in the control groups.

2.17 S1-nuclease based pulsed-field gel electrophoresis (S1-PFGE)

To investigate whether *mcr*-genes were located on plasmids or chromosome, endonuclease S1 pulsed-field gel electrophoresis (S1-PFGE) were assayed as previously described (Yong *et al.*, 2009, Toleman, 2018). In-gel hybridization was done with a *mcr*- probes labelled with ^{32}P (Stratgene, Amsterdam, Netherlands) with a random primer method according to manufacturers' instruction. The following is the step-by-step procedure for S1-PFGE

contributed by our colleague Dr. Toleman Mark (Toleman, 2018) with minor modifications and ingredients of solutions are provided in **Appendix 1.2**.

2.17.1 Agarose plugs preparation

Agarose plugs of bacterial strains (for *E. coli* only in this study) are prepared by using standard operating procedure, which is available on the Centres of Disease Control website: (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). First of all, tested isolates were streaked onto UTI plates and incubated at 37 ^{o}C overnight. Enough amount of bacterial pallets (usually one full 10µl- inoculating loop) were suspended them in 1 ml cell suspension buffer and mixed well. Then 400 µl of each cell suspension were transferred into a new 1.5ml Eppendorf tube, followed by adding 20 µl of Proteinase K solution (20mg/ml) into each Eppendorf tube. Then equal volume (400 µl) of 1% Seakem Gold agarose (Fisher Scientific, UK) were gently mixed into cell suspension and dispensed the mixture into plug mold immediately. After 15-20 minutes (when plugs are solid), they are placed into 5ml cell lysis buffer supplemented with 25 µl Proteinase K solution (20mg/ml, final concentration is 0.1 mg/ml). Plugs were digested in a 54-54oC shaker at 200 r.p.m. After for 2 h incubation, lysis buffer was discarded and plugs were washed by sterile water twice in 54-54 ^{o}C shaker for 10-15 minutes each time, and subsequently washed in pre-heat 1X TE buffer for another four times in 54-54 ^{o}C shaker for 10-15 minutes each time. After washing procedures, plugs can be stored in TE buffer with 1mM EDTA at 4 oC until needed.

2.17.2 S1 enzyme digestion

Plugs are placed into a 48-well plate individually, then 300 µl of 1/10 TE buffer was added and incubated at room temperature for 20 minutes, followed by adding 200 µl of 1X S1 buffer at room temperature for 20 minutes. After washing procedure, the S1 buffer is removed and 150U S1 enzyme (Invitrogen, Abingdon, UK) is added into each plug for 45

minutes at 37^{o}C, or keep in the 4 ^{o}C overnight. After digestion, each plug is cut in one-third, and then added to the gel. Ethidium bromide (20 µl of 10 mg/ml solution) is added to the gel running buffer 0.5X TBE. Electrophoresis was conducted on a CHEF-DR III apparatus (Bio-Rad, Hercules, CA, USA) under the following conditions: 6 V/cm at 14 ^{o}C, with an initial pulse time of 5 s and a final pulse time of 45 s for 18 h. The results are visualized after gel running on a UV trans-illuminator and photographed using a UVP geldoc II imaging system (UVP Cambridge, UK). Plasmid number and sizes are determined by comparison with the molecular size marker (PFG ladder, NEB, UK).

2.17.3 In Gel Hybridisation

Gels are dried by placing in a drying cabinet at 50 ^{o}C overnight between 2 sheets of blotting paper. Once dried, gels are re-hydrated by placing in 200 ml of deionized DNase free water in a flat-bottomed pyrex glass bowl. After 5 minutes, 200 ml of denaturing solution is added to denature the DNA in the gel at room temperature for 45 minutes, followed by adding 200 ml of neutralising solution and incubated for 45 minutes at room temperature. Once the neutralising solution discarded, the gel was placed in a hybridisation tube with 20ml of pre-hybridisation solution and incubated at 65 ^{o}C for 12 h, in order to block the gel before probing.

The probe is prepared by the random priming labelling method using the purified PCR product (ideally PCR product with 800-1000bp and 200ng in 15µl sterile water) (Prime-It II Random Primer Labelling Kit, Agilent Technologies, UK) and radio-active P^{32} labeled dCTP (PerkinElmer, London, UK), according to the standard protocol provided by the manufacture. Once the probe has been labelled, unincorporated dCTP32 and unlabelled nucleotides are removed using a sephadex G50 gravity flow gel filtration column (illustra^{TM} Nick^{TM} Columns Sephadex G-50 DNA grade, GE Healthcare Life Sciences, UK.): in brief, 60 µl of labelled probe is added to the gel filtration column, followed by adding 320 µl of 0.1M Tris buffer

(pH7.5). A new Eppendorf tube is then placed under the column and the labelled DNA is eluted with 430 µl of 0.1M Tris buffer. The labelled probe is then boiled for 6 minutes and then added to the pre-hybridised gel and left to hybridize at 65 ^{o}C overnight. Once hybridised, the probe is discarded and the gel is washed for 1 hour at 65 ^{o}C with 2X SSC 0.1% SDS (100mls) and the second washing is with 100mls of 0.1X SSC, 0.1% SDS at 65 ^{o}C for 1 hour. Once washed, gels are finally removed from the hybridisation tubes, washed with warm tap water for 1-3 minutes and blotted dry with blotting paper. They are then wrapped in clingfilm and placed against a sheet of Lumi-Film Chemiluminescent Detection film (Roche, Mannheim, Germany), in a film cassette for at least 24 hours before developing using standard film development and fixer solutions.

2.18 Confocal Laser Scanning Microscopy imaging using LIVE/DEAD® staining

E. coli TOP10 (*mcr-1*/pBAD), *E. coli* TOP10 (pBAD only) and *E. coli* TOP10 (n=4) were grown overnight in LB broth supplemented with 100 mg/L ampicillin (Fisher Chemical, UK) at 37°C (220 r.p.m.). Overnight cultures were standardised to OD_{600} 0.05 and inoculated (1:10; v/v) into 96-well glass-bottomed plates (Whatman®, UK) in LB broth for 16 h (37°C; 50 r.p.m.). The supernatant was gently removed and the biofilms were further incubated in fresh LB broth ± L-arabinose (0.2%, w/v) for 8 h. The supernatant was removed and the biofilms stained with 6% LIVE/DEAD® (v/v; BacLight™ Bacterial Viability Kit, Invitrogen) in phosphate buffered saline (PBS) prior to confocal laser scanning microscopy (CLSM) imaging (Leica TCS SP5) with ax63 lens. The CLSM z-stack images were analysed using COMSTAT image analysis software for quantification of biofilm biomass.

2.19 Whole genome sequencing and bioinformatics analysis

Total genomic DNA (gDNA) was extracted from an overnight culture (2ml) on a QIAcube automated system (Qiagen). Following extraction gDNA was quantified by fluorometric methods using a Qubit (ThermoFisher Scientific), with quality ratios of gDNA (A260/280 and 260/230) determined via Nanodrop (ThermoFisher Scientific). gDNA libraries are prepared for whole genome sequencing using the NexteraXT kit (Illumina), as described by the manufacturer. Paired end sequencing was performed using the Illumina MiSeq platform (MiSeq Reagent V3 Kit; 2x 300 cycles). For each *E. coli* isolate, at least 80x coverage was generated. Raw sequence reads were trimmed using Trim Galore and the genomes were de novo assembled into contigs using SPAdes (3.9.0) with pre-defined kmers set. Raw reads were also assembled with Geneious (10.0.9) de novo assembler, set at medium sensitivity for analysis of paired Illumina reads. Geneious was used to map both sets of contigs to reference genes identified by closest BLAST homology and was also used to annotate genes from closest homologues in NCBI Genome database.

2.20 Bioinformatic analysis

(i) MLST analysis. For each *mcr*-carrying E. coli isolate for which the whole genome sequence was available, wgMLST profiles were generated using the CGE platform (https://cge.cbs.dtu.dk/services/MLST/), coupled with the PubMLST.org database(Larsen *et al.*, 2012).

(ii) WGS analysis. Resistance genes and virulence factors were identified using Resfinder and VirulenceFinder within CGE (https://cge.cbs.dtu.dk/services/) (Zankari *et al.*, 2012). In addition, database for toxin-antitoxin systems (TAs) and metal resistance operons were created and determined using Geneious 10.0.7. Based on the genomic difference in their core

genome, phylogenetic tree was built using Harvest software and visualized using iTOL v3.3.1 (http://itol.embl.de/).

(iii) Plasmid analysis: Identification of plasmid incompatibility groups of *mcr*-bearing plasmids were performed using Plasmidfinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Carattoli *et al.*, 2014). Alignment and visualization of plasmids was performed with BRIG v0.9555. The majority of the isolates and plasmids described in this study were sequenced using a short read Illumina Miseq, which are unable to generate a high quality assembly of the plasmids due to the high number of repeat copies present in their sequence. Therefore, no phylogenetic analysis of the *mcr*-carrying plasmids was conducted in this study.

(iv) Genetic dataset of virulence factors in Klesiela pneumoniae: Briefly, using whole-genome sequence data, we have developed a database with publicly available genomes (Genbank accession Numbers are abbreviated as GB.No.) [NTUH-K2044 (GB.No. AP006725.1, AB117611.1), pK2044 (GB.No. NC_006625.1), pLVPK (GB.N. NC_005249.1), allantoin metabolism (GB.No. AB115590.1), SB3193 (GB.No. LK022716.1), *uge* CDS (GB.No. AY294624.1), Kp52.145 (GB.No. FO834906.1), SB4536_2858 (GB.No. HG518478.1), pO26-Vir (GB.No. NC_012487.1), IHE3034 (GB.No. AM229678.1), kvg operon (GB.No. AJ250891.2)] to determine the key virulence factors in *K. pneumoniae* strains. There are at least five groups of pathogenicity factors in this dataset, these include gene clusters associated with serum resistance (*traT*); adhesins (type I firmbrial operon *fimABCDEFGH*, and type III firmbrial operon *mrkBCDEF*); lipopolysaccharide (*wabGHN*); siderophore systems (enterobactin-encoding *entABCDEFS*, and aerobactin-encoding *iucABCD, iutA*) and capsular synthesis loci (*wzi/wzc* typing); the iron acquisition operons *kfuABC*, and *iroE*, urease-synthesis operon *ureABCDEFG* associated with gastric ulceration and urinary stone formation.

(v) Genetic dataset of TAs in MCRPEC strains: a total of 22 pairs of known TAs were created for the presence of TAs in our collection MCRPEC strains in Chapter 6. The Genbank accession Number of these TAs were provided as follow: *ccdA/ccdB* (GB.No. 6382276/6382275); *chpA/chpR* (GB.No. 8183452/8183453), *chpB/chpS* (GB.No. 14970161/ 14970992); *dinJ-yafQ* (GB.No. 8114747/8114741); *doc-phd* (GB.No. 2777474/2777473); *ghoS/ghoT* (GB.No. 948646/1450308); *hicA/hicB* (GB.No. 945989/946001); *higA/higB* (GB.No. 8115408/8116456); *hipA/hipB* (GB.No. 946064/946065); *hok-sok* (GB.No. 16833926/16833886); *mazE/mazF* (GB.No. 947245/947252); *mqsA/mqsR* (GB.No. 8116442/8116443); *pemK/pemI* (GB.No. 8319148/8139206); *relB/relE* (GB.No. 12674638/6062222); *stbD/stbE* (GB.No. 10549005/5850679); *tisA/tisB* (GB.No. 5061526/5061527); *vagC/vagD* (GB.No. 7324544/17500432); *vapB* (GB.No. 20492652); *yacA/yacB* (GB.No. 18252642/18252641); *yafN/yafO* (GB.No. 8179628/8179629); *yeeU/yeeV* (GB.No. 7148618/7148613); and *yefM/yoeB* (GB.No. 7149349/7148625).

(vi) Genetic dataset of heavy metal determinants in MCRPEC strains: 26 representative heavy metal resistance genes were created and the Genbank accession Number of these genes were provided as follow:

arsenic resistance genes: *arsA* (GB.No. 12884544), *arsB* (GB.No. 948011); *arsC* (GB.No. 12884546); *arsD* (GB.No. 1446554); and *arsR* (GB.No. 12884461).

copper resistance genes: *copA/incA* (GB.No. 24954233); *cusS* (GB.No. 945978); *pcoA* (GB.No. 5616826); *pcoB* (GB.No. 5616826).

mercury resistance genes: *merA* (GB.No. 18252634); *merC* (GB.No. 30522719); *merR* (GB.No. 16834528).

silver resistance genes: *sliA* (GB.No. 12880691); *sliB* (GB.No. 7145791); *sliC* (GB.No. 5616818); *sliE* (GB.No. 7145035); *sliF* (GB.No. 15486578); *sliP* (GB.No. 12756650); *sliR* (GB.No. 5616817); *sliS* (GB.No. 5616816).

Chapter 3

Balancing *mcr-1* expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms

3.1 Brief background

The global concern over colistin resistance has profound implications as to how we successfully manage and treat serious Gram-negative infections, particularly those caused by *Enterobacteriaceae* (Olaitan & Li, 2016, Jeannot *et al.*, 2017). Until recently, our understanding of colistin resistance was limited to chromosomal changes such as the *pmrA/pmrB* activation of *arnBCADTEF* and *pmrE*, that collectively modify lipopolysaccharide (LPS), the component of the Gram-negative outer-membrane by the addition of 4-amino-4-deoxy-L-arabinose (Olaitan *et al.*, 2014). However, the advent and subsequent reporting of plasmid mediated colistin resistance, *mcr-1*, in both animal and human health, has invoked commentaries announcing a return to the pre-antibiotic era. Since the first described *mcr-1* in November 2015, it has been reported in over 40 countries (see **Fig.1.5** in **Chapter 1**). Regrettably, as the main bacterial host of *mcr-1* is *Escherichia coli*, there are also significant public health[4] and environmental issues as MCR-1 positive *E. coli* (MCRPEC) has been reported from migratory birds (Wang *et al.*, 2017, Wang *et al.*, 2017), flies (Wang *et al.*, 2017), dogs (Wang *et al.*, 2017), imported reptiles (Unger *et al.*, 2017), rivers (Zurfuh *et al.*, 2016) and inevitably public water facilities (Sun *et al.*, 2017) (see **Fig.1.6** in **Chapter 1**). However, *mcr-1* encodes an enzyme that catalyzes the transfer of phosphoethanolamine onto a phosphate of the N-acetylglucosamine head group of lipid A in the bacterial outer-membrane, which may modify the structure of lipid A and alter its ability to induce the innate immune response and modify the clinical pathogenicity of bacterial

infections. Furthermore, colistin MICs displayed by MCRPEC are moderate (usually 2-8 mg/L) when compared to the level of colistin resistance (usually 8-256mg/l) mediated by, for example, increased expression of *pmrA*/*pmrB*, inferring that the expression of *mcr-1* is tightly controlled (Olaitan *et al.*, 2014, He *et al.*, 2017, Wang *et al.*, 2017). This, in part, is supported by recent studies on plasmid copy number where *mcr-1* were only found on plasmids of a relatively low copy number (Bontron *et al.*, 2016, Denervaud Tendon *et al.*, 2017). Here, I investigated the impact of *mcr-1* expression on bacterial morphological changes, fitness, competitiveness, immune stimulation and virulence. Furthermore, we analyse the fitness and virulence MCRPEC high-level colistin resistant mutants.

3.2 Results

3.2.1 Molecular models of colistin binding and *mcr-1*-catalyzed phosphoethanolamine transfer to lipid A

It has been well-known that the antibacterial activity of polymyxins depends on electrostatic attraction between cationic residue of polymyxin and the anionic phosphate head-groups of lipopolysaccride (LPS) in the outer membrane of GNB, resulting in disarrangement of the bacterial cell membrane (Newton, 1956, Falagas *et al.*, 2005) (see **Fig.1.3** in **Chapter 1**). A molecular model of polymyxin B binding to lipid A is displayed in **Fig.3.1**, which is based on the three-dimensional nuclear magnetic resonance (NMR) structure of polymyxin in complex with lipid A (Pristovsek & Kidric, 1999, Velkov *et al.*, 2010): the positively-charged Dab residues closely interact with the negatively-charged 1'- and 4'-phosphate groups of lipid A, reducing the net-negative charge of lipid A. The hydrophobic leucine residues and tail of polymyxin interact with the fatty acid tails of lipid A. This initial electrostatic interaction between polymyxin and LPS molecules in the outer membrane of the GNB leads to displace divalent cations, magnesium (Mg^{+2}) and calcium

(Ca^{+2}), which act as a bridge to stabilize the LPS molecules, allowing polymyxins to insert into the bacterial outer-membrane (Storm *et al.*, 1977, Yu *et al.*, 2015). As a result, this insertion of hydrophobic residues [($_{D}$-Phe^{6} -$_{L}$-Leu^{7} (polymyxin) or $_{D}$-Leu^{6} -$_{L}$-Leu^{7} (colistin)] and the fatty acyl chain acts to disrupt and destabilize the monolayer of outer-membrane, eventually the permeability of the cell wall to polymyxins is increased, that ultimately causes cell death.

However, *mcr-1* encodes an enzyme that catalyzes the transfer of phosphoethanolamine onto a phosphate of the N-acetylglucosamine head group of lipid A in the bacterial outer-membrane (**Fig.1A**) (Gao et al., 2016, Hinchliffe et al., 2017). Positively-charged phosphoethanolamine addition onto the 1'-PO_4 of lipid A likely interferes with the interaction of positively charged Dab8 and Dab9 side chains with the phosphate group, reducing colistin affinity to the outer-membrane of Gram-negative bacteria (**Fig.1C**).

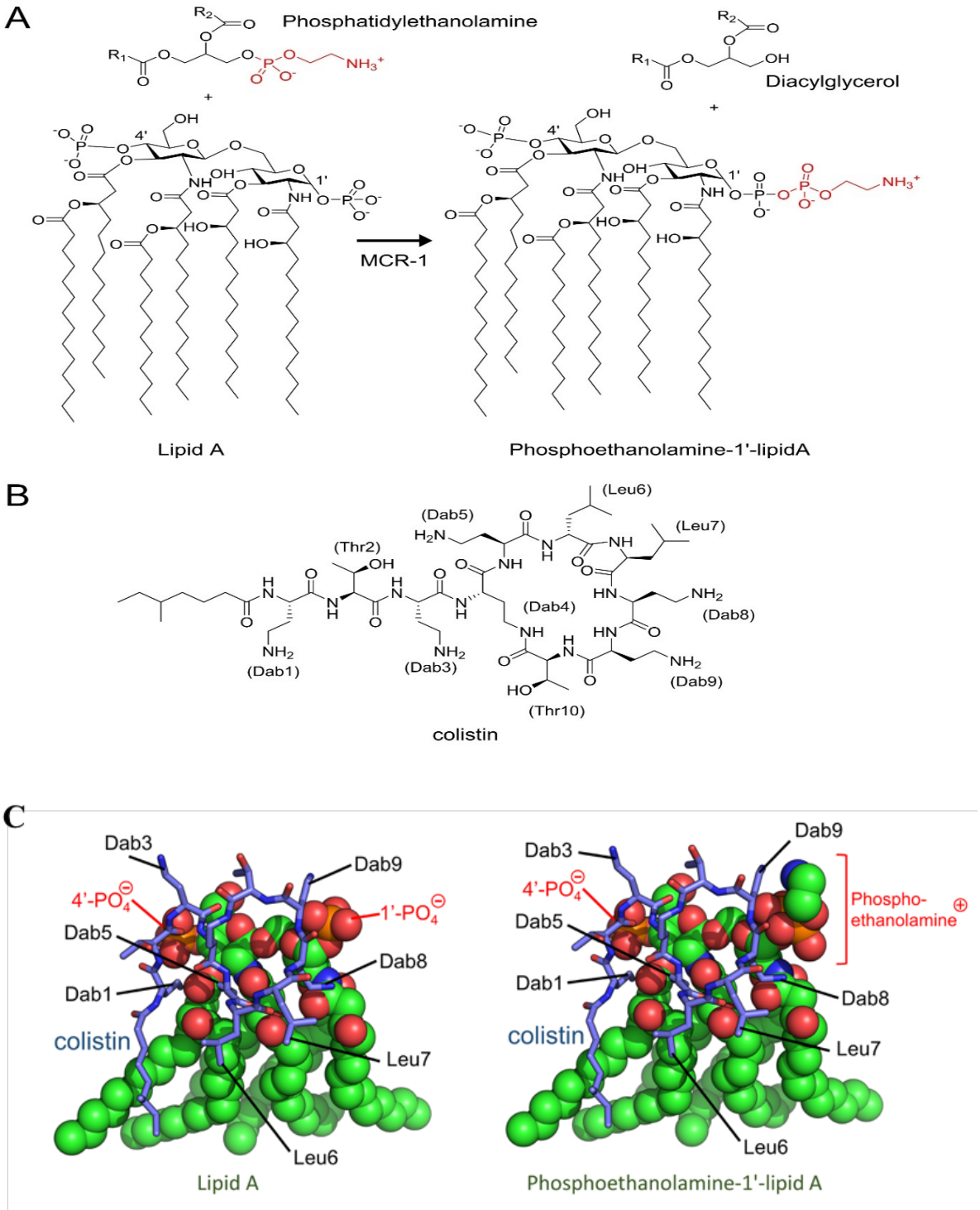


Fig.3.1 Molecular model of colistin binding to lipid A.

A, Schematic of phosphoethanolamine transfer to the 1-PO_4 group of hexa-acylated lipid A as catalysed by MCR-1.

B. Chemical structure of colistin, a polycationic peptide containing the hydrophobic tail and hydrophilic cyclic peptide made of amino acids L-threonine (Thr), D-leucine (Leu) and L-α,γ-diaminobutyric acid (Dab).

C, Models of colistin (blue sticks) binding to lipid A (*left*) or phosphoethanolamine-lipid A (*right*) (spheres coloured green, red, blue and orange for C, O, N and P atoms, respectively). The model is based on the NMR and docking studies of polymixin B binding to lipid A with lipid A coordinates from PDB 3fxi (Pristovsek & Kidric, 1999) and colistin coordinates adapted from the NMR structure of polymixin B bound to lipid A (Park *et al.*, 2009). The positively-charged Dab colistin residues closely interact with the negatively-charged 1' and 4' phosphate groups of lipid A, reducing the net-negative charge of lipid A. The hydrophobic leucine residues and tail of colistin A interact with the fatty acid tails of lipid A, allowing colistin A to insert into, and disrupt, the bacterial outer-membrane. (*right*), model of colistin binding to phosphoethanolamine-lipid A indicates addition of positively-charged phosphoethanolamine onto the 1'-PO_4 of lipid A likely interferes with the interaction of positively charged Dab8 and Dab9 side chains with the phosphate group, preventing colistin binding to the outer-membrane of Gram-negative bacteria. Figures were created using Pymol (https://www.pymol.org/).

3.2.2. Effects of *mcr-1* overexpression on bacterial growth and fitness

The *mcr-1* coding region obtained from pHNSHP45 was constructed into plasmid pBAD to generate strain *E. coli* TOP10 *mcr-1*/pBAD (*mcr-1*/pBAD) (see Methods **2.2** in **Chapter 2**). Laboratory strains and plasmids studied in this Chapter are list in **Appendix III Table 3.1**. To determine whether the expression of *mcr-1* affects bacterial growth rate and fitness, we examined growth curves, qRT-PCR and competition assays for *E. coli* TOP10 carrying the *mcr-1*-pBAD plasmid construct. The expression level of *mcr-1* was induced by increasing concentrations of L-arabinose and measured by qRT-PCR (**Fig.3.2B**). Maximal *mcr*-1 induction was observed at 0.02% L-arabinose, where *mcr*-1 expression was approx. 2-fold and 3-fold more than the arabinose concentrations of 0.002% and 0.2%, respectively. As shown in **Fig.3.2A**, after 8 h, the growth rates of *E. coli* strains induced by 0.2% and 0.02% (w/v) of L-arabinose showed approximately a 3-$\log_{10}$ unit decrease when compared to *E. coli*

TOP10 (*mcr-1*/pBAD) without L-arabinose. As the initial inoculum was approx. 7.5 $\log_{10}$ (CFU mL^{-1}), and this decreased to approx. 5 $\log_{10}$, it also suggests over-expression of *mcr-1* decreases cell populations. *In-vitro* competition experiments were also performed to determine the effect of *mcr*-1 expression on the relative fitness of *E. coli* (*mcr-1*/pBAD) with different *mcr-1* levels (see Methods **2.13** in **Chapter 2**). Results are shown in **Fig.3.2C** and indicate that increased *mcr-1* expression levels were associated with a significant fitness burden *in vitro*. For example, inducing high levels of *mcr-1* expression with 0.2% L-arabinose remarkably decreases relative fitness (average relative fitness 0.43, p=0.0022, using non-parametric Mann-Whitney test, see Methods **2.13** in **Chapter 2**) by more than 50% relative to uninduced controls (**Fig.3.2C** and **Appendix III Table 3.2**). Although the maximal expression of *mcr-1* was induced by 0.02% arabinose, the greatest fitness loss was observed in 0.2% arabinose-induced strain, this disparity is probably because bacteria viability is lower in 0.2% arabinose-induced strains showed in **Fig.3.2A**, when comparing to that in 0.02% arabinose-induced strains. Additionally, to eliminate these observations being singularly due to the overexpression of a random protein, our negative control, $bla_{\text{TEM-1b}}$, when overexpressed showed very similar growth curves to the uninduced *E. coli* cells (**Fig.3.3**).

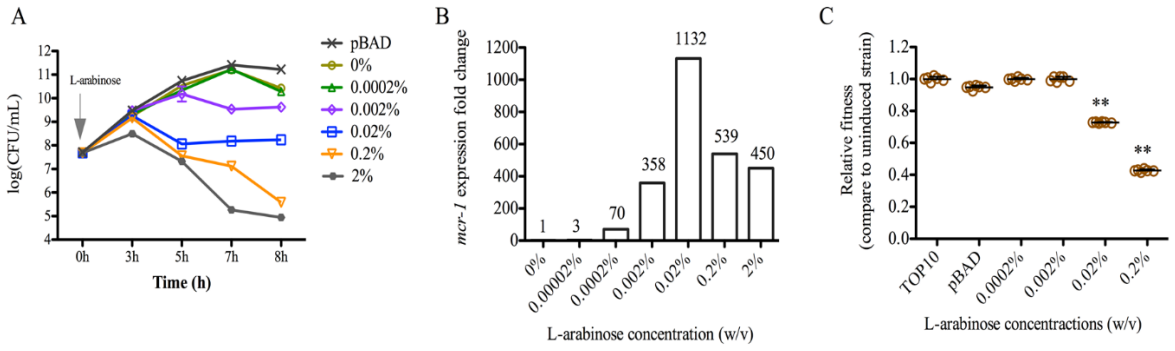


Fig.3.2 Effects of *mcr-1* overexpression on bacterial growth and fitness *in vitro*. A. Overproducing *mcr-1* causes variable effects on growth rate depending on the concentrations of L-arabinose (n=3). **B**, the expression levels of *mcr-1* gene induced by increasing concentrations of arabinose for 8 h, were measured by qRT-PCR (n=2). **C**, relative fitness of *mcr-1* overexpressing strain *mcr-1*/pBAD competing control strain pHT315 under increasing concentrations of L-arabinose (0.0002% vs 0%, 0.002% vs 0%, 0.02% vs 0%, 0.2% vs 0%). Error bars represent the S.D (n=6). The differences in fitness were tested using non-parametric Mann-Whitney test, ** indicates the p-values is less than 0.05. The average relative fitness and *p*-values are listed in supplementary Table 6.

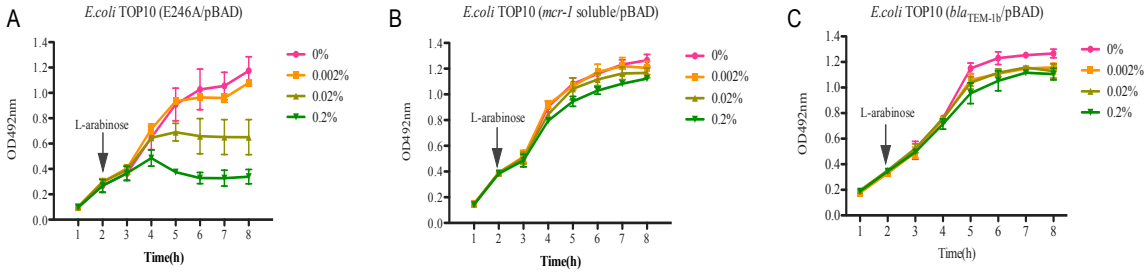


Fig. 3.3 *In vitro* growth curve of *mcr-1* derivatives and a negative control *E. coli* TOP10 strain carrying $bla_{\text{TEM-1b}}$. The means of three independent replicates were shown and the error bars represent the S.D (n=3).

3.2.3 Over-expression of *mcr-1* affects cellular morphology

As *mcr-1* encodes the transfer of phosphoethanolamine onto a phosphate of the N-acetylglucosamine head group of lipid A in the bacterial outer-membrane[9]. We employed transmission electron microscopy (TEM) and study the cellular morphology of *E. coli* (TOP10) under induced (0.2% [w/v] L-arabinose) and non-induced conditions (**Fig.3.4**). Both control strains, *E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD) without L-arabinose induction, showed normal cellular characteristics with a multi-layered cell surface consisting of a distinct, structured outer-membrane, a peptidoglycan layer in the periplasmic space, a normal cytoplasmic membrane and typically granular cytoplasm (**Fig.3.4A** and **Fig.3.4B**). Significant morphological changes were however, observed in *E.*

coli TOP10 (*mcr-1*/pBAD) treated with 0.2% L-arabinose for 8 h (**Fig.3.4C**). In particular, with the outer cell membrane cell envelope (**Fig.3.4F**). The outer-membrane region exhibited altered structural integrity, varying markedly in thickness and density it could not be differentiated from the cell wall or the cytoplasmic membrane (**Fig.3.4D, E** and **F**). TEM analysis on *E. coli* where *mcr-1* is over-expressed shows other gross cellular changes including complete loss of the "bacilli" morphology and absence of electron-dense material appeared as empty 'ghost' cells (**Fig.3.4C**). By comparison, *E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD) without L-arabinose induction possess homogeneous electron densities in the cytoplasm and exhibited unaltered multi-layers of cell membrane (**Fig.3.4A** and **B**) confirming that it is the high-level expression of *mcr-1* that has induced these gross morphological changes. In order to rule out effects caused by high concentration of L-arabinose, we examined *E. coli* TOP10 with pBAD alone (minus *mcr-1*) with 0.2% L-arabinose induction (**Fig.3.5**). Induction resulted in an intact cell wall with a well-define inner and outer-membrane, and a highly homogeneous electron density in cytoplasm were observed (**Fig.3.5)**, further supporting that cell membrane impairment is due to the expression of *mcr-1* gene only.

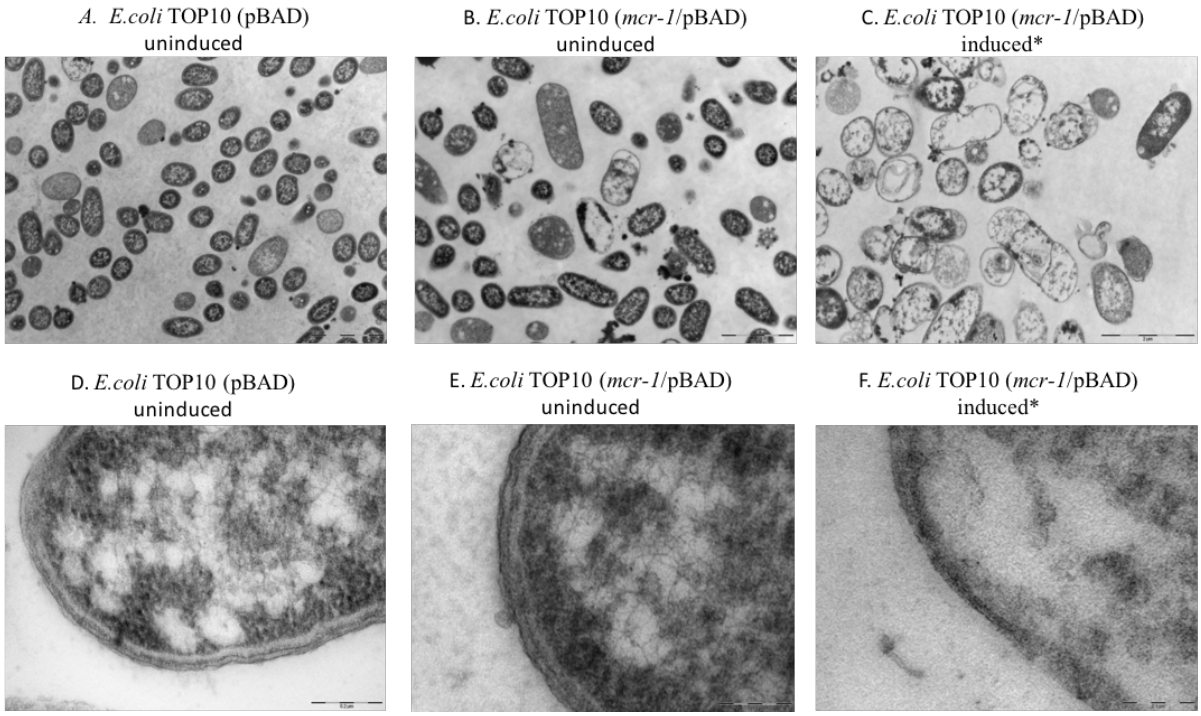


Fig.3.4 TEM micrographs of untreated and treated *E. coli*. In **A** and **B**, TEM micrographs of untreated control cells (*E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD) without L-arabinose induction, respectively) (see methods 2.12 in **Chapter 2**). Both control cells are intact with a well-define inner and outer-membrane, and showed a highly homogeneous electron density in cytoplasm region. **C**, TEM micrographs of *mcr-1* over-producing cells, the damaging outer-membrane and some completely lysed cells were observed.

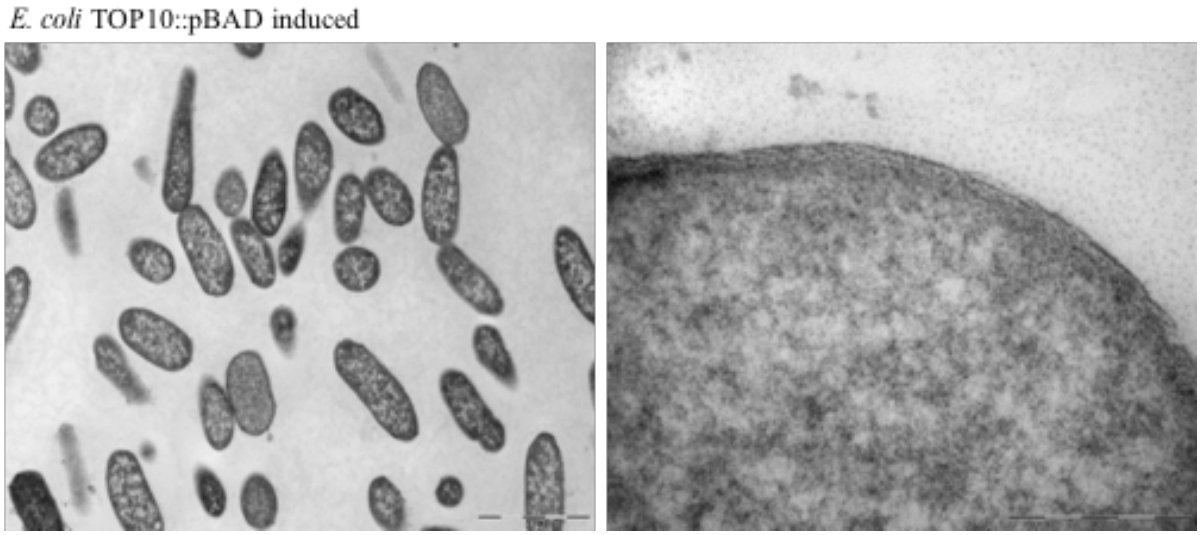


Fig.3.5 TEM micrographs of control cells: *E. coli* TOP10 with pBAD minus *mcr-1* with L-arabinose induction (0.2%) for 8 h (see methods 2.12 in **Chapter 2**), cells are intact with a well-define

inner and outer-membrane, and showed a highly homogeneous electron density in cytoplasm region. This result can rule out effects caused by high concentration of L-arabinose.

3.2.4. Effects of *mcr-1* overexpression on bacterial survival

As the growth curves from **Fig.3.2A** suggest that over-expression of *mcr-1* reduces cell counts and given its profound effect on the *E. coli* cell structure and morphology, we investigated the effects of *mcr-1* on cellular viability and biofilm assembly, using LIVE/DEAD® staining with confocal laser scanning microscopy (CLSM) imaging and COMSTAT analysis in *E. coli* TOP10 (*mcr-1*/pBAD) biofilms with/without 0.2% L-arabinose induction (**Fig.3.6**). The observed marked reduction of viability in bacterial cell overexpressing *mcr-1* (**Fig.3.6**), was contrast with the minimal reduction in bacterial viability observed in *E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD minus L-arabinose induction) (**Fig.3.6A** and **B**). COMSTAT analysis of the CLSM biofilm images revealed that relative dead/live biomass ratio (**Fig.3.6D**) of induced *E. coli* TOP10 (*mcr-1*/pBAD) was at least six times higher than *E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD) minus arabinose (0.33 versus 0.06; $p<0.001$, using one-way analysis of variance (ANOVA)). Morphological alterations were also apparent in CLSM imaging, where the live cells (green) in **Fig.3.6C** appeared more spherical and "bloated", in keeping with the changes observed in the TEM studies (**Fig.3.4C**). Overexpression of $bla_{\text{TEM-1b}}$ showed no evidence of increased cell death compared to that of *E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD) minus L-arabinose induction (**Fig.3.8** and **3.9**) providing further evidence that the cellular effects seen with increased expression of MCR-1 are not due to the fitness burden of protein expression.

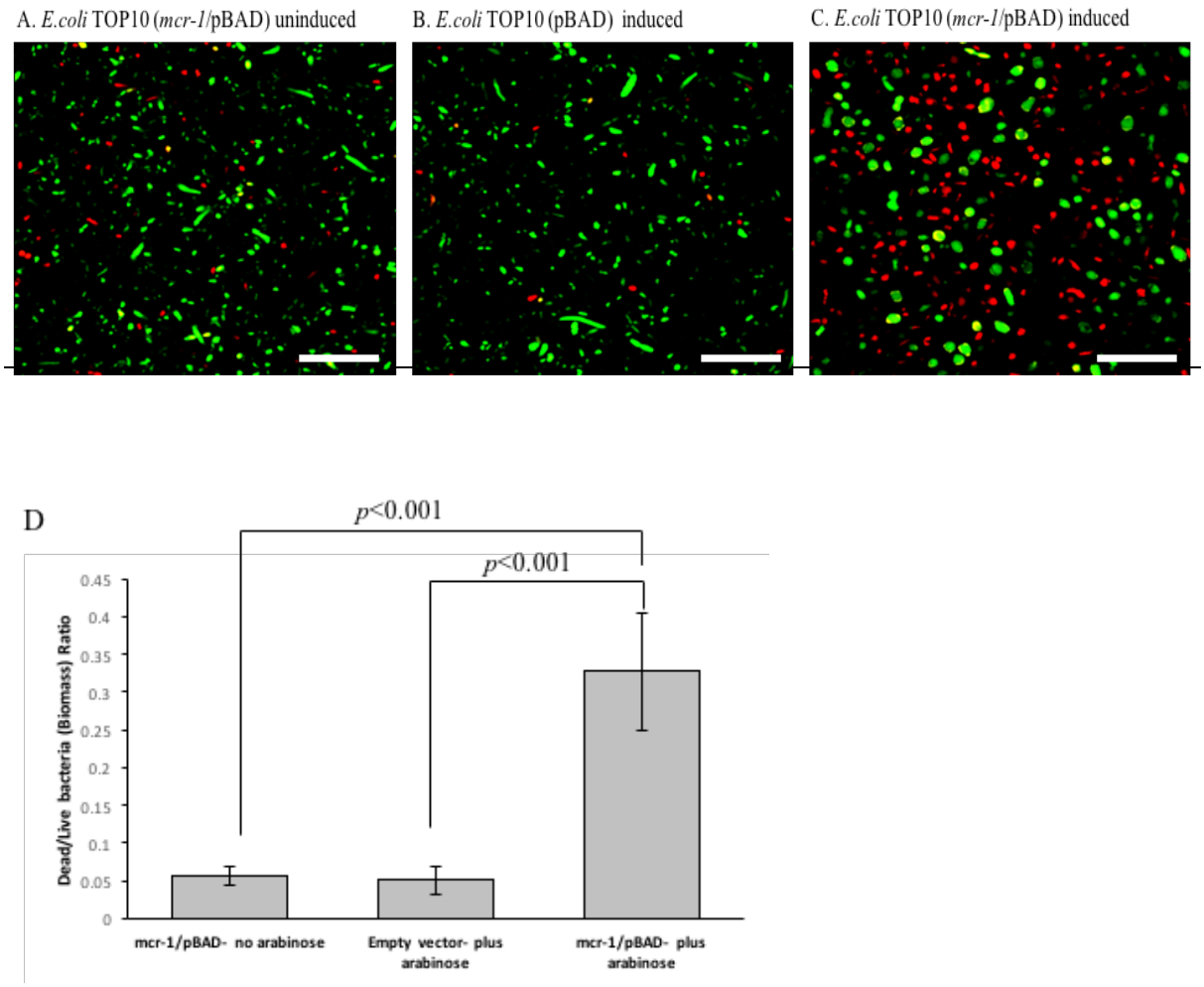


Fig.3.6 The toxic effects of *mcr-1* overexpression on cell viability. **A-C**, Confocal Laser Scanning microscopy images of cells treated with/without L-arabinose for 8 h and stained with LIVE/DEAD® (n=3). Live and dead cells presented green and red colour, respectively (see methods 2.18 in **Chapter 2)**. Scale bar is 15 µm. **D**, Ratio of Dead to Live bacteria (biomass) obtained from CLSM z-stack images through COMSTAT analysis of *E. coli* biofilms grown for 16 h in LB broth, followed by ± L-arabinose (0.2% w/v; 8 h) treatment, where the biofilms were stained with LIVE/DEAD® (n=4). The COMSTAT data was assessed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple comparisons post-hoc test. Statistical significance was set at $p < 0.05$.

3.2.5. *mcr-1*-mediated membrane-bound resistance mechanism

Our previous study has shown that MCR-1 is a membrane-bound enzyme consisting of five hydrophobic transmembrane helixes and a soluble form located in the periplasmic domain (Hinchliffe *et al.*, 2017). To examine whether the transmembrane domain or MCR-1

catalytic domain (typified by aspartate at position 246) plays a role on cell integrity and bacterial fitness, MCR-1 (E246A), and a MCR-1 soluble domain (residues 219-541 and lacking the N-terminal membrane-bound region) mutant, were cloned into a pBAD–hisA plasmid. Both constructed plasmids were transferred into TOP10 cells and induced with 0.2% (w/v) L-arabinose at exponential phase. Results show that increased expression of MCR-1 (E246A) produced a toxic effect on the *E. coli* cells as evidenced by decreased growth rate (**Fig.3.3**), moderate fitness loss (**Fig.3.7**) and significant membrane degradation (**Fig.3.8**). In contrast, the increased expression of the MCR-1 soluble domain (219-451) did not show any marked changes in growth rate, fitness loss or membrane architecture (**Fig.3.3, 3.7** and **3.8**). However, the toxic effects of the MCR-1 (E246A) mutant is moderate, compared to the marked effect of MCR-1 wild-type (**Fig.3.2, 3.4** and **3.6**). For example, increased expression of the full-length *mcr-1* gene caused at least three times higher bacterial death than *E.coli* with over-producing incomplete MCR-1 (E246A) (dead/live bacteria ratio, 0.33 versus 0.09) (**Fig.3.9**). Therefore, our data indicate that the fitness loss and the destruction of the membrane architecture is due to both the embedding of the protein in the *E. coli* outer membrane and the phosphoethanolamine modification of LPS. Phosphoethanolamine addition on Lipid A in MCRPEC has been observed by ESI-QTOF/MS (**Appendix III Fig.3.1**).

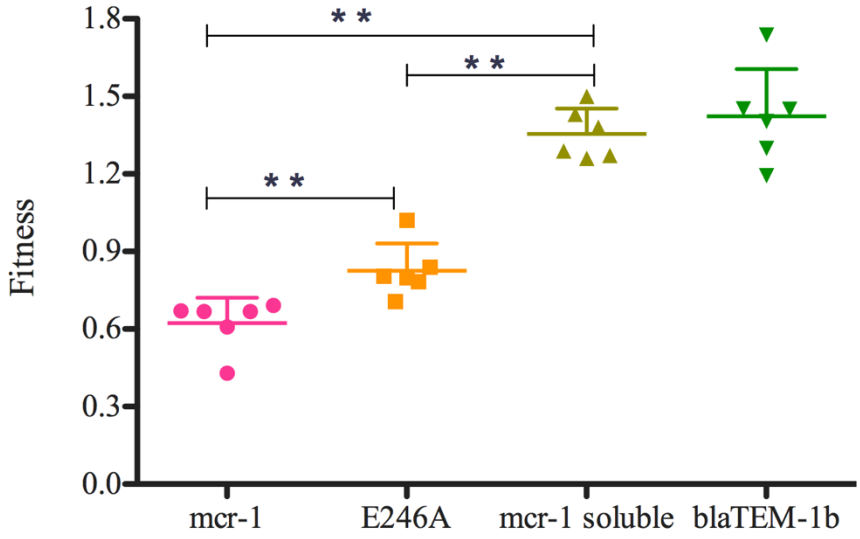


Fig.3.7 Relative fitness of E. coli TOP10 stains with full-length *mcr-1*, catalytically inactivated *mcr-1* (E246A) and *mcr-1* soluble domain. Fitness of the $bla_{\text{TEM-1b}}$-positibve strain as a negative control. Error bars represent the S.D (n=6). The differences in fitness were tested using non-parametric Mann-Whitney test, ** indicates the *p*-values is less than 0.05.

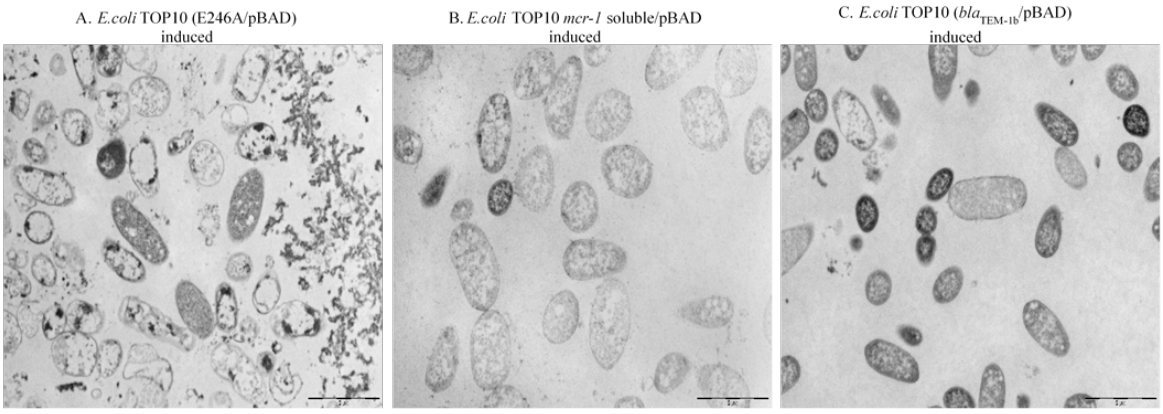


Fig.3.8 TEM micrographs of *mcr-1* mutants. A and B, TEM micrographs of *mcr-1* mutation implicated in its active site, E246A, and MCR-1 soluble domain, respectively. **C**, TEM micrographs of a negative control, $bla_{\text{TEM-1b}}$, a highly homogeneous electron density in cytoplasm region were observed.

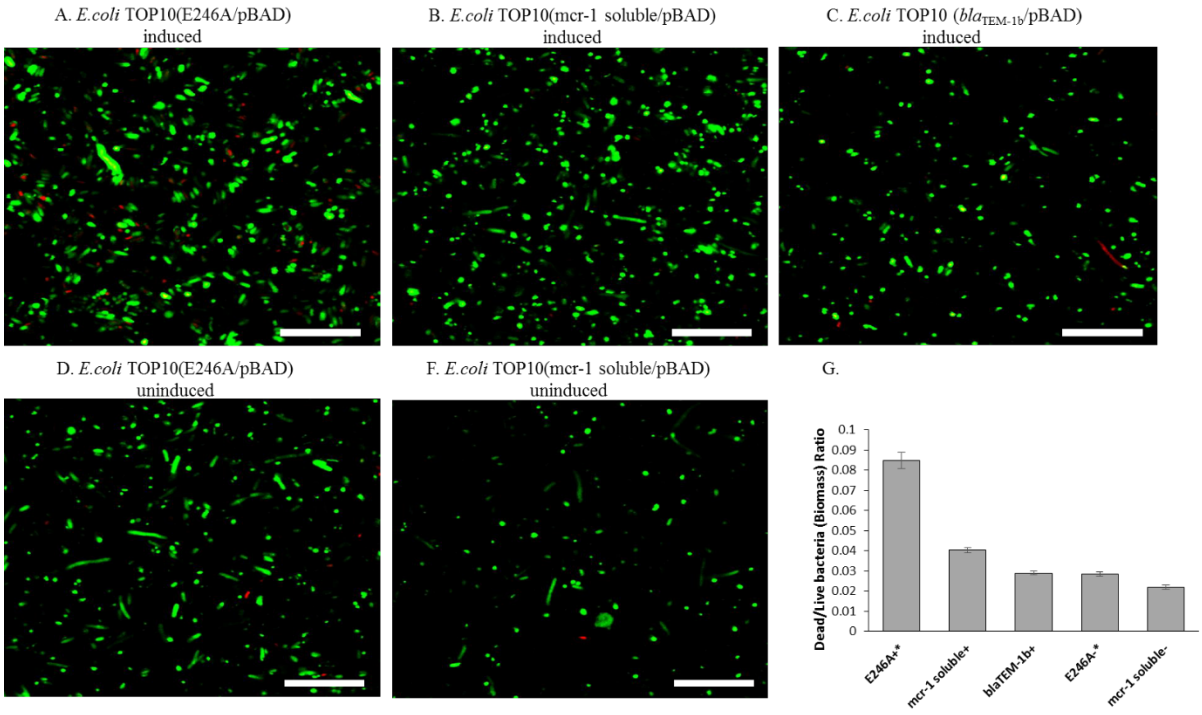


Fig.3.9 The toxic effects of *mcr-1* mutants on cell viability. **A-C**, Confocal Laser Scanning microscopy images of cells treated with L-arabinose (0.2% w/v; 8 h) and stained with LIVE/DEAD® (n=4). Live and dead cells presented green and red colour, respectively. **D**, Ratio of Dead to Live bacteria (biomass) obtained from CLSM z-stack images through COMSTAT analysis (n=4). The COMSTAT data was assessed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple comparisons post-hoc test.

3.2.6 *mcr*-1-mediated LPS modification reduced the stimulation of macrophage

mcr-1 encodes an enzyme that catalyzes the transfer of phosphoethanolamine onto a phosphate of the N-acetylglucosamine head group of lipid A, which may modify the structure of lipid A and alter its ability to induce the innate immune response and eventually modifying the clinical pathogenicity of bacterial infections. Therefore, to investigate whether the modified LPS of MCRPEC alters the activation of human macrophage THP-1, LPS-mediated human macrophage stimulation assays were undertaken. The macrophages were activated by serial concentrations (4.5, 0.45, 0.045 and 0.0045 ng mL^{-1}) of LPS extracted from *E. coli* TOP10 with pBAD minus *mcr-1*, and *E. coli* TOP10 (*mcr-1*/pBAD plus 0.2% L-arabinose). The production of IL-6 and TNF were assayed by using DuoSet® ELISA kit (R&D systems,

UK). The concentrations of IL-6 produced by macrophages induced by unmodified LPS were consistently higher than IL-6 levels produced by MCR-1 modified LPS at 8 and 24 h (**Fig.3.10A** and **B**). Additionally, TNF-alpha levels were also higher in macrophages stimulated by unmodified LPS than compared to modified LPS (**Fig.3.10C**).

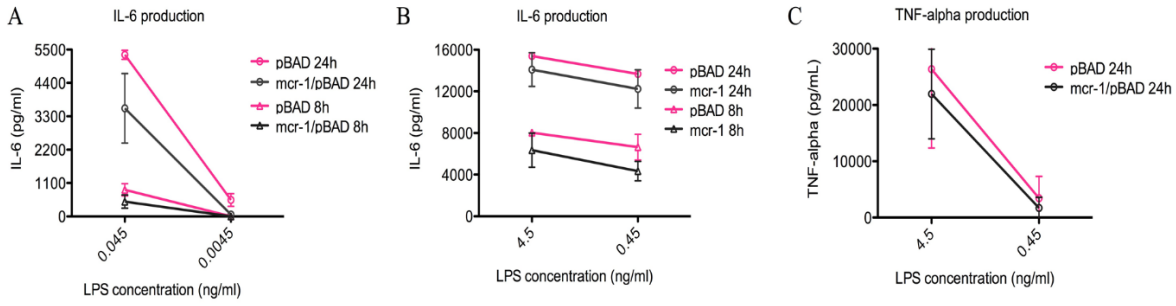


Fig.3.10 Expression of cytokines in LPS-mediated THP-1 macrophage. A, B and **C,** indicated IL-6 (n=2) and TNF-alpha (n=3) productions in the modified LPS (Grey) or normal LPS (Red)-treated cell culture, respectively. Error bars represented S.D.

3.2.7. Acquisition of high-level colistin resistance mutants

MCRPEC was first discovered on the premise that *E. coli* can rarely acquire colistin resistance by chromosomal mutations alone and that the levels mediated by *mcr-1* are moderate compared with other mechanisms[1, 2, 9]. Data previously published by us would suggest that the level of *in vivo* colistin resistance mediated by MCRPEC can protect the cells compared to control strains[9]. However, the data from **Fig.3.2-Fig.3.6** would suggest that the expression of *mcr-1* is also very tightly controlled because of the fitness cost of increased *mcr-1* expression. We have generated high-level colistin resistance mutants (HLCRMs) from wild type MCRPECs with low levels of colistin resistance (MIC 4-8 mg/l) (Yang *et al.*, 2017). We also exposed *E. coli* TOP10 (*mcr-1*/pBAD), *E. coli* TOP10 (pBAD) and *E. coli*

TOP10 in increasing concentrations of colistin. After a 14-day serial colistin challenge, *E. coli* TOP10 (*mcr-1*/pBAD) (in the absence of arabinose) displayed a 64-fold (from 0.5 to 32 mg/L) increase in colistin MICs (**Fig.3.11**). However, both *E. coli* TOP10 (pBAD) and *E. coli* TOP10 did not show any elevation in colistin MICs remaining at 0.25 mg L^{-1} +/- one dilution over the 14-day challenge. These data indicate that the increase in colistin resistance shown by *E. coli* TOP10 (*mcr-1*/pBAD) is singularly due to the presence of *mcr-1*.

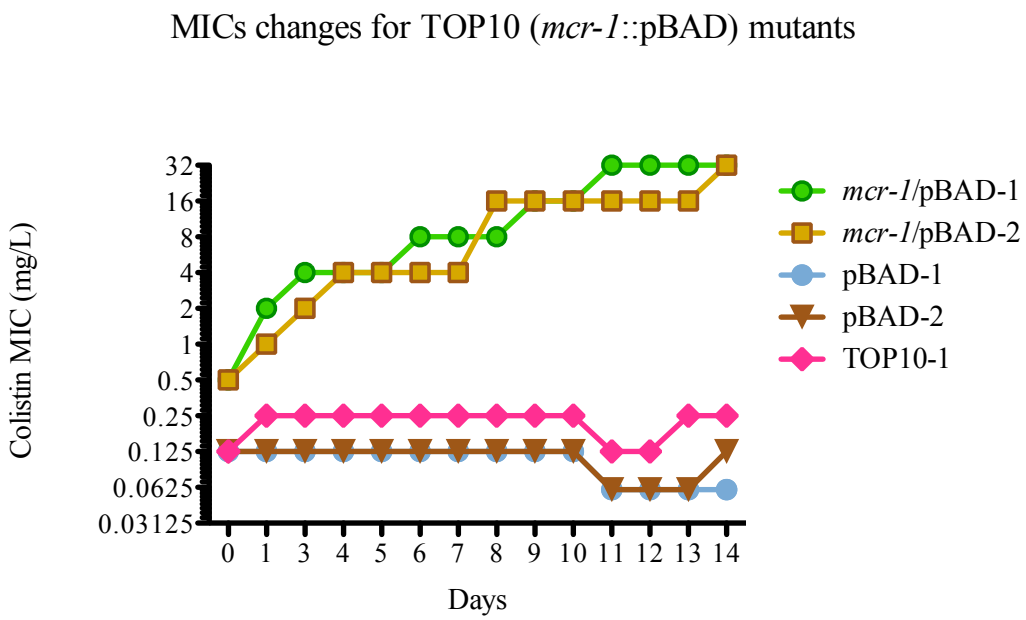


Fig.3.11 Acquisition and stability of high-level colistin resistance mutants. Changes in colistin minimum inhibitory concentrations (MICs) during 14-day challenging with increasing concentrations of colistin, in three laboratory strains

3.2.8 *in vitro* relative fitness of HLCRMs

Our data examining the physiological burden of *mcr*-1 would suggest that the HLCRMs might even be defective in fitness and pathogenicity. Therefore, to compare the growth rates of the MCRPEC isolates with their respective HLCRMs, we measured growth over a serial time course of HLCRMs strains that showed different resistance levels of resistance to

colistin at day 0, 3, 7, 11 and 14. The growth curves results suggested that day-8 and day-14 HLCRMs generated from *E. coli* TOP10 (*mcr-1*/pBAD) (both MICs of 16 and 32 mg L^{-1} for colistin, respectively), showed a decreased growth compared to that of their parental strains (**Fig.3.12A**).

Based on our data from bacterial growth curves, we propose there is substantial fitness disadvantage to the HLCRMs when compared to their MCRPEC parent strains. To test this hypothesis, *in vitro* competition assays were performed HLCRMs were directly competed against their MCRPEC parental strains. The fitness of the MRCPEC parent strains and HLCRMs were relative to the growth of the control strain which was fixed at 1.0 (see Methods). We examined the fitness cost for *E. coli* TOP10 (*mcr-1*/pBAD), *E. coli* TOP10 (pBAD) and *E. coli* TOP10 (**Fig.3.12B**). Day 3 and 4 HLCRMs of *E. coli* TOP10 (*mcr-1*/pBAD) had slightly less fitness (1.0 versus 1.1 of *E. coli* TOP10 (pBAD) and *E. coli* TOP10) and day 8 and 14 were less fit (0.83 versus 1.1, and 0.79 versus 1.1, respectively) (**Fig.2.12B**). Generally, our data shows a significant fitness burden with HLCRMs compared to their MCRPEC parent strains.

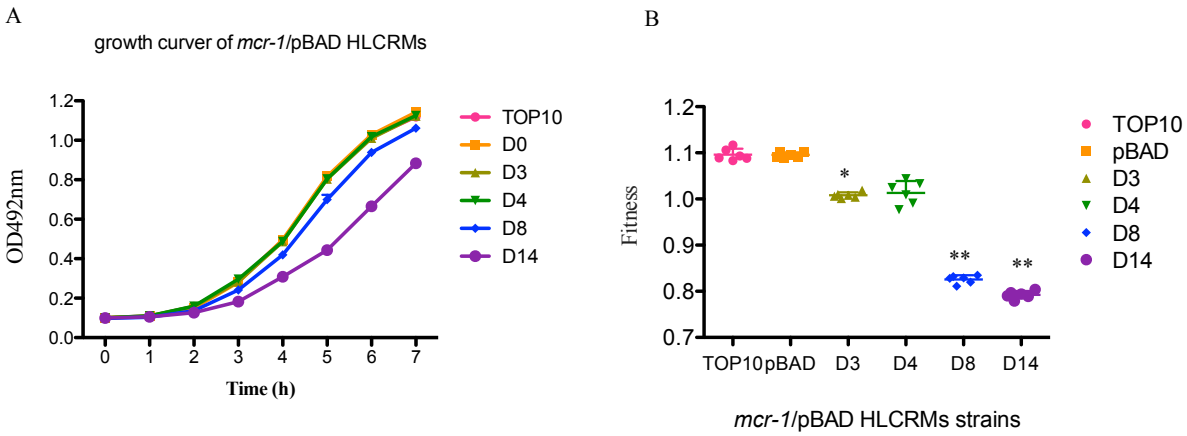


Fig.3.12 Growth curves and relative fitness of mcr-1/pBAD HLCRMs. A. indicated the growth rate of *E. coli* TOP10 (*mcr-1*::pBAD) and their derivatives (samples from serial time points: D0, D3, D4, D8 and D14). B. relative fitness of HLCRMs of *E. coli* TOP10 (*mcr-1*/pBAD) competing a control strain

pHT315. Error bars represent the S.D (n=6). The differences in fitness were tested using non-parametric Mann-Whitney test, ** indicates the p-values is less than 0.05. The average relative fitness and *p*-values are listed in **Appendix Table 3.2**.

3.2.9 Virulence reduction of MCRPEC

In wild-type strains, the level of colistin resistance mediated by *mcr-1* gene is often moderate (2-8 mg L^{-1}). The *in-vitro* generated HLCRMs possessed reduced fitness and virulence. We also determined that even the low level of colistin resistance mediated by *mcr-1* gene in clinical strains attenuates bacterial virulence in the *G. mellonella* infection model. We chose four non-MCRPEC clinical strains, belonging to different ST types: ST638 (ff112), ST589 (ff141), ST127 (ff156) and ST648 (CX37), which is one of diverse ST groups associated with *mcr-1* gene in China[10]. Three different *mcr-1* plasmids isolated from MCRPEC strains (PN16, PN23 and ZJ487) were transferred to these non-MCRPEC strains by electroporation. We have shown that the acquisition of *mcr-1* naturally occurring plasmids do not affect bacterial growth of the host (wild-type clinical strains) strain and so their fitness cost is modest **(Fig.3.13**). However, their virulence is markedly depleted (**Fig.3.14**) supporting our hypothesis that the loss of virulence shown by MCRPEC is not due to growth rate but by the activity of *mcr-1* on the *E. coli* outer membrane.

In order to rule out of other factors harbouring in wild-type plasmid such as virulence clusters or toxin-antitoxin systems, would affect bacterial virulence, a length of 1626bp of *mcr-1*-coding region was cloned into a pUC19 vector, generating a pUC19::MCR-1 plasmid. The pUC19::MCR-1 plasmid was then transferred to two non-MCRPEC clinical isolates (ff141 and ff156) by electroporation. As showed in **Fig.3.15**A and B, the acquisition of *mcr-1* in clinical strain ff156 caused a significant decrease of bacterial virulence. When compared to the parental strain ff141, the virulence loss was also observed in clinical strain ff141 carrying

pUC19::MCR-1 plasmid, even their differences are minimal, further suggesting that the impaired virulence due to the activity of MCR-1 on bacterial endotoxin lipid A.

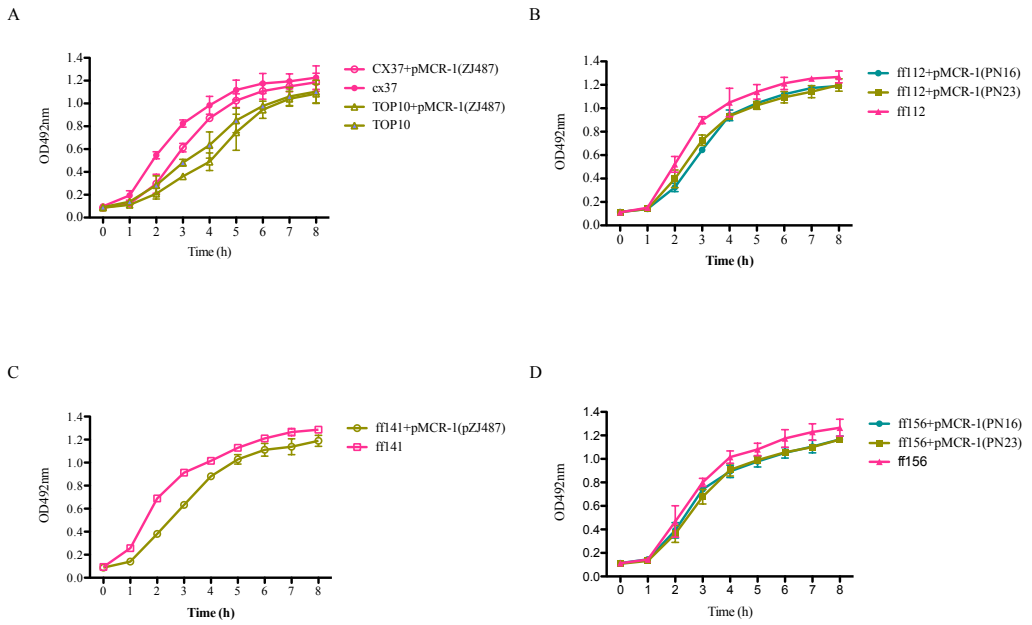


Fig.3.13 *In-vitro* growth curve of six MCRPEC strains and their counterpart non-MCRPEC. The means of three independent replicates were shown and the error bars represent the S.D (n=3).

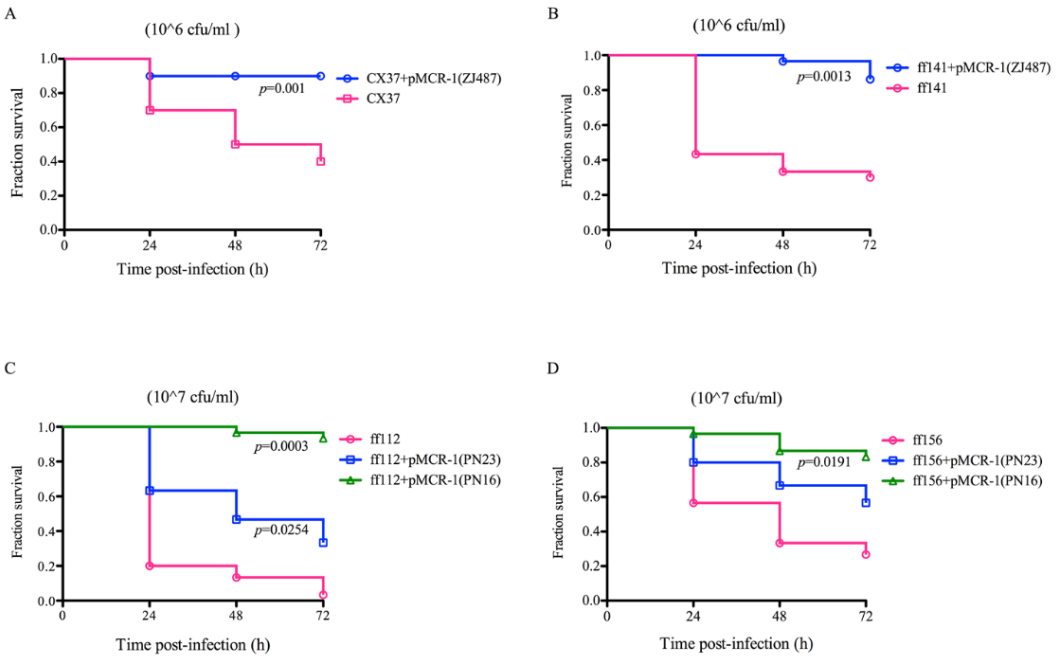


Fig.3.14 Kaplan-Meier plots showing the percent survival of *G. mellonella* over 72 h post-infection with MCRPEC and non-MCRPEC human clinical strains. Survival curves were plotted using the

Kaplan-Meier method (GraphPad Software). Error bars represent the S.D (n=3) and *p*-value for A (*t*=8.654, *d.f*=4), B (*t*=8.050, *d.f*=4), C [*t*=12.07, *d.f*=4 for strain ff112 with pMCR-1(PN16) and *t*=3.479, *d.f*=4 for strain ff112 with pMCR-1(PN23)] and D (*t*=3.801, *d.f*=4) were calculated by student *t*-test.

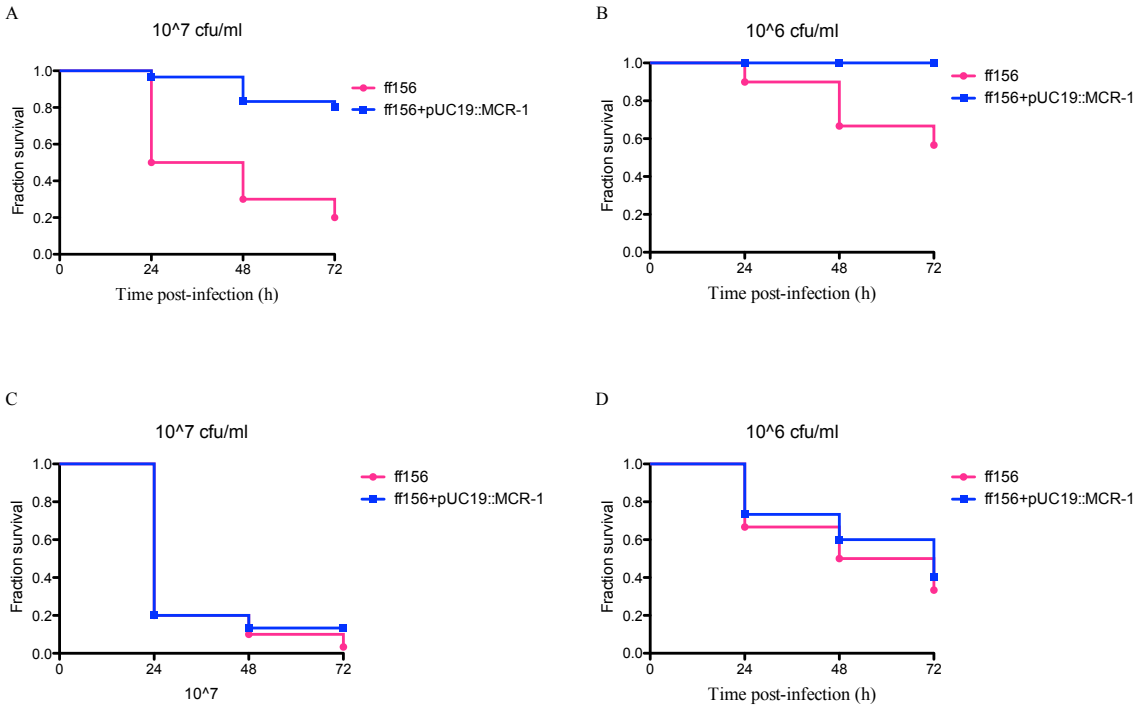


Fig.3.15 Kaplan-Meier plots showing the percent survival of *G. mellonella* over 72 h post-infection with MCRPEC and non-MCRPEC human clinical strains. Survival curves were plotted using the Kaplan-Meier method (GraphPad Software).

3.3 Brief discussion

Acquiring antibiotic resistance by mutation or horizontal gene transfer tends to be associated with a fitness cost (Andersson & Levin, 1999, Andersson & Hughes, 2010, Vogwill & MacLean, 2015). This cost plays a key in limiting the spread and maintenance of resistance in pathogen populations by generating selection against resistant strains under conditions where antibiotic doses are low, for example, during transmission between hosts

(Andersson & Hughes, 2010, MacLean *et al.*, 2010). However, recent studies have challenged this paradigm and provide evidence supporting the notion that, at least in some systems, the evolution of resistance can be associated with fitness advantages including the enhanced ability to cause disease (Roux *et al.*, 2015, Guillard *et al.*, 2016, Schroeder *et al.*, 2017). We have shown that the case for *mcr-1* imposing a fitness cost on its *E. coli* host is substantial. Increased expression of *mcr-1* not only impairs cell growth (**Fig.3.2A**) and diminishes bacterial fitness (**Fig.3.2C**), but also alters the cell's architecture (**Fig.3.4C**) and kills the bacteria (**Fig.3.6C**). Evolving colistin resistance by acquiring *mcr-1* therefore challenges bacterial populations with an evolutionary trade-off: high-levels expression of *mcr-1* provide protection against the antibiotic, but this increase in resistance compromises growth rate, fitness, membrane structural integrity and increases cellular death. This trade-off may explain several important phenomena. First, *mcr-1*-mediated colistin MICs are at best moderate (usually 2-8 mg L^{-1}) when compared to colistin resistance (8-256 mg L^{-1}) mediated by *pmrA*/*pmrB* (Olaitan et al., 2014, Liu et al., 2015, Wang et al., 2017). Secondly, we show that *mcr-1* plasmid copy number is very low (1-5 copies/cell), and this appears to protect the cell from too many copies of *mcr-1* (Yang et al., 2017*)*.

As a negative control, we show that the high-expression of $bla_{\text{TEM-1}}$ has negligible effect on *E. coli* fitness and survival (**Fig.3.3-3.9**), and therefore the effects witnessed with MCR-1 are due to its incorporation into the *E. coli* membrane or by its catalytic action – the phosphoethanolamine modification of LPS. It is interesting to note, that only a few mobile membrane-associated antibiotic resistance mechanisms have been characterised. One of them is the tetracycline efflux system, e.g. *tetA(B)* which is tightly regulated by its neighbouring gene, *tetR*. It has been shown that over-production of *tetA* is lethal, which is probably due to its effect on the membrane topology (Moyed *et al.*, 1983, Eckert & Beck, 1989, Coyne *et al.*, 2011). Unlike the *tetA*-like efflux pump system, *mcr-1* does not possess an adjacent repressor

to control its expression and its expression is control by other regulatory systems such as low plasmid copy number. Compared with the considerable burden of high expression of MCR-1 (**Fig.3.2-3.6**), the toxic effects of its two derivatives, MCR-1 E246A and MCR-1 soluble region, are moderate, which can be observed by some restoration of its outer membrane integrity and a reduction in death rate (**Fig.3.8** and **3.9**). However, as the toxic effects are more evident in MCR-1 E246A than the MCR-1 soluble region, therefore, cellular burden is due to both the embedding of the protein in the *E. coli* outer membrane and MCR-1 mediated PEtN modification of LPS.

Our data previously, and the stability data on HLCRMs, suggests that colistin resistance in MCRPEC and HLCRMs can be stable (Liu *et al.*, 2015, Yang *et al.*, 2017). Additionally, our data on *E. coli* TOP10 (*mcr-1*/pBAD) herein, shows that HLCRMs can be generated from a lab-strain, but only after acquisition of *mcr-1 s*uggesting the increase in colistin resistance is not dependent on the strain background which is further supported by the fact that all our selected wild-type MRCPECs readily generated HLCRMs. Finally, it is possible that colistin resistant strains will evolve compensatory adaptations that allow for *mcr-1* to be expressed at high levels at a low fitness cost. Compensatory adaptation is routinely detected *in vitro*(Andersson & Hughes, 2010), and there is some evidence that compensatory adaptation maintains otherwise costly resistance mutations *in vivo* (Comas *et al.*, 2012).

Due to its central clinical role in causing Gram-negative bacterial sepsis, lipid A as an immune modulator has been thoroughly scrutinised (Matsuura, 2013, Needham & Trent, 2013). However, despite the fact that both mechanisms of 4-amino-4-deoxy-L-arabinose and phosphoethanolamine LPS modification are well known, there have been very few systematic studies examining their impact on lipid A as an immune stimulant. Studies on *Salmonella* have examined the addition of phosphoethanolamine and found no significant change in virulence (Tamayo *et al.*, 2005). This data is supported by a recent study in *Haemophilus*

ducreyi that used knock-out deletions of phosphoethanolamine modifying genes (*lptA, ptdA* and *ptdB*) and concluded that the triple mutant was a virulent as the parent (Trombley *et al.*, 2015). A recent study by John and colleagues examined the phosphoethanolamine and its effect in *Neisseria meningitidis* by comparing invasive and carrier isolates and found more phosphoethanolamine and sialic acid substitutions from invasive strains suggesting that phosphoethanolamine-lipid A modification enhances virulence (John *et al.*, 2016). This finding also has been identified in non-*mcr-1* mediated colistin resistance in *K. pneumoniae*, where lipid A remodelling mediated by the *mgrB* mutation, resulted in increased colistin resistance enhancing the virulence of *K. pneumoniae* by decreasing the affinity of colistin and attenuating host defence response (Kidd *et al.*, 2017). These data are further supported by O'Brien and colleagues examining *Campylobacter jejuni* phosphoethanolamine modified lipid A, showed increased recognition of a human Toll-like receptor and increased commensal colonisation in mice (Cullen *et al.*, 2013). However, Zughaier *et al*. examined *Neisseria gonorrhoeae* phosphoethanolamine modified lipid A and showed that it reduced autophagy in human macrophages and useful mechanism to evade the host immune system (Zughaier *et al.*, 2015). Thus far, there are no similar studies on *E. coli* and this is the first examining the effect of *mcr-1* on *E. coli* fitness and virulence. The data presented in this study indicates that *E. coli* [in this case *E. coli* TOP10 (*mcr-1*/pBAD)] is forced to finely tune the expression of *mcr-1* or else the over-expression becomes toxic resulting in profound changes in the architecture of the outer-membrane, causing leakage of cellular cytoplasm (**Fig.3.4C**) and death (**Fig.3.6C**).

As *mcr-1* continues to spread globally, and the clinical impact is assessed, it will be interesting to examine how virulent MCRPEC compare with non-MCRPEC. In this study, *mcr-1* positive plasmids were transferred to non-MCRPEC clinical strains (ST638 (ff112), ST589 (ff141), ST127 (ff156) and ST648 (CX37)) by electroporation. We have shown that

the growth rate of all *mcr-1* positive transformants appears to be lower than that of their non-MCR parents, but the difference is not statistically significant (**Fig.3.13**). Compared to non-MCRPEC strains from phylogenetic group D (considered as second-most virulent ExPEC group), the mortality of *G. mellonella* showed a marked reduction after acquisition of a *mcr-1*-positive plasmid (**Fig.3.14**). To conclude, it would appear the acquisition of *mcr-1* by *E. coli* is a "poisoned chalice" -on the one hand *mcr-1* is required to provide protection in a colistin rich environment, yet acquisition compromises the bacterium's normal physiology; furthermore, over-expression results in acute "toxicity".

Chapter 4

The effect of the expression of two variant of *mcr-3* genes on bacterial fitness and competitiveness

4.1 Brief introduction

Since the discovery of *mcr-1* gene in late 2015, another seven different *mcr-like* genes and their variants have been subsequently found, namely, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7* and *mcr-8* (Xavier *et al.*, 2016, AbuOun *et al.*, 2017, Borowiak *et al.*, 2017, Garcia *et al.*, 2018, Wang *et al.*, 2018, Yang *et al.*, 2018). Compared to other *mcr-* variants (*mcr-2, -4, , -5, , -6, , -7* and *mcr-8*) which are reported sporadically in some countries (**Fig.1.5** in **Chapter 1**), *mcr-1* and *mcr-3*, are relatively common and distributed worldwide, but are especially prevalent in Europe and Asian countries including China (Ling *et al.*, 2017, Yin *et al.*, 2017) and Vietnam (Trung *et al.*, 2017). A recent study reported a considerably higher presence of *mcr-1, -2,* and *-3* genes in pigs and poultry from China when direct sampling was used rather than isolates growing in media supplemented with colistin, indicating that the prevalence of *mcr-like* gene family in many studies is probably underestimated, due to the limitation of bacterial isolation and current screening techniques (Wang *et al.*, 2017, Zhang *et al.*, 2018). Thus, the expanded group of *mcr-1*-like phosphoethanolamine transferases seems to provide multiple pathways for bacteria to evade the antimicrobial activity of colistin under human clinical and animal treatment.

Recently, concerns were raised regarding the increasing reports of *mcr*-mediated colistin resistance in Enterobacteriaceae, especially when combined with ESBL- or carbapenemase-producing isolates (Li *et al.*, 2016, Sun *et al.*, 2017). The emergence of this type of multi- or

pan-drug resistance poses severe therapeutic challenges due to the limited remaining effective antibiotics available against bacterial infections. Compared to the global prevalence of *mcr-1* has been widely reported (see **Fig.1.5** in **Chapter 1**), however, the prevalence and significance of a new *mcr*-variant, *mcr-3*, has not been fully examined. In June 2017, this new transferable *mcr-3* gene (with only 45% nucleotide homology to *mcr-1*), has been identified on an IncHI2-type plasmid in an *E. coli* isolate from pig feces from China. Shortly thereafter, *mcr-3* was found in animals, environmental and hospital settings worldwide including Denmark, Spain, Thailand and Vietnam (Hernandez *et al.*, 2017, Litrup *et al.*, 2017, Roer *et al.*, 2017, Zhang *et al.*, 2018). Co-occurrence of *mcr-1*- and *mcr-3*-positive plasmids has been identified in many samples from South Asia in my study and other research groups (Hernandez *et al.*, 2017, Liu *et al.*, 2017), highlighting that *mcr-3* may have spread widely. Notably, an NDM-5-producing *E. coli* has been identified to carry *two mcr-1*- and *mcr-3*-positive plasmids (Liu *et al.*, 2017), the combination of these resistance genes in clinical isolates results in limited treatment options.

Therefore, there is an urgent need to develop new methods to predict and prevent the new emergence of *mcr*-conferring colistin resistance. One of major factors determining the dissemination and maintenance of antibiotic resistance in a bacterial population is the fitness burden of resistance. The contribution of *mcr-1* expression to conferred fitness costs, virulence loss and changes in innate immune response in *E. coli*, have been fully discussed in Chapter 3. While the impact of *mcr-3* expression on bacterial fitness and virulence is still unknown. In addition, according to the *mcr*-positive *E. coli* (MCRPEC) isolates from human contributed by our collaborators from Vietnam, the nucleotide sequences of *mcr-3* genes were highly conserved, with two dominant variants, *mcr-3.1* and *mcr-3.5*, which differ by only three nucleotide substitutions. It remains unclear whether these mutations influence the level of colistin resistance, bacterial fitness and pathogenicity. Above all, there are three main

objectives in this chapter: (1) Characterization of isolates carrying new-variant *mcr-3* from human from Vietnam, using whole genome sequencing; (2) Investigation of the biological cost imposed by MCR-3 conferring colistin resistance, the maintenance and competitiveness between *mcr-1* and *mcr-3*-carrying plasmids; and (3) Provide evidence to support the potential evolution pathway of colistin resistance through step-wise accumulation of mutations in *mcr-3*.

4.2 Results

4.2.1 Characteristics of *mcr-3*-bearing *E. coli* from Vietnam collection

We have received 143 MCRPEC isolates originating from human stool from our collaborators in Vietnam. All of these MCRPEC isolates have been subjected to whole genome sequencing (see the details in **Appendix IV Table 6.2**). In this study, all 51 *mcr-3* positive isolates were chosen to be further analysed. Unsurprisingly, highly distinct *mcr-3*-linked *E. coli* isolates were observed, belonging to at least 25 STs including ST744 (n=6), ST10 (n=5), ST641 (n=3), ST206 (n=3) and ST101 (n=2) (**Table 4.1**). Based on SNPs in their core genome, phylogenetic tree analysis was generated by harvest software using their raw sequencing reads. The majority of these clinical isolates resided on distinct branches of the phylogenetic tree of 51 study genome (**Fig.4.1**), suggesting a high-level of diversity in the Vietnam *E. coli* population. Apart from *mcr-like* genes, MDR genotypes were identified in most of the studied isolates, with an average of 13.4 (range = 9 to 17) antibiotic resistance genes (**Table 4.1**). The most prevalent resistance genes found were *ampH*, bla_{ACC-1} encoding AmpC1-type β-lactamase, and bla_{ACC-2} encoding AmpC2-type β-lactamase, which were found in all isolates (n=5). Next most prevalent were resistance genes *dfrA* (n=44) and *sul3* (n=42), conferring resistance to trimethoprim and sulfonamides, respectively. Gene *qnrS1* encoding low-level fluoroquinolone resistance were also present in 23 isolates.

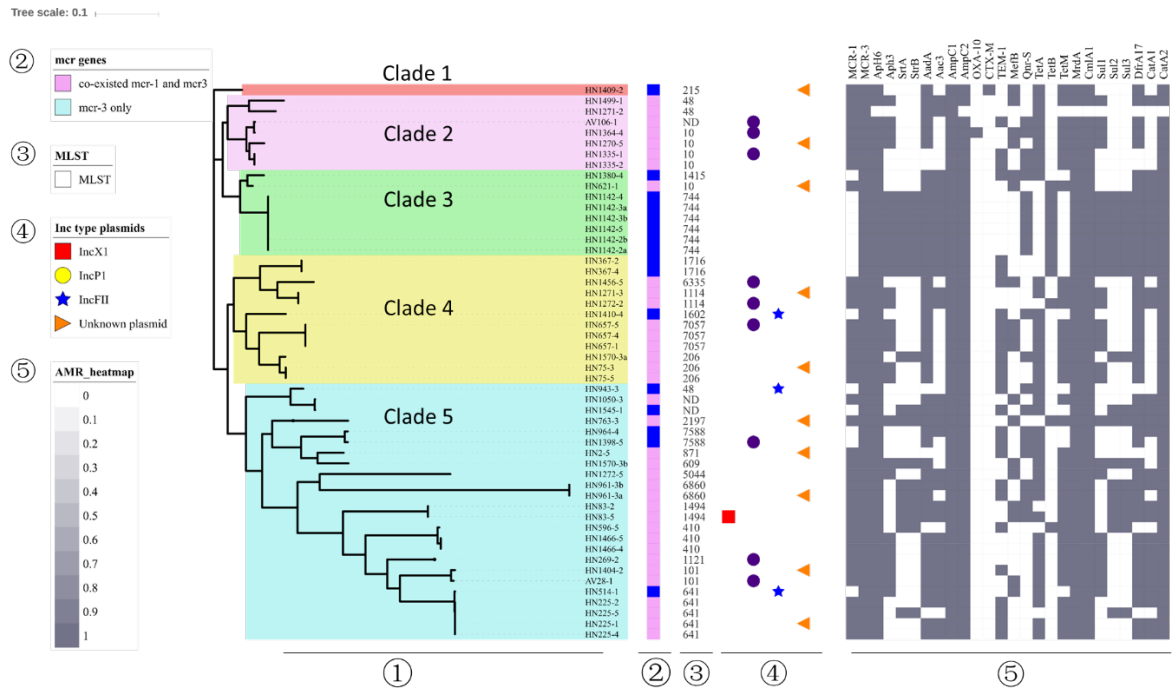


Fig.4.1 phylogenetic tree of 51 *mcr-3*-harboring isolates from Vietnam, was determined by Parsnp-harvest and visualized by iTOL. ① represents maximum-likelihood tree based on concatenated core genome sequences. Bacterial MLST ②, the presence of *mcr-* genes ③, *mcr-3*-linked Inc plasmid types ④ and AMR genes ⑤ , which are all analyzed by CGE website (http://www.genomicepidemiology.org/), are presented in this tree. Legends of each data set are also indicated in the left of this tree.

Table 4.1 characteristics of 51 *mcr-3*-positive *E. coli* isolates from Vietnam

Stain code	Source	Year	species	MLST	*mcr*-like genes	genetic location of *mcr-like* gene	
						***mcr-1* gene**	***mcr-3* gene**
AV106.1	human stool	2015	*E.coli*	ND	*mcr-1, mcr-3.5*	~40kb plasmid	~50kb IncP1
AV28.1	human stool	2015	*E.coli*	101	*mcr-1, mcr-3.5*	plasmid	IncP1
HN1050.1	human stool	2015	*E.coli*	NF	*mcr-1, mcr-3.1*	NA	NA
HN1142.2a	human stool	2015	*E.coli*	744	*mcr-3.1*	NA	NA
HN1142.2b	human stool	2015	*E.coli*	744	*mcr-3.1*	NA	NA
HN1142.3a	human stool	2015	*E.coli*	744	*mcr-3.1*	NA	NA
HN1142.3b	human stool	2015	*E.coli*	744	*mcr-3.1*	NA	NA
HN1142.4	human stool	2015	*E.coli*	744	*mcr-3.1*	NA	NA
HN1142.5	human stool	2015	*E.coli*	744	*mcr-3.1*	NA	NA
HN1270.5	human stool	2015	*E.coli*	10	*mcr-1, mcr-3.5*	~40kb plasmid	~50kb plasmid
HN1271.2	human stool	2015	*E.coli*	48	*mcr-1, mcr-3.1*	~50kb plasmid	~120kb plasmid
HN1271.3	human stool	2015	*E.coli*	1114	*mcr-1, mcr-3.5*	~50kb plasmid	~120kb plasmid
HN1272.2	human stool	2015	*E.coli*	1114	*mcr-1, mcr-3.5*	chromosome	~50kb IncP1
HN1272.5	human stool	2015	*E.coli*	5044	*mcr-1, mcr-3.5*	NA	NA
HN1335.1	human stool	2015	*E.coli*	10	*mcr-1, mcr-3.5*	chromosome	~50kb IncP1
HN1335.2	human stool	2015	*E.coli*	10	*mcr-1, mcr-3.5*	chromosome	~50kb
HN1364.4	human stool	2015	*E.coli*	10	*mcr-1, mcr-3.5*	chromosome	~50kb IncP1
HN1380.4	human stool	2015	*E.coli*	1415	*mcr-3* (D295E)	NA	
HN1398.5	human stool	2015	*E.coli*	7588	*mcr-3.5*	NA	IncP1
HN1404.2	human stool	2015	*E.coli*	101	*mcr-1, mcr-3.5*	chromosome	~50kb plasmid
HN1409.2	human stool	2015	*E.coli*	215	*mcr-3.1*	NA	~50kb plasmid
HN1410.4	human stool	2015	*E.coli*	1602	*mcr-3* (D295E)	NA	IncFII
HN1456.5	human stool	2015	*E.coli*	6335	*mcr-1, mcr-3.5*	chromosome	~50kb IncP1
HN1466.4	human stool	2015	*E.coli*	410	*mcr-1, mcr-3.5*	NA	NA
HN1466.5	human stool	2015	*E.coli*	410	*mcr-1, mcr-3.5*	NA	NA
HN1499.1	human stool	2015	*E.coli*	48	*mcr-1, mcr-3.1*	NA	NA
HN1545.1	human stool	2015	*E.coli*	ND	*mcr-3.1*	NA	NA
HN1570.3a	human stool	2015	*E.coli*	206	*mcr-1, mcr-3.5*	NA	NA
HN1570.3b	human stool	2015	*E.coli*	609	*mcr-1, mcr-3.5*	NA	NA
HN2.5	human stool	2015	*E.coli*	871	*mcr-1, mcr-3.5*	chromosome	~100kb plasmid
HN225.1	human stool	2015	*E.coli*	641	*mcr-1, mcr-3.1*	~120kb plasmid	~150kb plasmid
HN225.2	human stool	2015	*E.coli*	641	*mcr-1, mcr-3.1*	~120kb plasmid	~150kb plasmid

Stain code	Source	Year	species	MLST	*mcr*-like genes	genetic location of *mcr-like* gene	
HN225.4	human stool	2015	*E.coli*	641	*mcr-1, mcr-3.1*	~120kb plasmid	~150kb plasmid
HN225.5	human stool	2015	*E.coli*	641	*mcr-1, mcr-3.1*	~120kb plasmid	~150kb plasmid
HN269.2	human stool	2015	*E.coli*	1121	*mcr-1, mcr-3.5*	~40kb plasmid	~50kb IncP1
HN269.3	human stool	2015	*E.coli*	1121	*mcr-1, mcr-3.5*	~40kb plasmid	~50kb plasmid
HN367.2	human stool	2015	*E.coli*	1716	*mcr-3.5*	NA	NA
HN367.4	human stool	2015	*E.coli*	1716	*mcr-3.5*	NA	NA
HN514.1	human stool	2015	*E.coli*	641	*mcr-3.1*	NA	IncFII
HN621.1	human stool	2015	*E.coli*	10	*mcr-1, mcr-3.1*	~50kb plasmid	~50kb plasmid
HN657.1	human stool	2015	*E.coli*	7057	*mcr-1, mcr-3.5*	chromosome	~50kb plasmid
HN657.4	human stool	2015	*E.coli*	7057	*mcr-1, mcr-3.5*	chromosome	~50kb plasmid
HN657.5	human stool	2015	*E.coli*	7057	*mcr-1, mcr-3.5*	chromosome	~50kb IncP1
HN75.3	human stool	2015	*E.coli*	206	*mcr-1, mcr-3.5*	chromosome	~100kb plasmid
HN75.5	human stool	2015	*E.coli*	206	*mcr-1, mcr-3.5*	chromosome	~100kb plasmid
HN763.3	human stool	2015	*E.coli*	2197	*mcr-1, mcr-3.1*	chromosome	~90kb plasmid
HN83.2	human stool	2015	*E.coli*	1494	*mcr-1, mcr-3**	~50kb plasmid	~50kb plasmid
HN83.5	human stool	2015	*E.coli*	1494	*mcr-1, mcr-3**	~80kb plasmid	~50kb IncX1
HN943.3	human stool	2015	*E.coli*	48	*mcr-3* (D295E)	NA	IncFII
HN961.3a	human stool	2015	*E.coli*	6860	*mcr-1, mcr-3.1*	chromosome	~50kb plasmid
HN964.4	human stool	2015	*E.coli*	7588	*mcr-3.5*	NA	NA

**mcr-3* variant with two amino acid substitutions (M23V+G373V), ND indicates no determined, and NA means not available.

In addition, four *mcr-3* variants that differed by up to 3 nucleotide were identified in 51 isolates: *mcr-3.1*(n=19), *mcr-3.5* (M23V+E457A+T488I, n=27), *mcr-3* (D295E, n=3) and *mcr-3* (M23V+G373V, n=2). Most interestingly, co-occurrence of *mcr-1* and *mcr-3* genes have been found in 36 isolates from the MCRPEC collection (**Table 4.1** and **Fig. 4.1**). The genetic location of both *mcr-1* and *mcr-3* genes in randomly selected MCRPEC isolates were performed using PFGE with S1 nuclease, followed by hybridisation with *mcr-1* and/or *mcr-3* labelled probes. As shown in **Fig.4.2**, chromosomal and plasmid-borne *mcr-1* genes were detected in 34 isolates, with equivalent relative prevalence (55.9%, 19 each out of 34 the

MCRPEC isolates). In addition, more than one copy of *mcr-1* gene was found in several isolates; for example, the two isolates 1270.5 and AV106 both contained two *mcr-1* genes, one on a plasmid and a second in chromosome. In contrast, *mcr-3* is located on plasmids in single copy from different size plasmids, suggesting high plasticity of genetic backgrounds and horizontal gene transfer of *mcr-1* may facilitate its global spread.

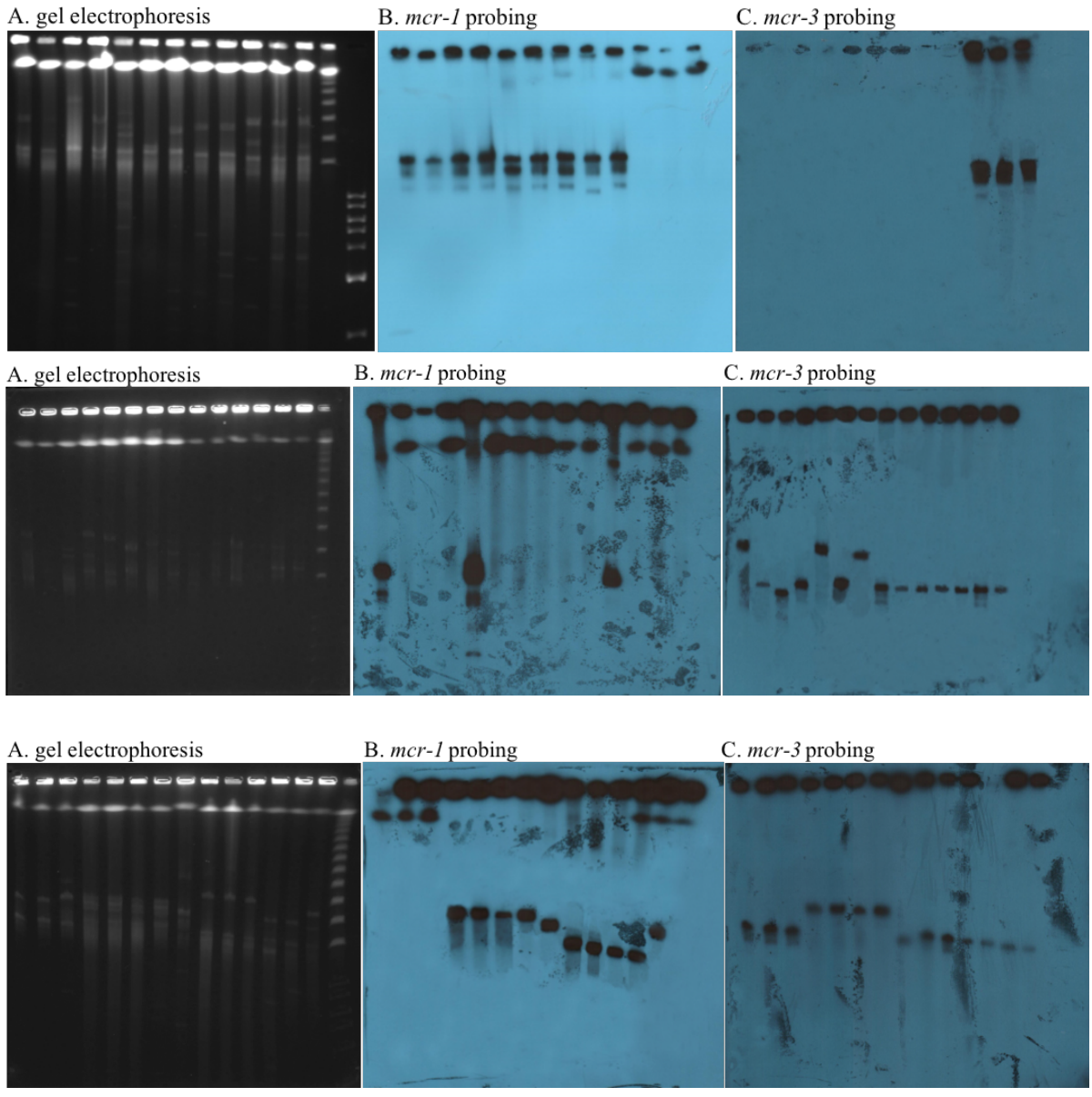


Fig.4.3 MCEPEC isolates were analysis by pulse-field gel electrophoresis (PFGE) with S1-nuclease, followed by ^{32}P hybridization with *mcr-1* or *mcr-3*-probe.

Labels of 1-12 lands in **A**: 1474.1; 1492.2; 1412.1; 1472.1; 1479.1; 1518.1; 1466.1; 1535.1; 1537.1; 1404.2; 1272.2, 1271.3.

Labels of 1-14 lands in **B**: 1271.1; 1456.5; 1409.2; 1404.2; 1050.1; 961.3a;763.3; 657.1; 657.4; 657.5; 621.1; 1364.4; 1335.2; 1335.1.
Labels of 1-14 lands in **C**: 75.5; 75.3; 2.5; 225.5; 225.4; 225.2; 225.1; 83.5; 83.2; 269.2; 269.3; 1270.5; AV106.1; AV28.1

Using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/), the Inc-types of *mcr-3*-linked plasmids were identified in thirteen isolates, including nine IncP1 plasmids, three IncFII plasmids and one IncX1 plasmid **(Table 4.1)**. Due to the limitations of short-read sequencing and the abundance of repeat regions in the plasmids, it is difficult to obtain the complete sequence of *mcr-3*-bearing plasmids. Fortunately, using primer walking (HN1364.4_Forword primer: CAGCTTGGAGACATGAAGCG; HN1364.4_Reverse primer: TGGCCGTGTACGTGTCATAC), followed by sanger PCR sequencing (Eurofins Genomics, Germany), one fully complete 51,958bp IncP1 plasmid has obtained (**Fig.4.3A**). Its nucleotide sequence is close to two *mcr-3*-carrying plasmids pMCR3_WCHEC1943 (GenBank accession No. MF678351.1, China), and pMCR3_WCHEC-LL123 (GenBank accession No. MF489760.1, China), with 97% coverage and 99% nucleotide identity. This plasmid has a typical IncP-type backnone coding replication initiation (*ssb-trfA* operon), conjugative transfer (*trb* gene cluster regulating the mating-pair formation, and *tra* cluster ecoding transfer protein) and maintenance modules (*kle* and *klc* genes promoting stability inheritance; *parB* gene activating the participation of plasmid copies into daughter cells) (Schluter *et al.*, 2007, Norberg *et al.*, 2011). The colistin resistance encoding region is approx. 4000bp *mcr-3-dgk* cassette, which is bracketed by two mobile transposons (Tn*3* transposon located at upstream and IS*6* transposon at downstream). Additionally, the backbone of this *mcr-3*-positive IncP1 plasmids in are highly conserved in another eight IncP1 plasmids identified in the studied strains (**Fig.4.3B**), suggesting that MCR-3_IncP_HN1364.4-like IncP plasmid is widely distrubuted in Vietnam. Due to its broad host-rangs, IncP plasmids can act

as a efficient vector to transfer *mcr-3* gene between different species, posting a great threat to human health.

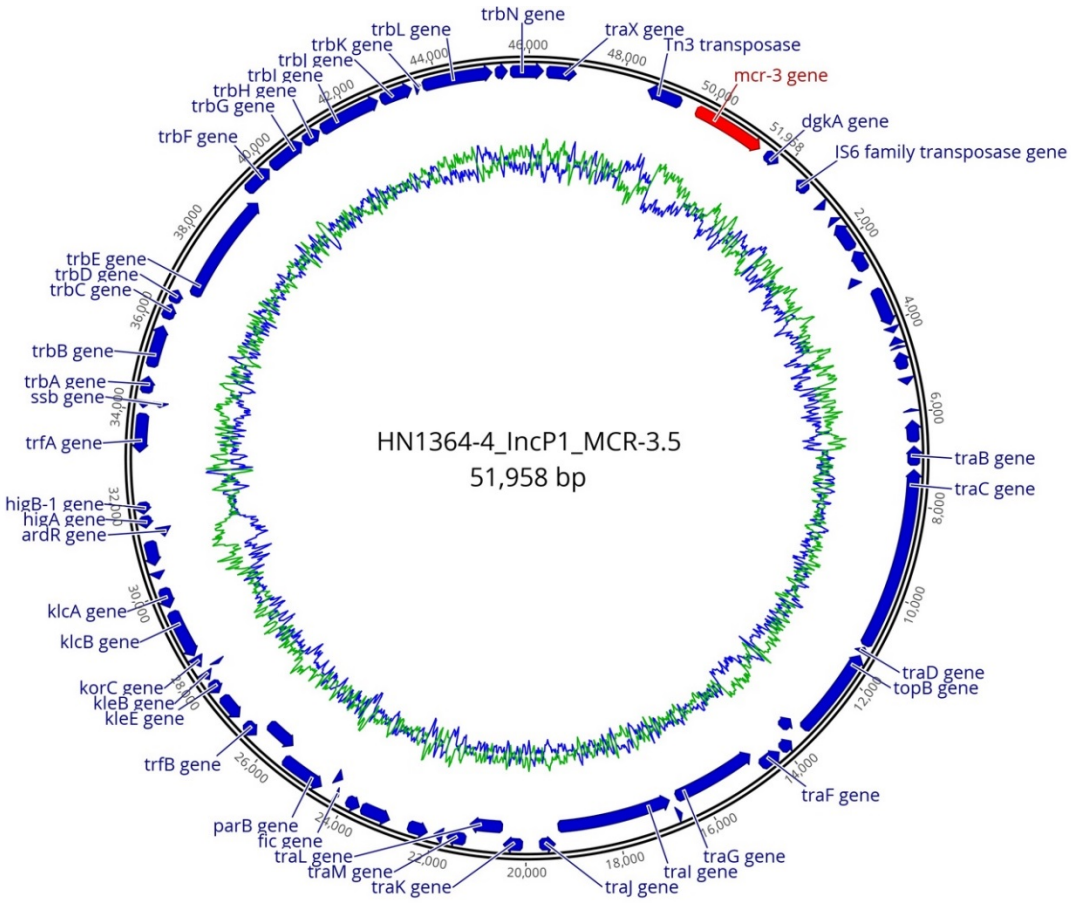


Fig.4.3A Circular presentation of the complete nucleotide sequencing of *mcr-3* containing IncP plasmid in *E. coli* strain HN1364-4 from human stool in Vietnam. Arrows indicate open reading frames (ORFs) with annotation, ORFs without annotation indicates hypothetical protein. Numbers indicate nucleotide positions. The inner circle represent CG (blue) /AT (green) content.

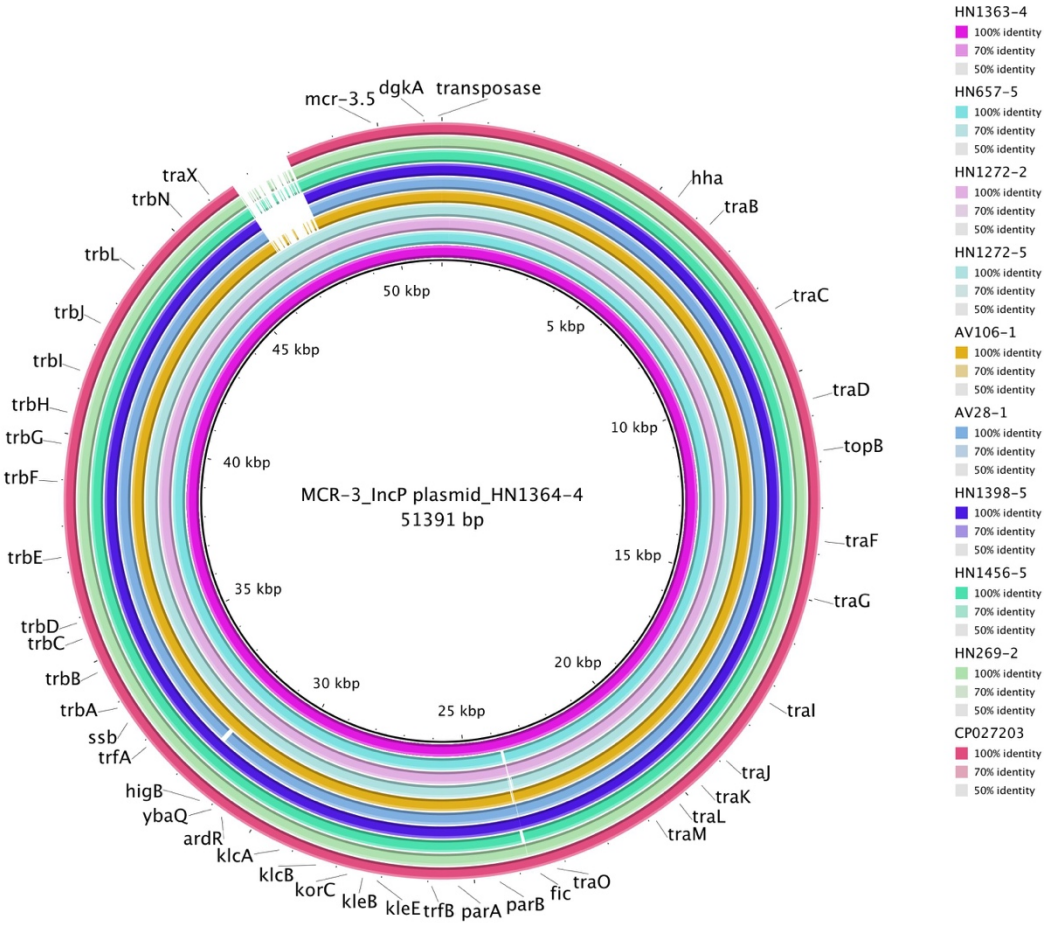


Fig.4.3B Alignment of 10 *mcr-1*-positive IncP1 plasmids and visualized using BLAST Ring Image Generator (BRIG v0.9555). First inner ring is the plasmid obtained from HN1364-4, used as reference for the alignment, name and size of the reference plasmid indicated in the middle of rings.

4.2.2 The fitness cost of *mcr-3*-mediated colistin resistance in *E. coli*

In chapter 3, the results show that *mcr-1*-mediated colistin resistance is associated with great fitness cost observed by growth arrest, cell viability and morphological changes. It is important to understand the effects of colistin resistance mediated by two new variants *mcr-3.1* and *mcr-3.5* on bacterial fitness. The *mcr-3.1* and *mcr-3.5* genes were cloned into an expression vector pBAD/hisA and transferred it into *E. coli* TOP10, generating *E. coli* TOP10 (*mcr-3.1*::pBAD and *mcr-3.5*::pBAD). Once expression was induced with 0.2% (w/v) L-arabinose, a markedly slower growth was observed in both *mcr-3*-strains, when comparing to their respective uninduced controls (**Fig.4.4)**, suggesting that the overexpression of both *mcr-3* variants is also associated with a reduced competitive ability against antibiotic-sensitive strains. Of note, the over-production of *mcr-3.5* seemed to be 'less toxic', observed by higher growth rates, compared to that of *mcr-1* and *mcr-3.1*. These results indicate that over-expression of *mcr-3* impose a lesser fitness cost, when compared to *mcr-1*-producing *E. coli*.

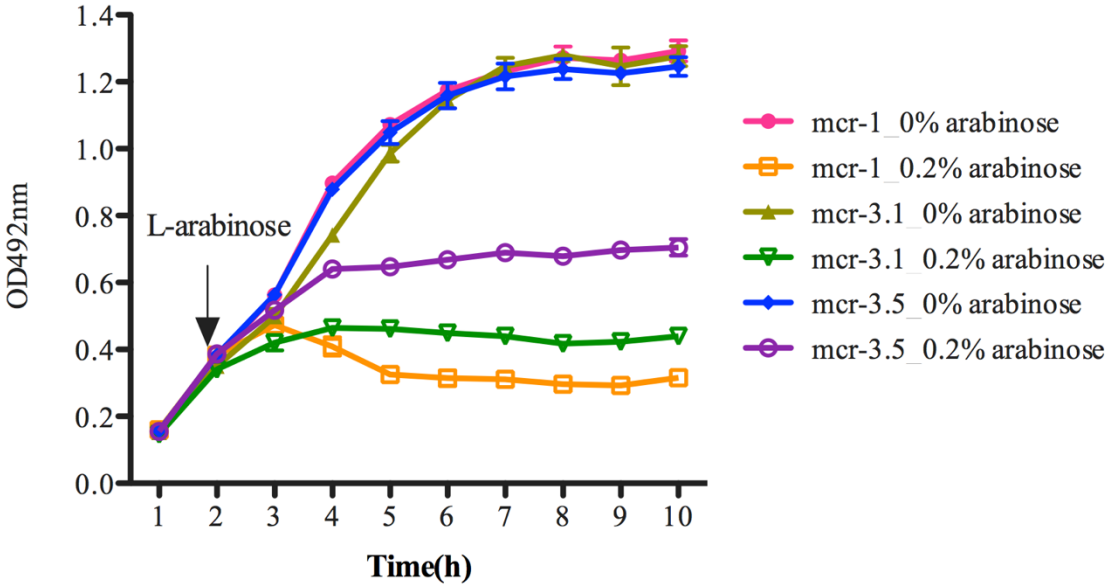


Fig.4.4 Effects of the expression of three *mcr*-variants, *mcr-1*, *mcr-3.1* and *mcr-3.5* on bacterial growth rate *in vitro* (n=3).

4.2.3 The effect of the expression of *mcr-3* genes on *E. coli* cell viability

Based on the above growth rate and cell population, *mcr-3*-carrying *E. coli* may provide a relative fitness advantage over *mcr-1*-positive isolates. LIVE/DEAD® staining with confocal laser scanning microscopy (CLSM) imaging and COMSTAT analysis was used to assess cell viability. After 8h induction of gene expression with 0.2% (w/v) L-arabinose, a minimal reduction (less than 9%) in bacterial viability was observed in both *mcr-3*-producing (*mcr-3.1* and *mcr-3.5*) *E. coli* TOP10 strains, compared with the over-producing *mcr-1 E. coli* TOP10 cells (**Fig.3.4** in Chapter 3). COMSTAT analysis of the CLSM biofilm images revealed that relative dead/live biomass ratio of induced *E. coli* TOP10 (*mcr-3.1*/pBAD) was approximately three times higher than induced *E. coli* TOP10 (*mcr-3.5*/pBAD) (9% vs. 3%) (**Fig.4.5**). The above data suggest that both *mcr-3* novel variants (*mcr-3.1* and *mcr-3.5*) have evolved to attenuate the toxic effect of this gene's expression, and supports the above fitness

results. Furthermore, the three amino acid substitutions in *mcr-3.5* may play an important role in bacterial compensatory mutation that can alleviate the fitness burden and toxicity imposed by over-producing *mcr-3.1*, without altering the level of colistin resistance.

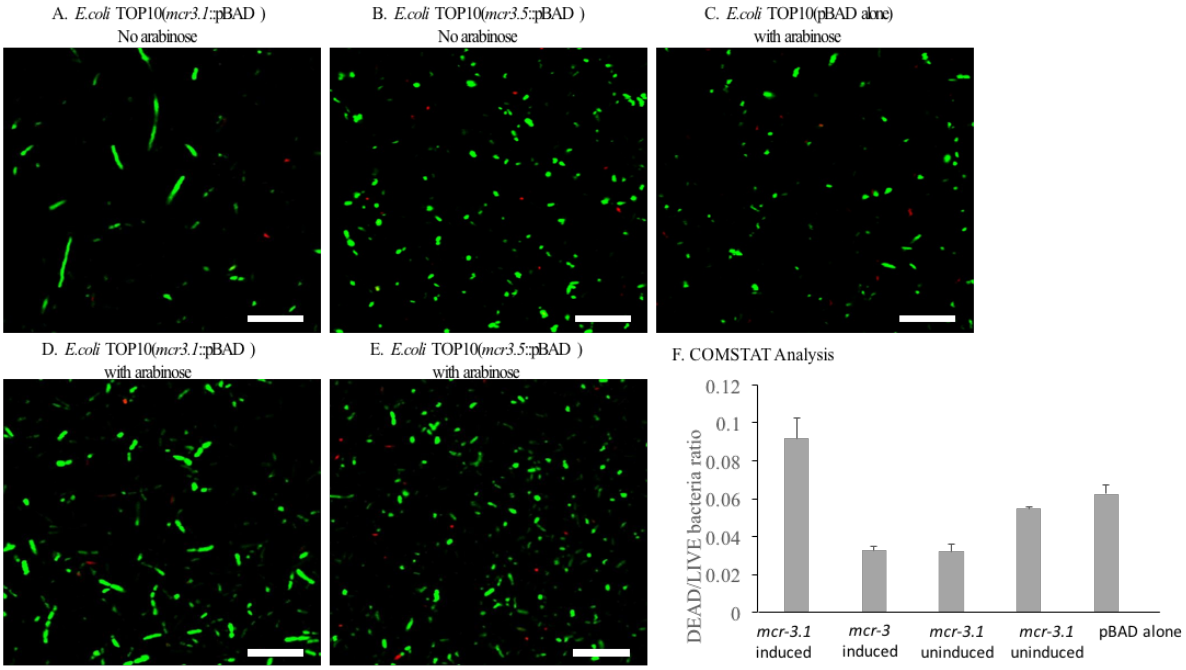


Fig.4.5 The toxic effects of *mcr-3* overexpression on cell viability. **A-D**, Confocal Laser Scanning microscopy images of cells treated with/without L-arabinose and stained with LIVE/DEAD® (n=3). Live and dead cells presented green and red colour, respectively. Scale bar is 15 µm. **D**, Ratio of Dead to Live bacteria (biomass) obtained from CLSM z-stack images through COMSTAT analysis of *E. coli* biofilms grown for 16 h in LB broth, followed by ± L-arabinose (0.2% w/v; 8 h) treatment, where the biofilms were stained with LIVE/DEAD® (n=4). The COMSTAT data was assessed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons post-hoc test.

4.2.4 Over-expression of *mcr-3* significantly effects cellular morphology

MCR-3 belongs to *mcr-1*-like phosphoethanolamine transferase group, which can catalyze the transfer of phosphoethanolamine onto the phosphate group of lipid A in the bacterial

outer-membrane (Hinchliffe *et al.*, 2017). I investigated whether both *mcr-3.1* and *mcr-3.5* affect bacterial morphological changes by using transmission electron microscopy (TEM). Without *mcr-3* expression, *E. coli* TOP10 (pBAD alone) showed normal cellular characteristics with a multi-layered cell surface and identical electron density **(Fig.4.6).** However, following *mcr-3* gene expression induction by 0.2% (w/v) arabinose for 8 h, a loss of electron density and impaired cell wall integrity were observed in both *mcr-3.1*- and *mcr-3.5*-producing *E. coli* TOP10 relative to empty pBAD vector transformed control cells (**Fig.4.6**). These findings are consistent with my previous findings of the influence of *mcr-1* overexpression on bacterial morphology. Ergo, it is the high-level expression of *mcr* genes (*mcr-1*, *mcr3.1* and *mcr-3.5*) that induced these gross morphological changes.

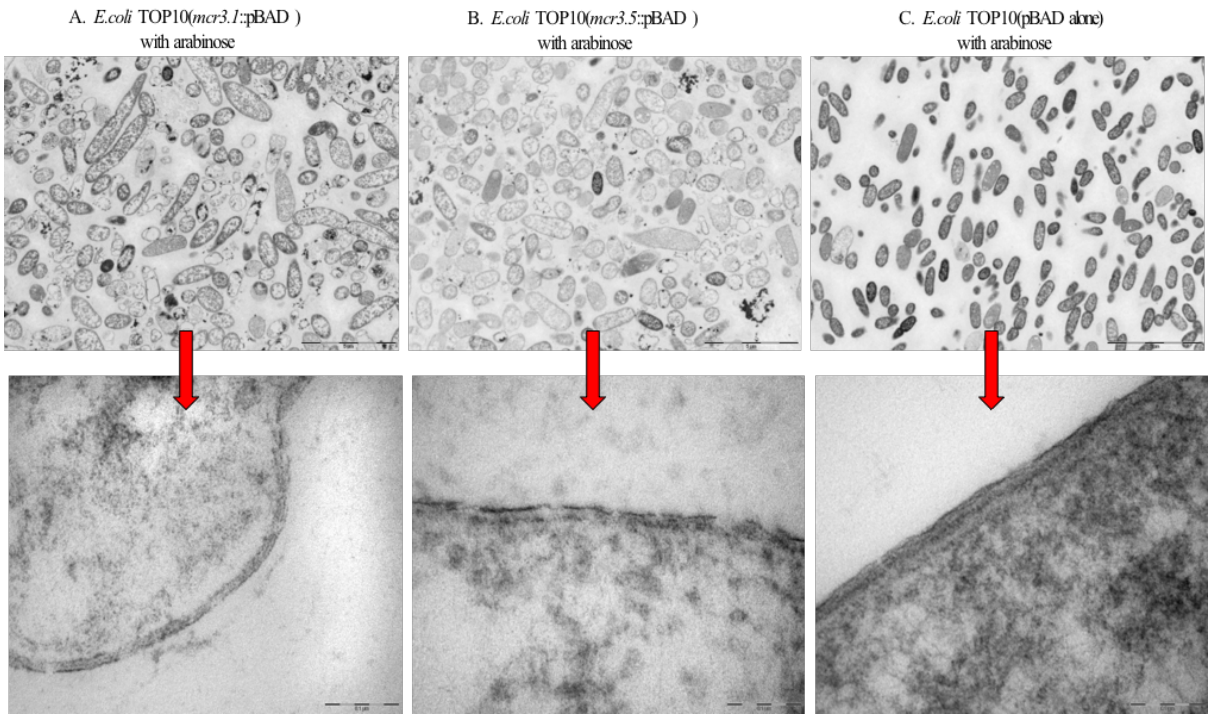
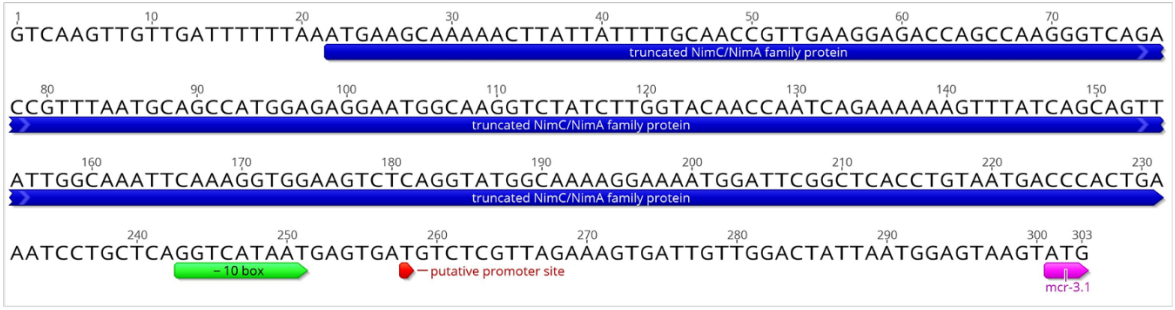


Fig.4.6 TEM micrographs of *mcr-3.1* and *mcr-3.5* over-producing cells in A and B, respectively, the damaged outer-membrane and some completely lysed cells were observed. C. TEM micrographs of of control strain TOP10 (pBAD alone) *E. coli*, exhibited the intact outer membrane structure and highly homologous electron density.

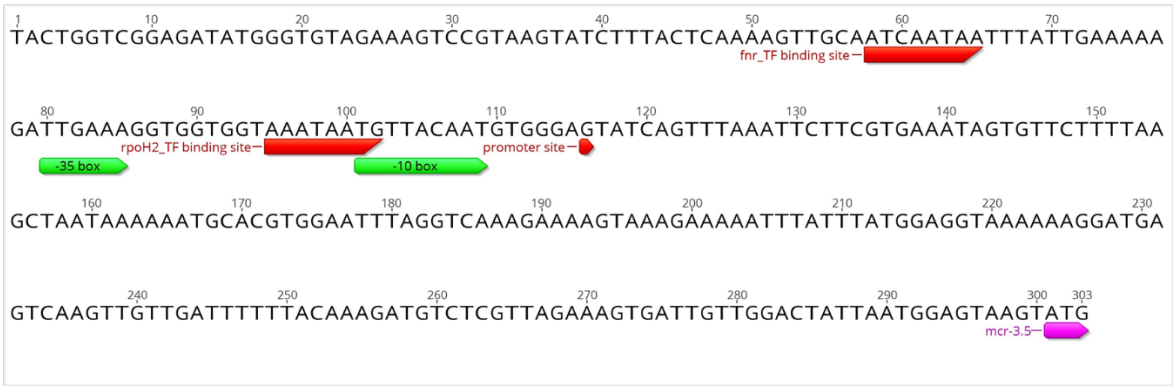
4.2.5 Transcription activity of *mcr-3-linked* promoters

So far, the regulation of *mcr-3* gene expression is still unknown and the impact of *mcr-3* linked promoter on gene expression needs to be further explored. Based on the Illumina sequencing data, putative *mcr-3*-linked promoter sequences are either intact or truncated (**Fig.4.7**). It would be interesting to compare the transcription activities in these two types of promoters. Unfortunately, no truncated *mcr-3*-linked promoters was found in my MCRPEC collection, the mcr-3-linked truncated promoter was obtain from plasmid pWJ1 (GenBank accession No. KY924928) (Yin *et al.*, 2017). Promoter sequencing was analysed online (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). Primers were designed and approximately 500bp and 300bp DNA fragment containing *mcr-1* and *mcr-3* putative promoters, respectively, was cloned into the pGLOW-TOPO vector (ThermoFisher, UK), as per manufacturer's instructions, resulting in two constructs P_*mcr-1*-pGLOW and P_*mcr-3_intact*-pGLOW (see methods **2.2.5** in **Chapter 2**). Equal background fluorescence emission was seen in the cultures carrying negative control plasmid (empty pGLOW-TOPO vector). Transcriptional activity of both *mcr-3*-linked promoters was measured by the GFP reporter assay using flow cytometry. Unexpectedly, **Fig.4.8** shows that GFP expression driven by an intact promoter was approx.10-fold higher than that of *mcr-1* promoter; however, the GFP expression observed under the *mcr-1* promoter appeared the same as the negative control pGLOW vector. Further analysis of the incomplete promoter sequence indicates that the promoter sequence has been truncated by a *nimC/nimA* gene (**Fig.4.7**), resulting in lack of transcription factor binding site. It is necessary to further analyse the effect of mcr-3-truncated promoter on *mcr-3* expression.

A. Truncated *mcr-3.1*-promoter sequence (GenBank No. KY924928)



B. Intact *mcr-3.5*- promoter sequence from MCRPEC collection



C.

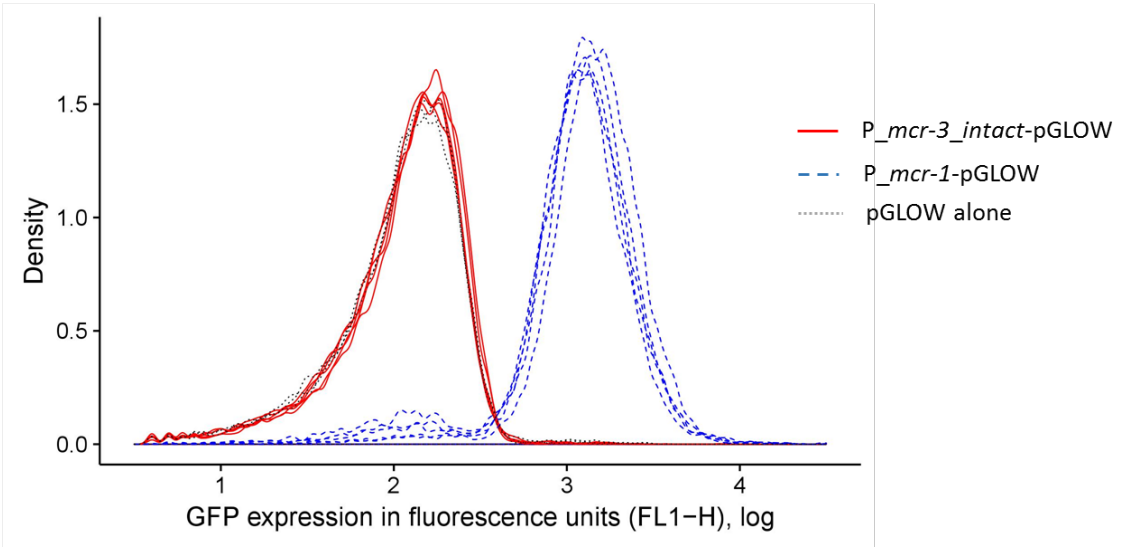


Fig.4.7 A and B, *mcr-3*-linked promoter sequencing analysis. 'TF' binding site indicates transcription factor binding sites, which present the most essential elements in a promoter (Browning & Busby, 2004). In (A), *mcr-3*-linked promoter sequence were truncated by *nimC/nimA* gene and no TF binding sites were found in the promoter. In (B), *mcr-3*-linked promoter sequence seems to be intact. (C) Transcriptional analyses of the MCR-3 protein. The strength of GFP expression driven from two different *mcr-3*- and *mcr-1*- linked promoter constructs in strain TOP10 *E. coli*. To measure bacterial-

GFP activity, log-phase cultures of *E. coli* TOP10 carrying the transcriptional fusions of either P_*mcr-3.5-intact*-pGLOW or P_*mcr-1*-pGLOW were sampled, followed by GFP signal measurement using flow cytometer. The data presents five biological replicates. The data suggested that the transcriptional level of the intact promoter is appreciably 10-fold higher than that of the incomplete promoter.

4.2.6 Compensatory adaptation to the deleterious effects of *mcr-3* genes in *E. coli*

The above results of bacterial growth curves suggested that there is a relative beneficial fitness to the isolates carrying *mcr-3* genes, especially for *mcr-3.5*, when compared to carrying *mcr-1*. To test this hypothesis, *in vitro* competition experiments were performed using flow cytometry (see methods in **2.13.1** in **Chapter 2**). The relative fitness is shown in **Table 4.2** and as anticipated from above observations, the expression of *mcr-3.5* has a fitness advantage over than that of *mcr-3.1***.** These results indicate that the high-level expression of both *mcr-3.1*- and *mcr-3.5*- was associated with a fitness burden *in vitro* (average relative fitness is 0.73 and 0.85, respectively), compared to its relative uninduced control *E.coli* TOP10 (*mcr-3*/pBAD) (**Fig.4.8**). Fitness costs can often be ameliorated by compensatory mutations, without losing antibiotic resistance. In contrast to a dramatic fitness loss for overexpression of *mcr-3.1*, evidenced by lower growth rate, *mcr-3.5* appears to have genetically evolved through three second-site compensatory mutations, which may play an important role in decreasing the deleterious effect of expression of *mcr-3.1*. To test this hypothesis, a total of six single and/or dual site mutations were constructed using Q5 Site-Directed mutagenesis kit (NEB, UK), resulting in a total of eight *mcr-3* variants constructed (**Table 4.2**). Except for the single mutation V23M, which confers the same fitness cost to the strain carrying *mcr-3.1*, the rest of six mutants showed a higher fitness over *mcr-3.1*-harboring strain (**Fig.4.8** and **Table 4.2**). Mutant T488I has already been found in patients with bloodstream infection (Roer *et al.*, 2017), moreover, as described above, 51 *mcr-3*-

positive *E. coli* isolated from human stool in Vietnam, 27 isolates were *mcr-3.5* while 19 were *mcr*-3.1 positive (Table 4.1). In combination with epidemiological data, the fitness data indicates that *mcr-3.5* might become the predominant *mcr-3* variant in Vietnam.

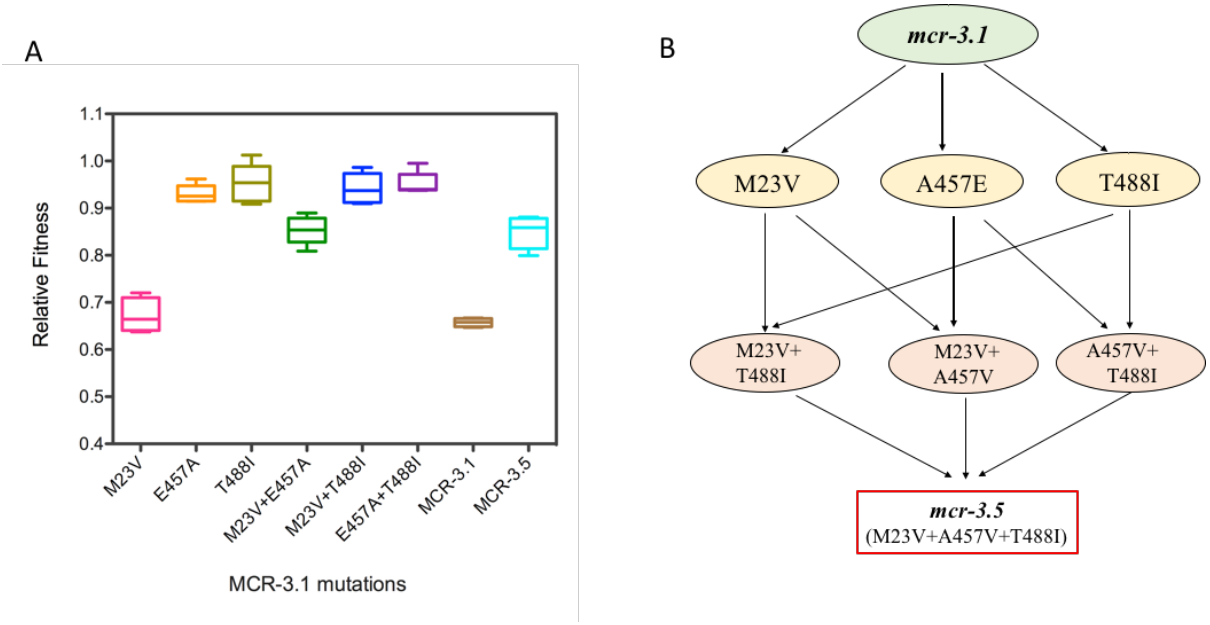


Fig.4.8 A. average relative fitness of eight *mcr-3* variants. All data presents six biological repeats and error bars represent the SD (n=6). **B.** The adaptive landscape of colistin resistance *mcr-3.5* conferred by three amino acid substitutions in the sequence of *mcr-3.1* gene.

Table 4.2 Eight possible evolutionary trajectories of *mcr-3* variants constructed by site-directed mutagenesis (see methods 2.2.4 in **Chapter 2**)

Plasmid templates	Primers' name	Targeted mutations	Relative fitness
mcr-3.5/pBAD	WT(M23V+A457E+T488I)	M23V+A457E+T488I	0.85
mcr-3.5/pBAD	V23M	A457E+T488I	0.95
mcr-3.5/pBAD	E457A	M23V+T488I	0.84
mcr-3.5/pBAD	I488T	M23V+A457E	0.85
mcr-3.5/pBAD	V23M+ E457A	T488I	0.95
mcr-3.5/pBAD	V23M+ I488T	A457E	0.83
mcr-3.5/pBAD	E457A+ I488T	M23V	0.73
mcr-3.1/pBAD	WT		0.73

4.2.7 Competitive ability of *mcr-1* and *mcr-3*-plasmids

The above observations raise an interesting puzzle: Why do bacteria need two different *mcr*-like genes, both with the same activity to reduce colistin affinity to the bacterial outer membrane? The above data examining the effect of the expression of *mcr*-like genes on bacterial growth rates, morphological changes and cell viability, indicates that *E. coli* carrying *mcr-3.5* in particular, has fitness advantages over *E. coli* carrying *mcr-1* gene. In order to test this hypothesis, two *in vitro* competition models were built. Firstly, three non-duplicate clinical strains harboring both *mcr-1* and *mcr-3* plasmids were chosen to examine the dynamics and abundance of both *mcr*-genes during 14-day serial passaging in the presence and absence of colistin. In this experiment, qPCR was performed to quantify the relative changes of *mcr-1* and *mcr-3* genes in each culture. As shown in **Fig.4.9,** the abundance of both *mcr-1* and *mcr-3* gene/plasmids was significantly decreased in colistin free

medium (p<0.001), indicating that both plasmids confer fitness cost and plasmid-free cells are more successful when the selective pressure of colistin is removed. However, there were decreases in *mcr-1* or *mcr-3* carriage in the presence of colistin as well, just less marked. The stability of *mcr-1* plasmid seemed to be slightly higher in the presence of colistin than that in antibiotic-free medium. Most importantly, the relative fitness of *mcr-3* was higher over time. I plotted the y axis as log10 (MCR3/MCR1), therefore the slope of this regression gives the relative fitness of the two strains (**Fig.4.9**). In this case, there is no significant difference in the presence of colistin, indicating that both relative fitness of *mcr-1* and *mcr-3* plasmids are consistent under colistin selective pressure (4 mg/L). However, a marked change is observed in the antibiotic-free condition (p=0.023), which suggests that *mcr-3* plasmid probably has a higher fitness even in the absence of the antibiotic (**Fig.4.9**).

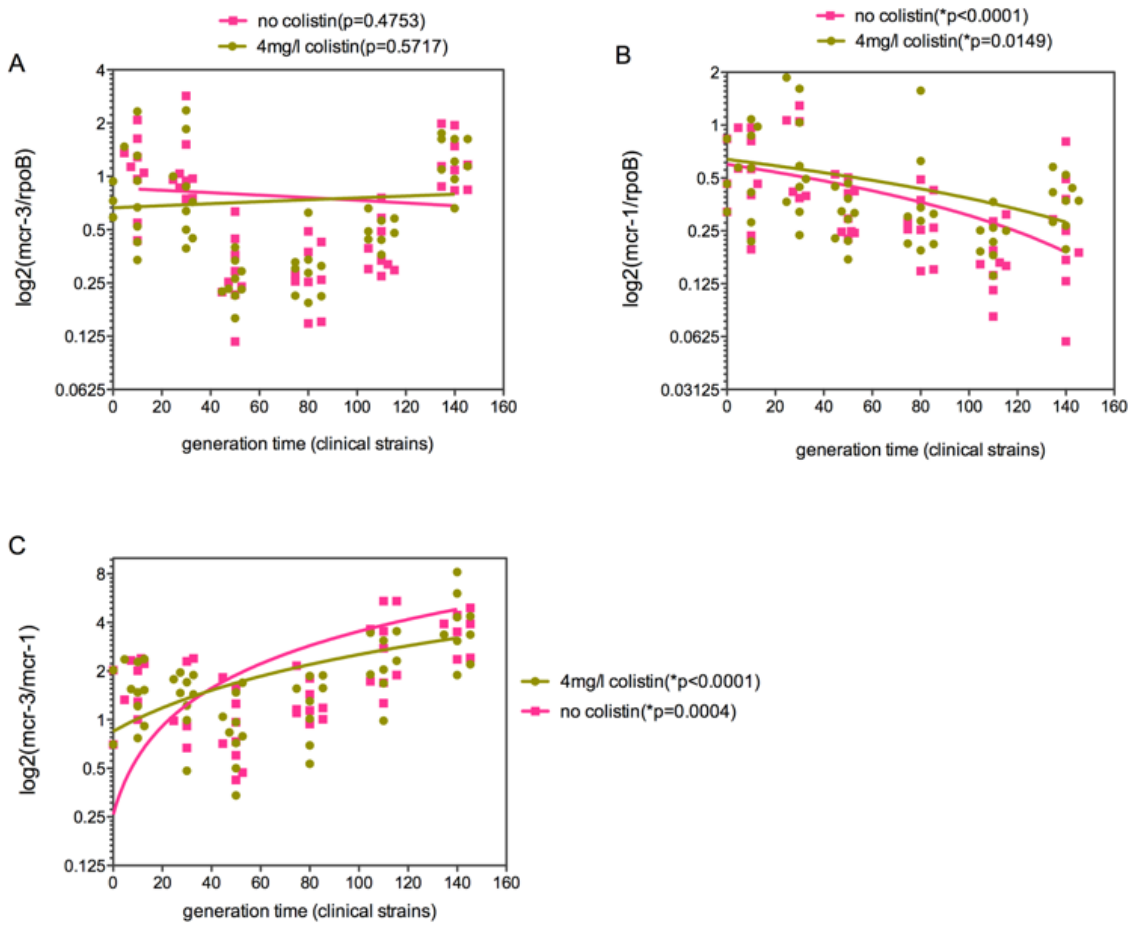


Fig.4.9 Change in the relative abundance of *mcr-1* and *mcr-3* gene/plasmid in clinical *E. coli* strains, which naturally carry both *mcr-1* and *mcr-3* plasmids. (A) and (B), the dynamic changes of both *mcr-1* and *mcr-3* gene in monocultures, respectively. (C) The relative frequency of *mcr-3* gene to *mcr-1* gene in both different treatments (with or without colistin). These estimates were calculated by linear regression. Standard errors were based on the regression and all p values of the regressions were indicated in the figures.

In order to rule out other influencing factors in clinical strains, a pair of *mcr-1*- and *mcr-3*-carrying plasmids were transferred to J53 strain by conjugation. The strains were allowed to adapt over the course of 80 generations to serial transfer culture in colistin free or 2 mg/l colistin LB broth. As controls, monocultures of each plasmid were also processed in the same way. **Fig.4.10** shows that the abundance of both plasmids declined strikingly in the monocultures. Plasmid abundance tended to be higher when the medium was supplemented

with 2mg/l colistin, than that of colistin-free medium. Furthermore, initial plasmid abundance was higher for *mcr-1* than *mcr-3*, possibly reflecting a difference in plasmid copy number. Interestingly, the rate of decline in both *mcr-1*- and *mcr-3* was essentially constant across the 14-day passage (**Fig.4.10**). The changes in the relative frequency of *mcr-3* abundance relative to *mcr*-1 in mixed cultures can be observed in **Fig.4.10C**. The dynamics of both plasmid bearing strains was decreased, moreover, the *mcr*-3 strain had a higher fitness than the *mcr-1*-carrying strain in the presence of colistin. This is consistent with the above observations of clinical strains with both *mcr*-like-carrying plasmids. Taken together, both *mcr-1* and *mcr-3*-carrying plasmids impose a fitness burden in the host, and *mcr-3* plasmid in both clinical and laboratory strains has fitness advantage over *mcr-1*-plasmid in the presence of antibiotic.

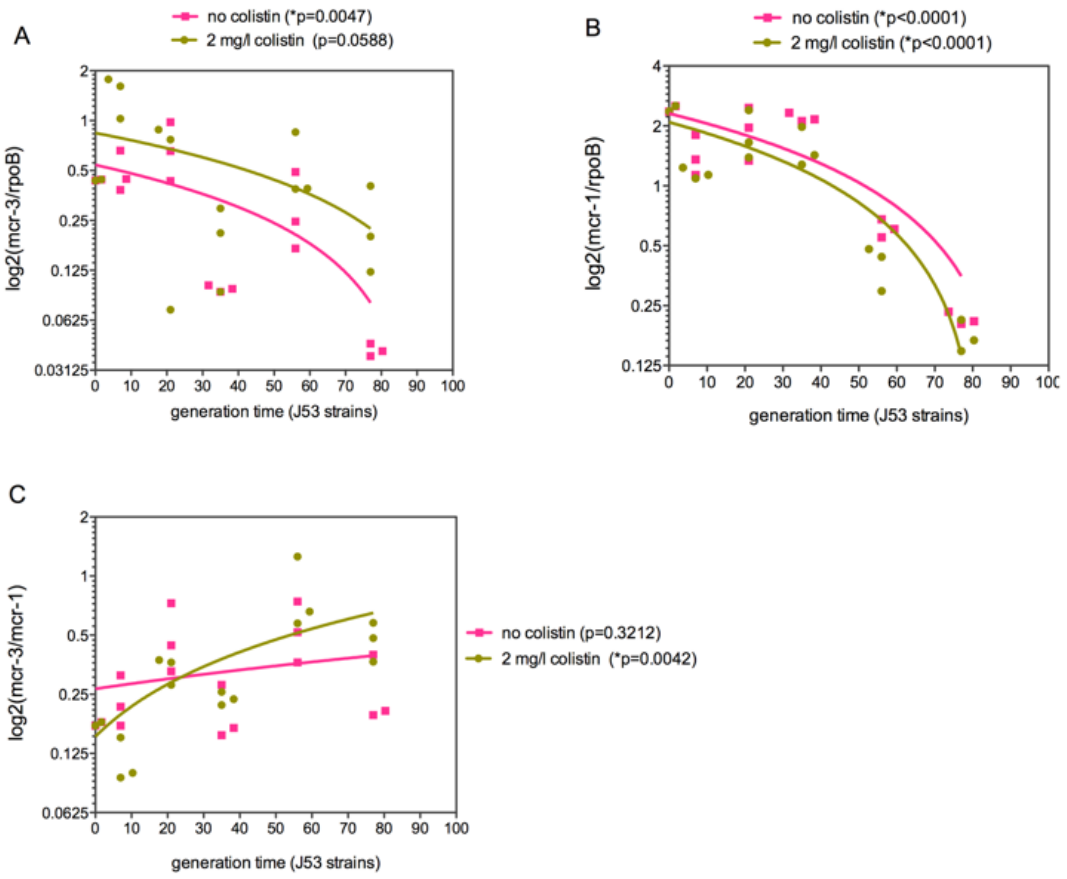


Fig.4.10 The relative abundance and stability of *mcr-1* and *mcr-3* gene/plasmid in laboratory *E. coli* strain J53. (A) and (B), the dynamic changes of both *mcr-1* and *mcr-3* gene in monocultures,

respectively. (C) The relative frequency of *mcr-3* gene to *mcr-1* gene in both different treatments (with or without colistin). These estimates were calculated by linear regression. Standard errors were based on the regression and all *p* values of the regressions were indicated in the figures.

4.3 Brief Discussion

Currently, attention towards antibiotic resistance is growing, reflected by increased global reports of antimicrobial resistance by surveillance projects, and diversity of the underlying mechanisms studied by global scientific researchers. There is no doubt that colistin is the main driver for the emergence of colistin resistance. Large quantities of colistin have been used in food-producing animals as therapeutics or growth promoters in some countries (Carrique-Mas *et al.*, 2015, Liu *et al.*, 2015, Health, 2016), and colistin is still considered to be of vital importance to treat human infections (Kluytmans, 2017). Bacteria always can find some strategies to continue growing even under such selective pressure, generating some potential trajectories of evolution through step-wise accumulation of resistance mutations, or acquiring resistance genes to colistin. The presence of varied concentrations of colistin in different settings such as hospital, community, animal farms and environment, provide many adaptive landscapes for bacteria to evolve genotypes of colistin resistance, therefore resulting in the diversity of genotypes in bacterial populations. Up to the preparation of this thesis, there are eight groups of plasmid-borne *mcr-like* gene conferring low-level resistance to colistin, have been identified. It should be noted that few data are available on the prevalence of *mcr-like* genes including *mcr-2, -3, -4, -5, -6, -7* and *-8*, other than *mcr-1* in human samples. However, it is still unclear to what extent these *mcr*-like genes influence the evolution of colistin resistance. We are currently expanding this research programme by studying whether the carriage of *mcr*-like genes would affect bacterial evolution to high-level resistance to colistin under antibiotic pressure, and the genetic variations between mutants

from the same ancestor during *in vitro* evolutional pathway, which may enable us to identify associated genes driving antibiotic resistance.

In order to better understand the genetic reservoirs and transfer trajectories of antibiotic resistance genes, we need to determine the characteristics of plasmids linked to resistance genes, such as efficiency of replication, the range of hosts, conjugative transfer rate and plasmid stability. It has been reported that over 90% of *mcr-1*-linked plasmids belonged to three narrow host-range plasmids, IncX4, IncHI2 and IncI2, to a lesser extent, other incompatibility groups such as IncFII, IncFIB or IncHIA/B, also have been identified (Matamoros *et al.*, 2017). Worryingly, in this study, *mcr-3* gene from nine *E. coli* strains were identified on plasmids carrying IncP1, which is widely distributed in GNB, including *E. coli*, *Pseudomonas* spp. and *Klebsiella aerogenes* (Popowska & Krawczyk-Balska, 2013). The association of *mcr*-like genes and the broad-host range plasmid is of increasing concern, due to its highly efficient conjugative transfer and ability to replicate in a broad range of hosts. Future studies are required to determine the risk factors on the spread of *mcr*-like genes located on these plasmids.

Predicting evolutionary paths to antibiotic resistance is key to understanding and preventing the rapid emergence of drug resistance, and antibiotic resistance can evolve through the sequential accumulation of multiple resistance-conferring mutations in a single gene (Hartl, 2014). Given the relatively small number of mutation in the same gene or different genes that might contribute to bacterial compensatory adaption, it would be helpful to understand adaptive evolution pathways by constructing all possible combination of these mutations and determining their contribution to adaption. For example, Palmer and colleagues have identified multi-peak adaptive landscapes for trimethoprim resistance by constructing all combinatorial alleles of seven mutations in dihydrofolate reductase (Palmer

et al., 2015). The epistasis interactions increase the accessibility of each path, and more indirect rather than direct pathways were observed, where mutations are adaptively gained and later adaptively lost or changed (Palmer *et al.*, 2015). The complex interactions allow the evolution of trimethoprim resistance to proceed through multiple adaptive steps, hindering our ability to predict or control the emergence and spread of trimethoprim resistance. So far, more than 10 variants were identified in *mcr-3* gene, with few nucleotide mismatches (0-8 amino acid substitutions) (Zhang *et al.*, 2018). Two variants of *mcr-3, mcr-3.1* and *mcr-3.5* possess three nucleotide mismatches, with two choices for each, then there are 2^3 different combinations. Therefore, in this study, I have characterized the adaptive fitness landscape among *mcr-3* variants by constructing six possible combinatorial alleles of these mutant sites, followed by assaying their relative fitness by flow cytometry. I observed that six out of eight mutation combinations increased bacterial fitness, compared to the strain carrying the parent *mcr-3.1* gene, which was first discovered in porcine *E. coli* from China. Except for *mcr-3.1* and *mcr-3.5* identified in 19 and 27 *mcr-3*-posistive strains, respectively, another two mutated combinations (double mutants M23V+G373V, and single mutant D295E) were found in our collection. Our data suggests that the process of mutational gain in *mcr-3* gene may increase the opportunity of evolutionary pathways leading to the widespread colistin resistance in ever changing environments. However, it remains unclear which adaptive evolution pathway is affected by differences in amino acid sequence that do not alter the substrate binding or the catalytic activity of the enzyme, but may play an essential role in protein folding and the proper orientation of residues in and around the active site. The answer to this question needs to be further explored.

This study describes 51 *mcr-3* positive human *E.coli* isolates from Vietnam, and 39 of these isolates are especially relevant as they were positive for both *mcr-1* and *mcr-3*. Thus, it raises the question: Why do bacteria need to carry two *mcr*-like genes, although both *mcr-*

bearing plasmids conferring fitness burden with or without selective pressure? As shown in our competition model, both *mcr-1* and *mcr-3*-bearing plasmids had a significant fitness cost reflected by declining abundance of both genes during serial passages. Therefore, some strategies need to favor the plasmid-carrying hosts, allowing the resistant plasmids to persist in the population. Integrating the *mcr-1* gene into their chromosome seems to be a good strategy to maintain the *mcr-1* gene in the bacterial host. In this study, chromosome-located *mcr-1* gene was identified in 18 out of 51 *mcr-3*-bearing isolates, therefore, there is no chance for these bacteria to easily remove *mcr-1* gene conferring colistin resistance. Taken together, there are at least four ways for bacteria to persist colistin resistance mediated by *mcr*-like genes: i) The fitness burden caused by *mcr-3.1* expression was reduced by epistatic mutations within the gene. ii) The *mcr-1* gene was integrated into chromosome, in order to increase the stability of *mcr-1* gene in bacterial population. iii) The combination of *mcr-1* and *mcr-3* genes in the same strain provides greater maintenance capacity for colistin resistance. iv) Diversity of *mcr*-linked genotypes were found in microbial populations. If the consumption of colistin does not reduce in livestock in particular, bacteria are continually exposed to a colistin-rich environment, thus, there is a strong reasons to expect bacteria to evolve new *mcr*-like variants: *mcr-6*, *mcr-7* (Yang *et al.*, 2018), *mcr-8* (Wang *et al.*, 2018) ad infinitum.

Chapter 5

Dissemination of highly virulent *mcr-1* positive *Klebsiella pneumoniae* clone by *Chrysomya* spp. (common blowfly): an under rated factor in the AMR contagion story

5.1 Brief background

There is growing public health concern for environmental dispersal of antibiotic resistance. Flies have been recognized as potential reservoirs participating in the spread of antibiotic-resistant pathogens among animals, environments and human (**Fig.5.1**). It has been demonstrated that houseflies are involved in the mechanical transmission of nosocomial infections with multidrug resistant bacteria in hospital environments, such as *Shigella* spp.; *Escherichia coli*; *Klebsiella* spp. and *Enterobactor* spp. (Graczyk *et al.*, 2001). Recent studies reported flies are also responsible for the spread of *mcr-like* genes conferring colistin resistance in pig farms in Germany (Guenther *et al.*, 2017), as well as in a Chinese university hospital with high-prevalence of *mcr-1* (51.1%) and *mcr-3* (44.4%) (Zhang *et al.*, 2018), suggesting that blow flies may servers as a reservoir for *mcr* genes. Among them, *mcr-1* was most commonly found in *E. coli* isolates, although several other Enterobacteriaceae including *K. pneumoniae* have been detected in other sources such as animals (Wang *et al.*, 2018) and human clinical isolates (Li *et al.*, 2018). *K. pneumoniae* is known to be a leading cause of hospital-acquired infections, such as pneumonia, post-surgical wound and urinary tract infections (Holt *et al.*, 2015). It is especially problematic in hospitals when resistance to colistin, the last-resort antibiotic, leaves very limited therapeutic options. More attention need to be paid to the transmissions of *mcr-1*-positive Enterobacteriaceae (MCRPE), in *E. coli* and

K. pneumoniae in particular, in order to provide a better understanding to control their threat to human health.

In this study, we screened blowflies (*Chrysomya* spp.) from different areas near the city of Phitsanulok, Northern Thailand, for the presence of AMR genes and in particular, *mcr-1*, using whole genome sequencing (WGS). In total, 48 *mcr-1*-positive isolates were recovered, consisting of 17 *mcr-1*-positive *Klebsiella pneumoniae* (MCRPKP) and 31 *mcr-1*-positive *Escherichia coli* (MCRPEC) strains. The 17 MCRPKP were shown to be clonal (ST43) and by indistinguishable PFGE patterns and few single poly nucleomorphs (SNPs) by WGS analysis. In *in-vitro* models, the MCRPKP were shown to be highly virulent. In contrast, 31 recovered MCRPEC isolates are varied, belonging to 12 different MLST groups shared with those causing human infections. The majority of *mcr-1* genes are located on IncX4 plasmids (29/48, 60.42%), sharing an identical plasmid backbone. WGS-based analysis of *mcr-1*-carrying isolates from blowflies (*Chrysomya* spp.) provide evidence that flies serve as a potential 'hot spots' for the spread of *mcr-1*-mediated colistin resistance including hyper-virulent MDR *Klebsiella pneumoniae* isolates. These findings highlight the contribution of flies to the AMR contagion picture in low- and middle-income countries and the challenges of tackling global AMR.

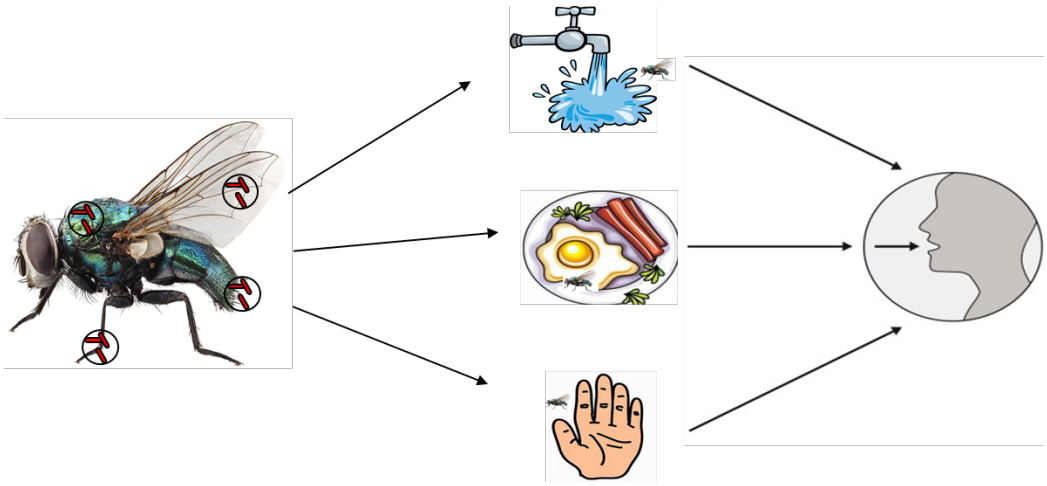


Fig. 5.1 Multiple routes of bacterial transmission between humans and animals through flies. Flies can

transmit microbes from place to place through their exoskeleton (such as legs and wings) and defecation (updated from (Junqueira *et al.*, 2017, Onwugamba *et al.*, 2018). Since flies share their habitat with both animals and humans, transmission of antimicrobial resistance is therefore possible: Flies can 'pick' bacteria on their legs and wings up from sewage and animal feces, and then deposit their microbial 'passengers' in elsewhere including food, water, meat in local markets or hands. Red sticks on the body of blowfly indicate bacteria.

5.2 Results

5.2.1 Details of *mcr-1*-positive isolates recovered from Blowflies

In early 2016, a total of 300 blowflies were trapped at three different areas in Northern Thailand, 100 blowflies from each area: local market in urban community, rural area and suburbs around Phitsanulok. These locations are approximately 10 kilometers apart. The flies were individually pulverized in enriched peptone water for 30 min and aliquots of these suspensions (100 µl) were plated on Eosin-Methylene-Blue (EMB) agar supplemented with 2 mg/l colistin and incubated at 37^{o}C overnight. One to three representative colonies with different colours from each agar plates were purified and the *mcr-1* gene identified by PCR. The *mcr-1*-positive bacteria were sub-cultured in liquid nutrient broth for 18h before DNA extraction for species identification and whole-genome sequencing. In total, we recovered 48 *mcr-1*-positive isolates from 300 collected blowflies (16.0%, each isolate represents one blowfly), consisting of 31 *E. coli* and 17 *K. pneumoniae*. Among them, four MCRPE strains are recovered from local market in the urban community, 16 and 28 are recovered from rural and urban areas, respectively. In addition, minimum inhibitory concentrations (MICs) of colistin for 48 *mcr-1*-bearing isolates was performed using broth microdilution, in accordance with the guideline of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Reference strain *E. coli* ATCC25922 served as a quality control. Colistin MICs for all MCR-1-producing isolates range from 4-16 mg/l (**Table 5.1**).

Table 5.1. Characteristics of 48 MCRPE strains recovered from blowflies in Northern Thailand

Strain ID	Species	Isolated area	colistin MIC	wgMLST	Inc plasmid type (size: kb)	Conjugation frequency
PN100	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$2.1x10^{-5}$
PN104	*K. pneumoniae*	Rural area	16	43	IncX4(~31,778)	$1.5x10^{-6}$
PN105	*K. pneumoniae*	Suburb area	8	43	IncX4(~31,778)	$1.5x10^{-5}$
PN106	*K. pneumoniae*	Suburb area	8	43	IncX4(~31,778)	$2.1x10^{-5}$
PN107	*K. pneumoniae*	Rural area	16	43	IncX4(~31,778)	$9.45x10^{-7}$
PN110	*K. pneumoniae*	Rural area	16	43	IncX4(~31,778)	$2.08x10^{-6}$
PN114	*K. pneumoniae*	Suburb area	8	43	IncX4(~31,778)	$7.63x10^{-6}$
PN118	*K. pneumoniae*	Suburb area	8	43	IncX4(~31,778)	$2.44x10^{-5}$
PN120	*K. pneumoniae*	Rural area	4	43	IncX4(~31,778)	$2.0x10^{-5}$
PN77	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$1.5x10^{-6}$
PN79	*K. pneumoniae*	Rural area	4	43	IncX4(~31,778)	$3.13x10^{-5}$
PN81	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$2.86x10^{-5}$
PN84	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$3.0x10^{-5}$
PN95	*K. pneumoniae*	Suburb area	8	43	IncX4(~31,778)	$1.0x10^{-5}$
PN96	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$7.11x10^{-7}$
PN97	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$2.31x10^{-6}$
PN98	*K. pneumoniae*	Suburb area	4	43	IncX4(~31,778)	$8.03x10^{-7}$
PN123	*E. coli*	Local market in urban community	4	2345	IncHIA	$1.44X10^{-7}$
PN33	*E. coli*	Local market in urban community	4	10	NA	$1.33x10^{-3}$
PN93	*E. coli*	Local market in urban community	4	162	IncX4	ND
PN103	*E. coli*	Rural area	8	10	IncHIA	$3.1x10^{-7}$
PN109	*E. coli*	Rural area	16	1244	IncX4(~31,778)	$7.6x10^{-4}$
PN111	*E. coli*	Rural area	8	457	NA	ND
PN116	*E. coli*	Rural area	4	648	NA	ND
PN119	*E. coli*	Rural area	8	549	NA	ND
PN74	*E. coli*	Rural area	16	10	IncX4(~31,778)	ND
PN75	*E. coli*	Rural area	4	58	IncHIB	ND
PN87	*E. coli*	Rural area	4	549	NA	ND
PN88	*E. coli*	Rural area	4	10	IncX4(~31,778)	ND
PN91	*E. coli*	Rural area	4	181	IncX4(~31,778)	$6.67x10^{-5}$
PN101	*E. coli*	Suburb area	4	58	NA	ND
PN102	*E. coli*	Suburb area	4	648	NA	ND
PN108	*E. coli*	Suburb area	8	549	IncX4(~31,778)	ND
PN112	*E. coli*	Suburb area	8	181	IncX4(~31,778)	ND
PN117	*E. coli*	Suburb area	8	201	IncX4(~31,778)	$5.94x10^{-3}$
PN121	*E. coli*	Suburb area	8	5487	NA	ND
PN122	*E. coli*	Suburb area	8	549	NA	ND
PN124	*E. coli*	Suburb area	8	181	IncX4(~31,778)	$5.33x10^{-4}$
PN126	*E. coli*	Suburb area	4	648	IncHIB	NS
PN127	*E. coli*	Suburb area	4	648	IncHIB	$1.85x10^{-7}$
PN73	*E. coli*	Suburb area	4	648	IncHIB	NS
PN76	*E. coli*	Suburb area	4	58	IncHIA_IncHIB	$8.70x10^{-8}$
PN78	*E. coli*	Suburb area	4	10	IncX4(~31,778)	ND
PN80	*E. coli*	Suburb area	8	218	NA	$1.14x10^{-4}$
PN83	*E. coli*	Suburb area	8	10	IncX4(~31,778)	$6.27x10^{-7}$
PN85	*E. coli*	Suburb area	4	2705	NA	NS
PN86	*E. coli*	Suburb area	8	218	IncX4(~31,778)	ND
PN92	*E. coli*	Suburb area	4	10	NA	ND

NA, not available; ND, not determined; NS, not successful

In July 2017, our collaborators discovered a new *mcr*-variant, *mcr-3* (Yin et al., 2017). MCR-3, belongs to the family of phosphoethanolamine transferases, only exhibited approximately 45% and 47% nucleotide sequence identity to *mcr-1* and *mcr-2*, respectively. For the screening of this new *mcr-3* genes, I received 148 *mcr-1* negative, colistin-resistant isolates recovered from blowflies in Thailand. Seven out of 148 isolates are *mcr-3*-positive. When comparing with *mcr-3.1*, which is available in NCBI, the *mcr-3*-like gene belongs to *mcr-3.5* with three difference amino acid mutations: Met23 to Val (position 67: **A**TG to **G**TG), Ala457 to Glu (position 1370: G**C**A to G**A**A) and Thr488 to Ile (position 1463: A**C**C to A**T**C). To elucidate the genetic mechanism of *mcr-3*-mediated colistin resistance, whole genomic sequencing were also performed on these seven MCRPEC strains isolated from Thailand (see full details in **Appendix II** and **Chapter 6**).

5.2.2 WGS-based analysis of *mcr-1*-positive isolates

A total of 48 *mcr-1* positive isolates were sequenced using Illumina Miseq platform. In brief, total gDNA was extracted from an overnight culture (2 ml) on a QIAcube automated system (Qiagen, Germany), and genomic DNA libraries are constructed using the Illumina MiSeq platform (MiSeq Reagent V3 Kit; 2x300 cycles). The CGE platform (http://www.genomicepidemiology.org/) were used for analysis of multi-locus sequence typing (MLST). As a result, the distinct *E. coli* isolates belonged to 12 STs (**Table 5.1**): ST10 (n=7), ST648 (n=5), ST549 (n=4), ST58 (n=3), ST181 (n=3), ST218 (n=2), ST201 (n=1), ST162 (n=1), ST457 (n=1), ST1244 (n=1), ST2345 (n=1), ST2705 (n=1) and ST5487 (n=1).

Most interestingly, all 17 *K. pneumoniae* isolates belonged to ST43, then we further determined the clonal relationship of 17 ST43 *K. pneumoniae* isolates using pulse-field gel electrophoresis identified with *speI* restriction enzyme, according to the CDC instruction (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). The

indistinguishable PFGE patterns were observed in all *K. pneumoniae* isolates mainly obtained from local market in urban community and rural area (**Fig. 5.2**). Moreover, phylogenetic tree analysis for 17 strains based on their raw sequencing reads showed that their core genome differed only by a few SNPs (n<10), suggesting clonal dissemination of ST43 *K. pneumoniae* isolates in blowflies from Thailand.

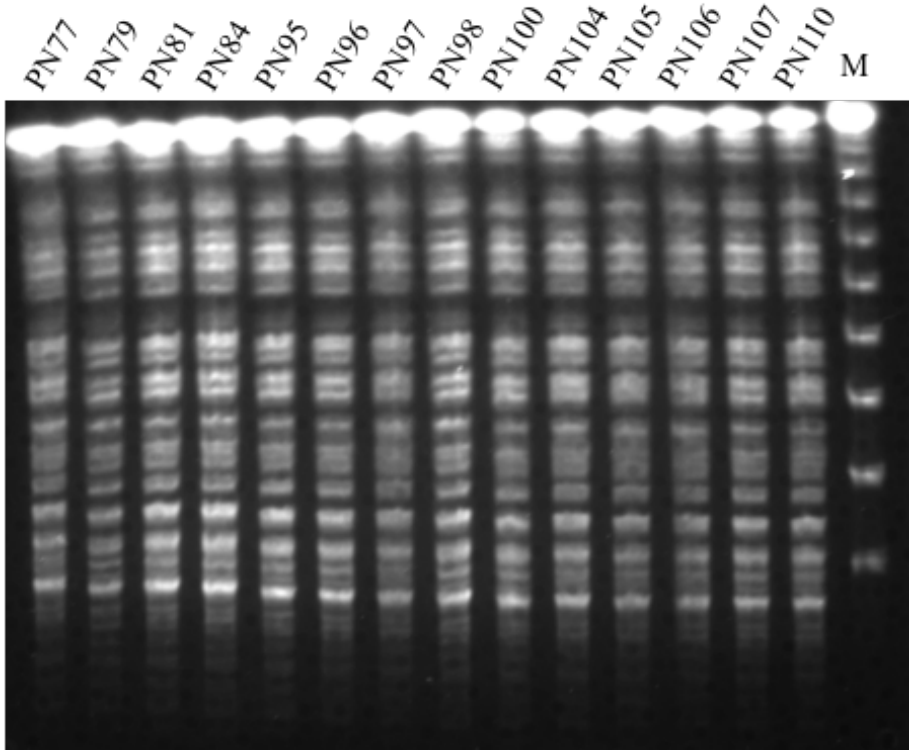


Fig.5.2 PFGE patterns of 14 MCRPKP strains from blowflies. The node of isolates are displayed at the top of the PFGE pattern images.

Analysis of genomic AMR genes, showed significant variations in resistance gene content. Firstly, the CGE services were used for identification of acquired resistance genes (ResFinder, https://cge.cbs.dtu.dk/services/ResFinder/). Apart from *mcr-1* gene, multiple antibiotic resistance genes were identified in the studied isolates, with the average number 8.34 and 7.82 in *E. coli* strains and *K. pneumoniae* strains, respectively. In the 31 *E. coli*, 24 different resistance genes were identified, conferring resistance to nearly all current available antibiotics, such as ß-lactams, aminoglycoside, chloramphenicol, fluoroquinolones and

sulfonamide (**Fig.5.3)**. The most prevalence AMR gene is the ß-lactamase-encoding *ampC* (n=30, 93.75%), followed by *aadA* gene conferring streptomycin resistance (n=28, 87.50%). Plasmid-mediated fluoroquinolone resistance genes, *qnrS1*, *oqxA* and *oqxB*, are present in 20, 3 and 3 isolates, respectively. Several ß-lactamase-producing genes, bla_{TEM}, bla_{ampH}, bla_{mrdA} $bla_{\text{CTX-M-55}}$ are detected in 21 (65.63%), 16 (50.0%), 16 (50.0%) and 6 (18.75%) isolates, respectively. In contrast, a total of 14 AMR genes were found in MCRPKP. Fluoroquinolone resistance genes (*qnrS1*, *oqxA* and *oqxB*), $bla_{\text{TEM-1b}}$ and tetracycline resistance gene *tetA* are identified in all *K. pneumoniae*, followed by bla_{SHV} encoding SHV β-lactamase present in 14 isolates. Three aminoglycoside modifying enzymes, AAC(3')-Iia (n=2) and APH (n=3) are detected. *dfrA* and *sul2* conferring sulfonamide resistance are only found in one MCRPKP strain.

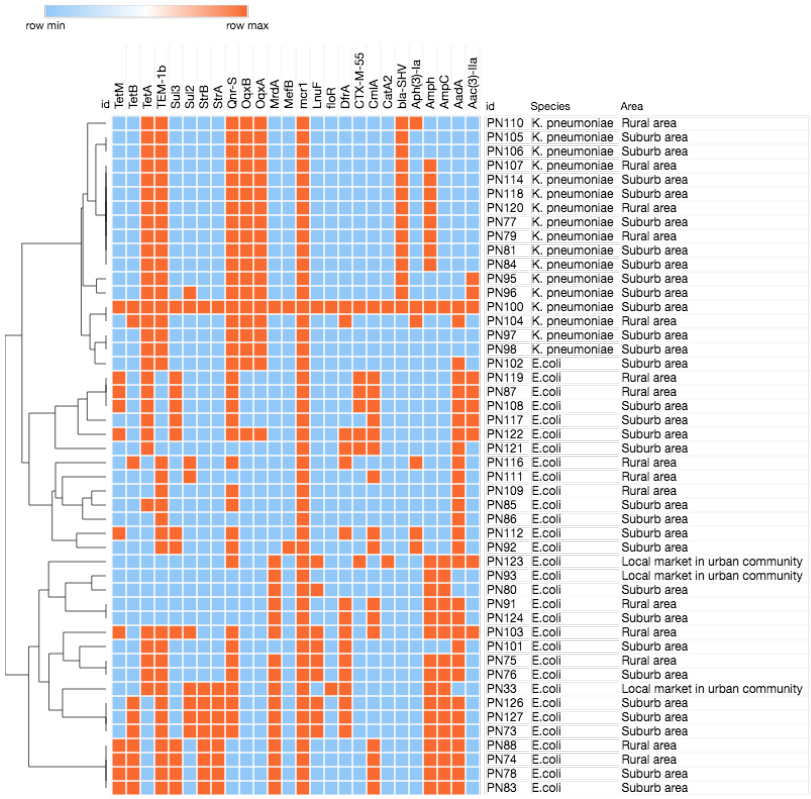


Fig.5.3 Heat-map analysis of antibiotic resistance genes in 48 MCRPE isolates recovered from blowflies, using Morpheus website version (https://software.broadinstitute.org/morpheus/). The presence or lack of AMR genes is colored in orange or light blue, respectively.

5.2.3 Plasmid types and transferability of *mcr-1* gene

De novo bacterial genome assembly was performed and the *mcr-1*-carrying contigs were analyzed. Replication origins are located in the *mcr-1* contigs, allowing to analyze incompatibility groups of these plasmids by using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/). 35 out of 48 isolates are able to identify replicon sequence type: IncX4 (n=29, 12 *E. coli* and all 17 *K. pneumoniae* isolates), IncHI1A (n=2), IncHI1B (n=3) and IncHI1A-IncHI1B (n=1). Ten randomly chosen *mcr-1*-bearing IncX4 plasmids obtained from *K. pneumoniae* isolates were probed with *mcr-1* gene using S1-PFGE. As shown in **Fig.5.4,** 10 *mcr-1* genes were all located on a ~32-kb IncX4 plasmid. PCR was performed to fill the gap in the *mcr-1*-carrying contigs with primers (PN_IncX4_F: CGACCTTTAAGTCGTATTTGCAAGT; PN_IncX4_R: ATTGCGCCCGTAGTTCGCTA), followed by sanger sequence (Eurofins, Germany). The complete plasmid sequence were conducted by *de novo* assembly using Geneious (10.0.7). As a results, complete sequences of 26 IncX4-*mcr-1*-carrying plasmids were achieved. Alignment of ~32-kb IncX4-*mcr-1*-carrying plasmid visualized by BRIG showed that all *mcr-1*-carrying plasmids share the most parts of the reference plasmid Incx4-pMR0617mcr (GenBank accession number CP024041), including the typical region encoding ~11kb T4ss conjugation system and a toxin-antitoxin system *hicAB*, further suggesting that IncX4 plasmids acts as a reservoir of *mcr-1* gene (**Fig. 5.5** and **Fig. 5.6**).

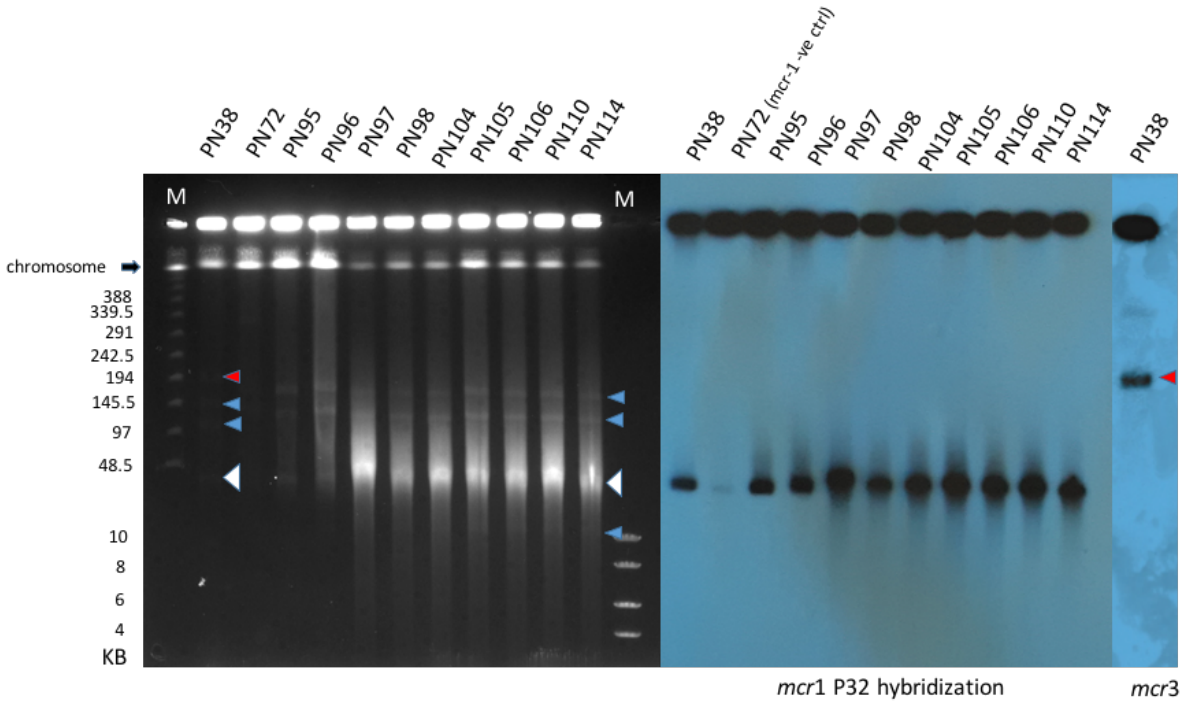


Fig.5.4 PFGE analysis of MCR-1-producing strains digested with S1 nuclease (right) and hybridization with *mcr-1* gene probe (right).

The transferability of *mcr-1*-bearing plasmids were assessed by conjugation and *E. coli* J53 as a recipient. We randomly selected 31 *mcr-1*-positive isolates as donors, containing at least four different *mcr*-1-linked Inc-type plasmids: IncX4 plasmids (n=25), IncHI1B plasmids (n=3), IncHI1A (n=2) and IncHI1A_HI1B (n=1) (**Table 5.1**). We performed mating assays with sodium-azide resistance *E. coli* J53 as the recipient strains. Briefly, overnight cultures of *mcr-1*-producing donors and the recipient strain were 1:2 mixed and incubated in 37 ^{o}C for 16-20 h. After incubation, we subsequently ten-fold serial diluted the mixed culture in sterile saline and aliquoted 100 µl of diluted culture onto selective agar plates containing with 2 mg/l colistin and 150 mg/l sodium azide. The *mcr-1*-positive transconjugants were confirmed by PCR and transfer frequency was calculated by the number of transconjugants per recipient. As a results, 12 out of 14 *mcr-1*-bearing plasmids were successfully transfered to *E.coli* J53, IncX4-*mcr-1* plasmids are able to transferred into the recipient with higher

frequency (mean $1.46x10^{-3}$ in *E. coli* and mean $2.11x10^{-5}$ in *K. pneumoniae*), comparing with other four *mcr*-1-related IncHI1 types ($2.77x10^{-7}$), IncHI1A (mean $2.27x10^{-7}$), IncHI1B ($1.85x10^{-7}$) and IncHI1A-IncHI1B ($8.7x10^{-8}$) (**Table 5.1**).

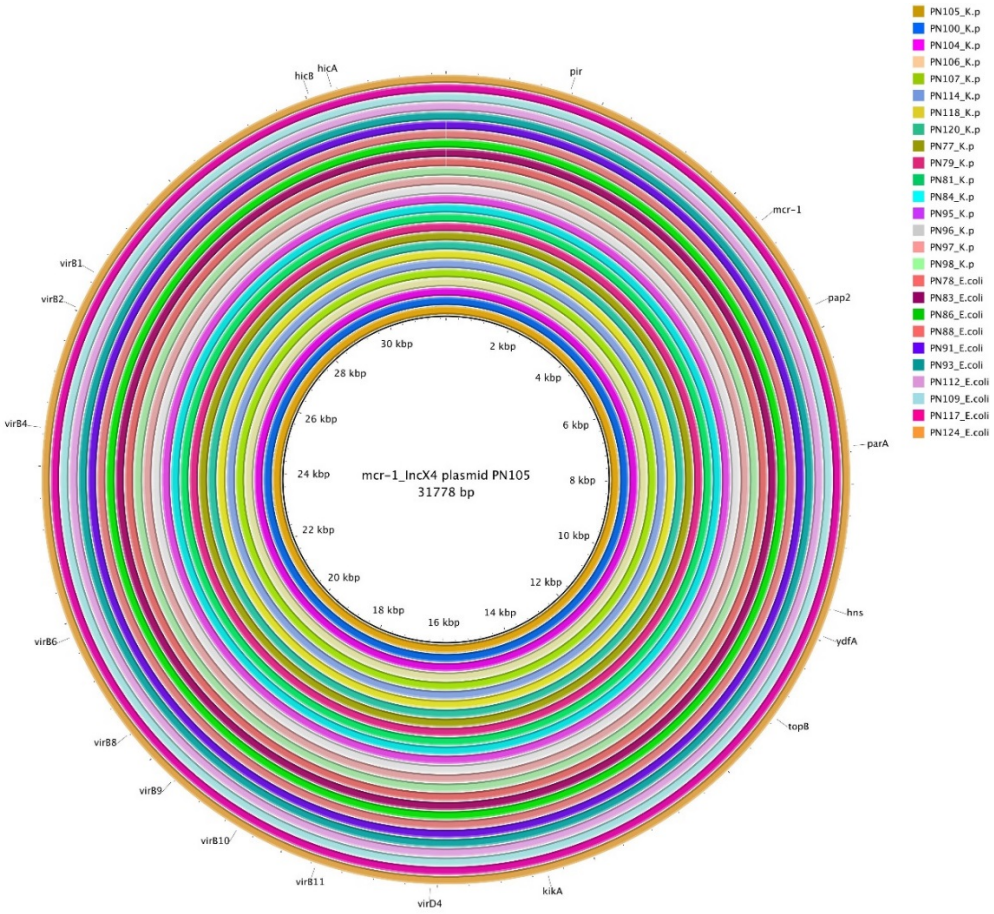


Fig.5.5 Alignment of 26 *mcr-1*-complete plasmids and visualized using BLAST Ring Image Generator (BRIG v0.9555). First inner ring is the plasmid obtained from PN105, used as reference for the alignment, name and size of the reference plasmid indicated in the middle of rings.

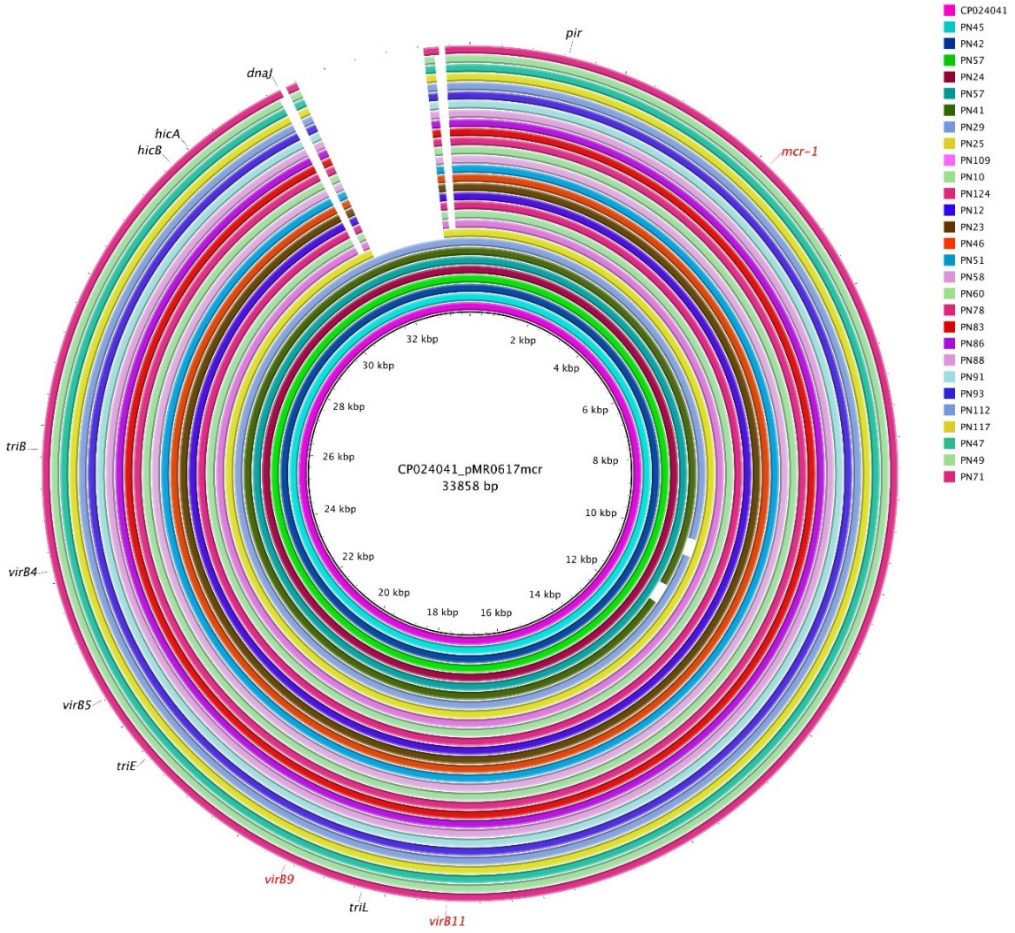


Fig.5.6 Alignment of 29 *mcr-1*-complete plasmids and visualized using BLAST Ring Image Generator (BRIG v0.9555). First inner ring is the plasmid used as reference for the alignment, GenBank accession number and size of the reference plasmid indicated in the middle of rings. These mcr-1-carrying plasmids are recovered from different sources, namely, companion animals (PN10 and PN11), poultry (PN23, 24, 25 and 29), human feces (PN45, 42, 57, 41, 46, 51, 58, 60, 47, 49 and 71), excepting 10 from blowflies (PN78, 83, 86, 88, 91, 93, 112, 117, 109 and 124). Besides those strains from blowflies fully described in Table 1, the rest of strains showed in this figure were also obtained from Thailand (**Appendix II Table 2.1**).

5.2.4 Virulence factors in MCRPKP strains and virulence loss in *G. mellonella* models

K. pneumoniae is now recognized as an urgent threat to human health because of the emergence of MDR strains associated with hospital outbreaks, a number of virulence factors of *K. pneumoniae* are significantly associated with bacterial pathogenicity. Our database includes a set of virulence genes: capsular biosynthesis genes (*wzy/magA*, *K2A*) (Yu *et al.*,

2008, Lin *et al.*, 2012); mucoid factor regulator (*rmpA, rmpA2*) (Cheng *et al.*, 2010); allantoin metabolism operons (*allABCDRS*, *gcl* and *glxRK*) (Chou *et al.*, 2004); an iron-uptake system (*kfuABC*) (Ma *et al.*, 2005); two-component operon (*kvgAS*) (Lai *et al.*, 2003); gene clusters for siderophores dependent iron acquisition (aerobactin *iucBCD-iutA*, yersiniabactin *ybtAEPQSTUX-irp1-irp2-fyuA*, colibactin *clbBCDEFGHHIJKLMNOPQR*, salmochellin *iroBCDN*, enterobactin *entABCDEF*) (Hsieh *et al.*, 2008); gene clusters of type I and type III fimbriae (*fimABCDEFGH* and *mrkABCDFHIJ*, respectively) (Di Martino *et al.*, 2003, Struve *et al.*, 2008); outer membrane lipoprotein (*ycfM*); serum resistance factor (*traT*) (El Fertas-Aissani *et al.*, 2013); hemolysin transport protein (*hlyABCD*) (Thomas *et al.*, 2014); urease operon (*ureABCDEFG*) (Liu & Bender, 2007)and type IV secretory system gene cluster (*virB1* to *B11*) (Juhas *et al.*, 2008). Annotation of genes with 75% identity to reference sequences was performed by Geneious (10.2; Biomatters Ltd.). Capsular (KL) loci were evaluated using Kaptive platform (http://kaptive.holtlab.net/jobs) (Wyres *et al.*, 2016). The detailed of this dataset are available in method **2.21** in **Chapter 2**.

There are at least five groups of pathogenicity factors found in the 17 MCRPKP isolates, these include gene clusters associated with serum resistance (*traT*); adhesins (type I firmbrial operon *fimABCDEFGH*, and type III firmbrial operon *mrkBCDEF*); lipopolysaccharide (*wabGHN*); siderophore systems (enterobactin-encoding *entABCDEFS*, and aerobactin-encoding *iucABCD*, *iutA*) and capsular synthesis loci (wzi/wzc typing, 412/K16). Similarly, the iron acquisition operons *kfuABC*, and *iroE*, urease-synthesis operon *ureABCDEFG* associated with gastric ulceration and urinary stone formation, and gene *ycfM* encoding surface protein are identified in all MCRPKP isolates (Table 5.2).

Table 5.2 Virulence determinants in 17 MCRPKP strains obtained from blowflies in Thailand

virulence factors	virulence genes	MCRPKP strains																
		PN 120	PN 118	PN 110	PN 114	PN 107	PN 106	PN 105	PN 104	PN 100	PN 98	PN 97	PN 96	PN 95	PN 84	PN 81	PN 79	PN 77	PN 38*
Fimbria type I	*fimABCDEFGH*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fimbria type III	*mrkBCDEF*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
iron acquisition systems	*iroE*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
kfu iron uptake system	*kfuABC*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Aerobactin	*iucABCD iutA*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Enterobactin	*entABCDEFS*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
salmochellin	*iroB*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
yersiniabactin		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
colibactin	*clb*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serum resistance	*traT*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
urease	*ureABCDEFG*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LPS synthesis	*wabGHN*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
surface protein	*ycfM*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
allantoinase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rmpA / rmpA2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
two-component system	KvgAS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wzi/K-typing	wzi/wzc	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	412/ L61	412/ K61	412/ K61	412/ K61	412/ K61	412/ K61	385 / K22

*PN38 strain serves a control strain, is a clinical strain from human feces in Thailand.

('+', positive; ' -', negative)

As shown in **Table 5.2**, at least 10 different virulence factors were found in all 17 ST43 MCRPKP strains, which prompted us to investigate the virulent potential of these isolates by using a *G. mellonella* model. The effect of different inoculum of 10 randomly selected ST43 MCRPKP strains was assessed in the *Galleria mellonella*. As a reference isolate, *K. pneumoniae* K1 is known as a hypervirulent strain. As shown in **Fig. 5.7** (after 12 hour post-infection), with an inoculum of approx. $1x10^6$ CFU, 100% of mortality was observed on both K1 strain and 10 ST43 MCRPKP strains. With an inoculum of $1x10^5$ CFU survival was 70% with K1 strain and 0% with 10 ST43 MCRPKP strains. Finally, with an the inoculum of $1x10^4$ CFU resulted in 100% survival by K1 strain and approx. 20% by ST43 MCRPKP strains. The consistency between genotypic virulence factors and *G. mellonella* infection model data suggest that the ST43 MCRPKP strains recovered from blowflies are highly virulent clones.

Based on our previous study, the acquisition of a *mcr-1*-carrying plasmid leads to virulence loss in *E. coli* strain. In this study, three *mcr-1*-carrying plasmids were transferred into a clinical susceptible *K. pneumoniae* strain ff101 and a KPC-positive *K. pneumoniae* strain p35, followed by infection of *G. mellonella* larvae with an inoculum of ~$1x10^5$ CFU. As shown in **Fig. 5.8** two *K. pneumoniae* strains ff101 and p35 caused more than 80% and 90% of mortality after 72h infection, respectively. After acquiring of IncX4-*mcr-1* plasmid, the survival rates of larvae increased to 40% - 80% with strain ff101, and survival rates of five-fold higher from ~10% to ~50% with strain IncX4-*mcr-1*-carrying p35 strain at 72h after infection. These data suggest that IncX4-*mcr-1* plasmid are responsible to reduce bacterial virulence.

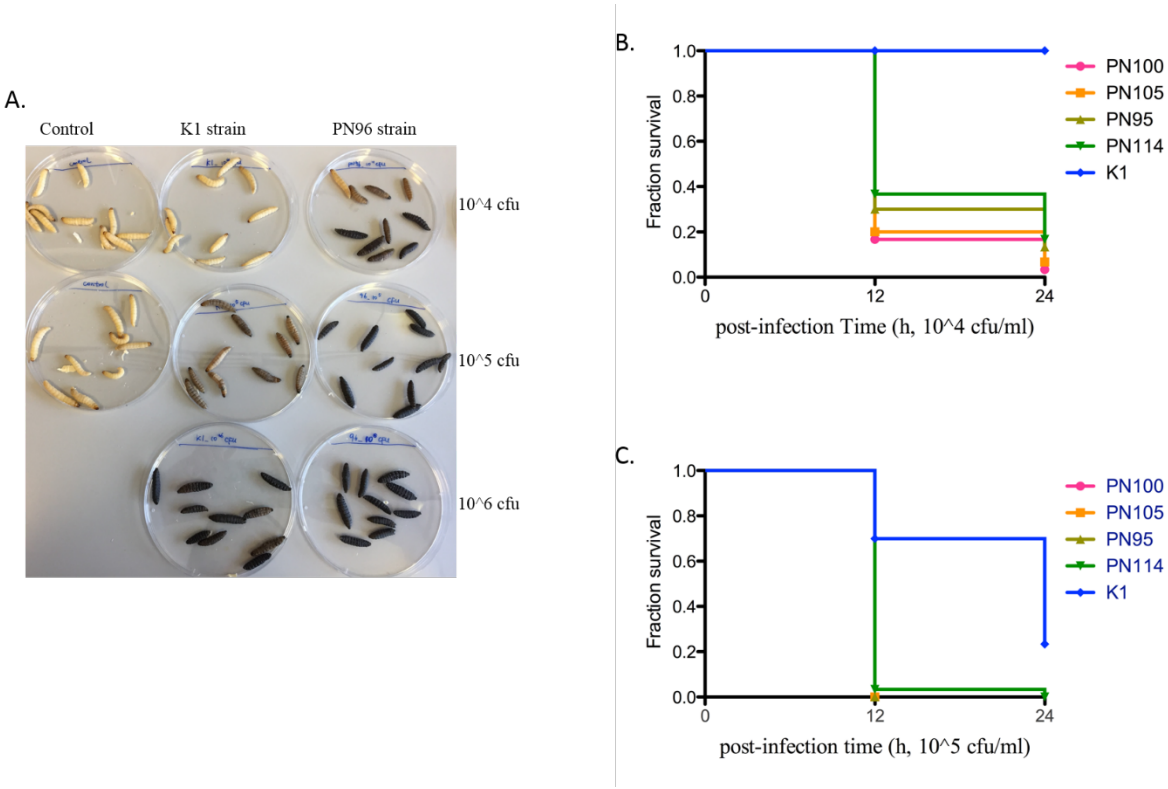


Fig.5.7 A. The image of *G. mellonella* over 12 h post-infection with ST43 MCRPKP strains and a clinical reference strain K1. B and C, Kaplan-Meier plots showing the percent survival of *G. mellonella* over 24 h post-infection with the 10^4 CFU/ml (B) and 10^5 CFU/ml (C) inoculum of MCRPKP and strain K1. Survival curves were plotted using the Kaplan-Meier method (GraphPad Software).

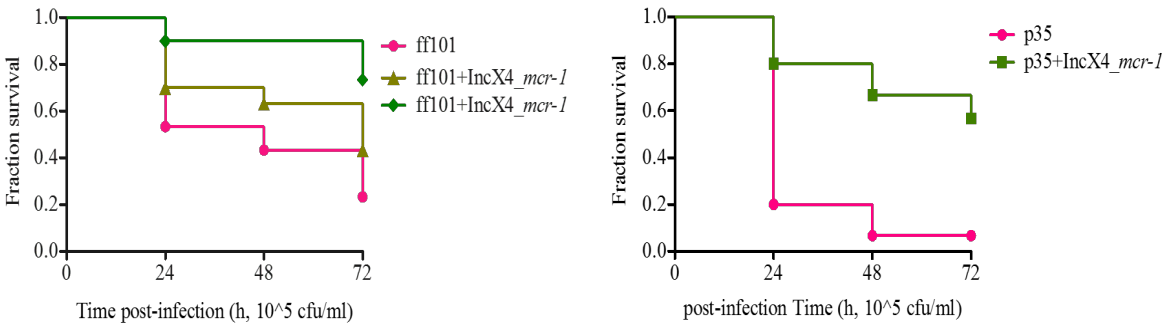


Fig. 5.8 A and B, Kaplan-Meier plots showing the percent survival of *G. mellonella* over 72 h post-infection with the 10^5 CFU/ml inoculum of clinical susceptible *K. pneumoniae* ff101 and clinical KPC-positive *K. pneumoniae* p35, with or without *mcr-1*-carrying plasmid. Survival curves were plotted using the Kaplan-Meier method (GraphPad Software).

5.3. Discussion

The *mcr-1* gene first discovered in *E. coli*, which has become the major host of *mcr-1*, and has been found in most continents crossing more than 40 countries. So far, MCRPE isolates have been mainly recovered from animal samples compared to environmental and human clinical samples, there is a dearth of evidence linking blowflies to the transmission of *mcr-1* in Thailand. Widely considered as important epidemiologic factors for the spread of antibiotic resistance bacteria in different environments, blowflies involved in the mechanical transmission of *mcr-1* is an immense public health concern. In our study, 16% of blowflies possessed *mcr-1*, predominantly located on IncX4 plasmids (29/48, 60.42%) with high conjugant frequency (up to $5.93x10^{-3}$ in this study). These data suggest that flies serve as an important transport host for *mcr-1*.

To date, no less than 14 different plasmid incompatibility types have been identified, and approximately 35.2% of *mcr-1*-carrying plasmids belong to IncX4 plasmids found from *E. coli* isolates from humans, animals and the environment (Matamoros *et al.*, 2017). Interestingly, identical nucleotide sequences of 27 *mcr-1*-carrying IncX4 plasmids in our study, share a similar plasmid backbone to that obtained from human and animal samples in Thailand, suggesting that this IncX4-type plasmid serves as a common *mcr-1* carrier and responsible for the dissemination of *mcr-1* gene in Thailand.

WGS analysis provided comprehensive information about the *mcr-1*-carrying bacteria and their phylogenetic relationship. Twelve different STs were identified in 31 MCRPEC strains, which is consistent with other studies affirming that MCRPEC isolates are highly diverse, but *E. coli* ST10 being the most dominant clade linked to *mcr-1* (n=7, 22.58%). *E. coli* ST10 has been frequently recovered from meat products (Cohen Stuart *et al.*, 2012), food-borne animals (Cortes *et al.*, 2010) and human clinical samples (Oteo *et al.*, 2010), has been

strongly associated with human infections and ESBL-production (Manges & Johnson, 2012). ESBL-producing *E. coli* ST10 has also been shown to be the most predominant lineage obtained from a military medical center in America (Manges *et al.*, 2017). *E. coli* ST10 is highly represented among MCRPEC isolates (Matamoros *et al.*, 2017), recovered from human (Bernasconi *et al.*, 2016), animals (Xavier *et al.*, 2016) and environmental sectors worldwide (Sun *et al.*, 2017). Apart from *mcr-1*, a variety of other acquired resistance genes was detected in the MCRPE isolates (mean 8.05, ranging from 4 to 15), including plasmid-mediated quinolone resistance genes (*qnrS*, *oqxA* and *oqxB*) and ESBL genes, bla_{SHV} and $bla_{\text{CTX-M}}$. Furthermore, a higher number of acquired resistance genes has been found in *E. coli* ST10 isolates (mean 12, ranging from 9 to 15), compared to other STs. Therefore, *E. coli*, notably that related to ST10, may act as *mcr-1* gene pool, facilitating the spread of *mcr-1* in clinical and environmental settings.

Comparison with highly reported MCRPEC isolates, the prevalence of MCRPKP is rare. In a recent study, *mcr-1*-positive *E.coli*, *Providencia* spp and *Enterobacter cloacae* strains were recovered from houseflies and blowflies in China, but no *mcr-1*-carrying *K. pneumoniae* strain was reported (Zhang *et al.*, 2018). Sporadic *mcr-1*-positive isolates of *K. pneumoniae* have been identified mainly from patients (Li *et al.*, 2016, Rolain *et al.*, 2016), animal samples (Kieffer *et al.*, 2017) and environmental sectors (sewage water) (Ovejero *et al.*, 2017). The fact that 17 MCRPKP isolates (35.42%, 17/48) recovered from blowflies is of particular concern, as *K. pneumoniae* is widely considered as a leading cause of hospital-acquired infections. Most interestingly, all 17 MCRPKP isolates belonged to ST43, *K. pneumoniae* ST43 has been widely reported in clinical sectors, and associated with epidemic ß-lactamases CTX-M-15 and OXA-181 (Lascols *et al.*, 2013, Kayama *et al.*, 2015). The highly identical PFGE patterns and phylogenetic tree (SNP<10) further proves the clonal relationship of ST43 MCRPKP among these blowflies. This scenario is worrying, because

there is evidence that blowflies acting as one of environmental dispersals of bacterial pathogens to human and animals, are strongly associated with outbreak of enteric pathogens in rural area in developing countries (Echeverria *et al.*, 1983, Olsen, 1998).

It has been proposed that antibiotic resistance in pathogenic bacteria poses a growing threat to human health, by increasing the mortality rate and economic burden associated with bacterial infections (Shankar, 2016). These ST43 MCRPKP isolates also contain at least four major virulence determinants responsible for disease progression: capsular synthesis loci (wzi/wzc typing, 412/KL16); lipopolysaccharide; siderophores (Enterobactin and aerobactin) that are responsible for binding ferric iron in the host cell; and adherence factors (fimbria type I and III) that allow bacteria to attach to the host cell surface (Podschun & Ullmann, 1998, Paczosa & Mecsas, 2016). The virulence potential of these isolates, as evidenced in a *G. mellonella* model, suggest that ST43 MCRPKP are virulent clones circulating via blowflies in Thailand. Taken together, apart from the acquisition of IncX4-*mcr-1* plasmids, these ST43 MCRPKP strains are virulent, multidrug resistant and transmissible, providing evidence of their threat to public health.

Chapter 6

Genomic insights into MCR-1 and MCR-3-producing *Escherichia coli* isolates, an urgent threat to public health

6.1 Brief introduction

The recently reported *mcr*-conferring colistin resistance in Enterobacteriaceae, frequently associated with multiple resistance genes including $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ (Li *et al.*, 2018), has been described as a global crisis and an impending return to the pre-antibiotics *era*. *mcr* genes, especially *mcr-1* and *mcr-3* genes are mainly found in *Escherichia coli*, which is not only ubiquitous in the environment, but also can cause a range of infections in humans such as most community acquired urinary tract infections, asymptomatic bacteriuria, intra-abdominal and soft tissue infections (Rodriguez-Bano *et al.*, 2004). Multiple resistance to currently available antimicrobials in *E. coli* poses considerable therapeutic challenges, and resistance to colistin, considered as a last-resort therapeutic option, is even more worrying. So far, there is a lack of knowledge regarding *mcr*-positive *E. coli* (MCRPEC), regarding transmission, pathogenicity, resistance evolution and the global spread of MCRPEC clones. The drivers and maintenance of antimicrobial resistance was hitherto, thought to be antimicrobials themselves; however, increasingly we are becoming aware that antimicrobial resistance is as much to do with genetic maintenance systems e.g. toxin-antitoxin systems (TAs), as it is to do with the presence of the drug (Yang & Walsh, 2017). TAs are remarkable systems that parasitize the bacteria and hold it hostage. TAs are also extremely varied and are a testimony to the dexterity and plasticity of genetic systems to adapt and evolve (Yang & Walsh, 2017). Although yet to be fully established, TAs are becoming increasingly numerous

and associated with antimicrobial genes present on the same plasmid; thereby, providing maintenance of the antimicrobial resistance mechanism in the absence of the drug. Apart from antibiotic resistance genes and TAs, other genetic factors, like virulence factors and heavy metal resistance, are closely linked to bacterial survival and pathogenicity.

Therefore, it is important to improve our understanding of the increasing development of colistin resistance, and whole genome analysis is a necessary step to unveil the genetic basis of *mcr*-carrying isolates. In this study, we sequence the genome of over 210 human, environment and animal isolates carrying *mcr*-genes, from Vietnam and Thailand, which divide into three distinct groups, *mcr-1*-bearing *E. coli*, *mcr-3*-postive *E.coli* and *mcr-1/3*-positive *E.coli*. Herein, I provide a detailed genomic framework for MCRPEC, for example, characteristics of phylogenetic group of *mcr*-harboring clones, distinct sets of accessory genes including TAs, heavy metal resistance and virulence traits, and diversity of antibiotic resistance genes. These data show that the main host of *mcr*-genes, *E.coli*, are highly diverse, this is partially due to a population structure of conserved/dominant lineages associated with unique combinations of genes encoding virulence factors, host adaptation and mobile genetic elements.

6.2. Results

6.2.1. Dataset

A total of 219 MCRPEC isolates, isolated from a variety of samples, were contributed by our collaborators in Thailand and Vietnam. All isolates are given in **Appendix II**. All isolates from Vietnam are isolated from patient faeces (n=136). Samples from Thailand were collected from different sources (n=83), including human (n=22), blow flies (n=40), environmental water (n=9), poultry feces (n=7), pet feces (n=3), cattle feces (n=1) and poultry meat (n=1). Genomic DNA was extracted and sequenced via Illuminia HiSeq (See

methods in **2.20** in **Chapter 2**). The wgMLST, PlasmidFinder, ResFinder and VirulenceFinder tools from the Center for Genomic Epidemiology were used for WGS data analysis, full details of all analyses are provide in methods **2.21** in **Chapter 2**.

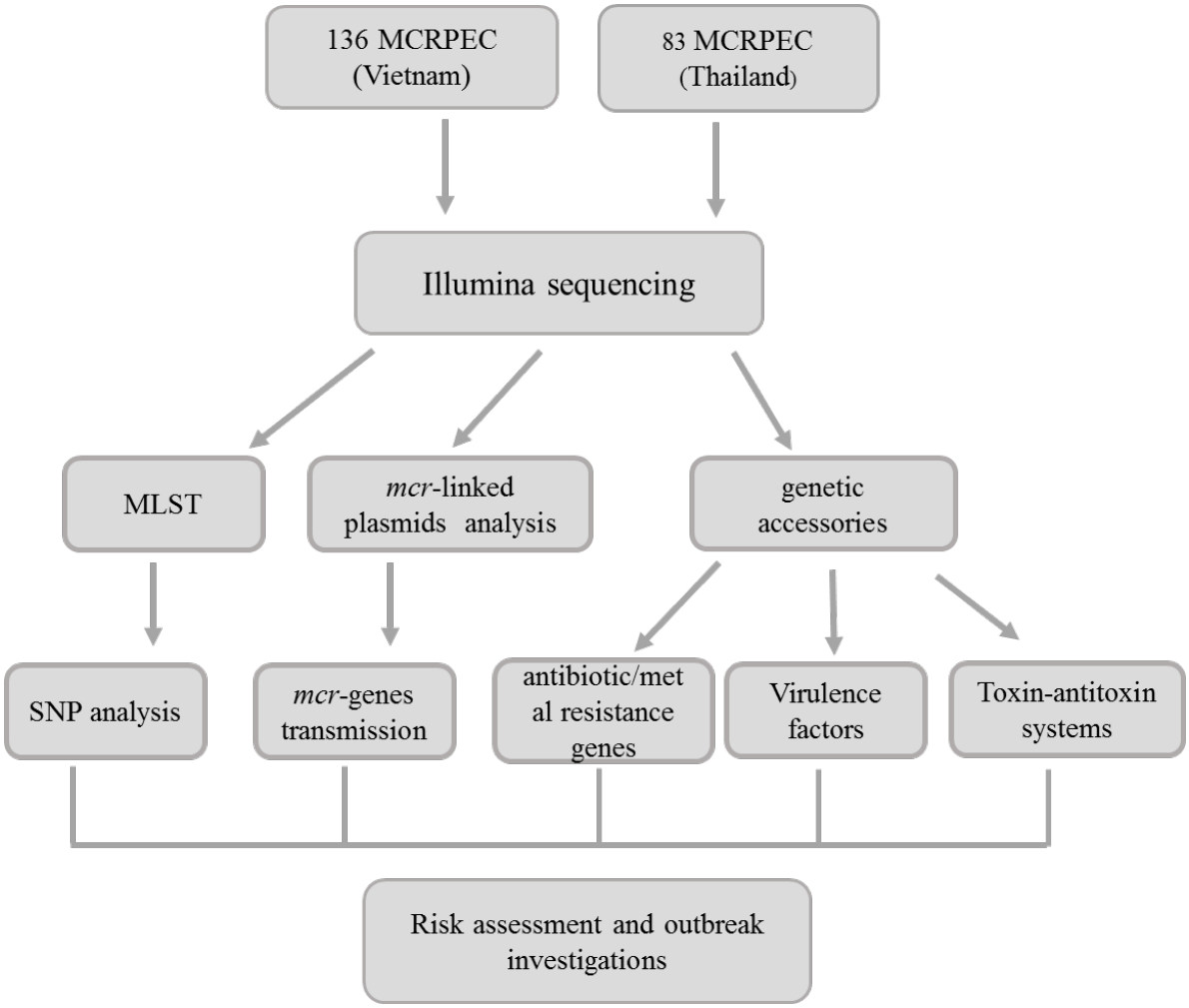


Fig. 6.1 database structure and workflow of sample collections from various sources in Vietnam and Thailand. MCRPEC represents *mcr*-positive *E. coli* and the list of samples were shown in Appendix II.

6.2.2. Multi-locus sequence typing of MCRPEC isolates

As *E. coli* is the most frequent host for *mcr* genes, especially for *mcr-1* and *mcr-3*, I sought to determine the clonal diversity of the accumulation of MCRPEC from South Asia. Diverse sequence types (74 STs in total) have been found among 219 MCRPEC isolates, while 19

isolates are belonged to unknown ST clones. The most common ST clone is ST10 (n=24, 24/219), which includes 13 from Vietnam and 11 from Thailand; followed by ST48 (n=11). The number and diversity of strains from Thailand were greater than those from Vietnam, for example, Vietnam have 54 ST clones from 141 strains (38.3%), compared with 31 STs found in 75 isolates from Thailand (41.3%). There are only 11 STs shared from both Vietnam and Thailand; namely, ST10, ST34, ST48, ST58, ST101, ST206, ST218, ST410, ST617, ST685, ST746 and ST2705, furthermore, they are linked to both *mcr-1* and *mcr-3* isolates, excepting ST746 and ST2705 associated with *mcr-1* isolates only. Importantly, a total of 57 isolates bearing both *mcr-1*- and *mcr-3*-plasmids share 26 different ST clones, including the two most frequent ST groups ST359 (n=7) and ST10 (n=6). ST10-like *E. coli*, globally distributed in human and animals, particularly for ESBL- and AmpC-producing isolates (Ewers *et al.*, 2012), is closely associated with MCRPEC strains. As shown in **Fig.6.2**, the majority of MCRPEC strains recovered from Vietnam and Thailand, belong to ST10-like group, which is consistent with other studies (Matamoros *et al.*, 2017). It is noted that partial several *mcr*-carrying isolates from human (n=13), blowfly (n=9) and animals (n=1), shared the identical sequence type, ST10, suggesting interspecies transmission of plasmid-borne *mcr*-genes among different habitats, but more clear evidence is needed. Core-genome-based phylogenetic analysis was employed in all MCRPEC strains (**Fig.6.3** and **Fig.6.4**). The MCRPEC strains from Thailand were divided into four lineages and mainly fell into yellow lineage (n=54/83) shown in **Fig.6.3**. Similarly, five lineages were found in MCRPEC strains from Vietnam, and the main lineages are indicated in blue (n=40/81) and green (n=24/81) (**Fig.6.4**). Taken together, the highly diverse clones of MCRPEC as observed above, suggest that the acquisition and spread of *mcr*-genes is not only dependent on strain background.

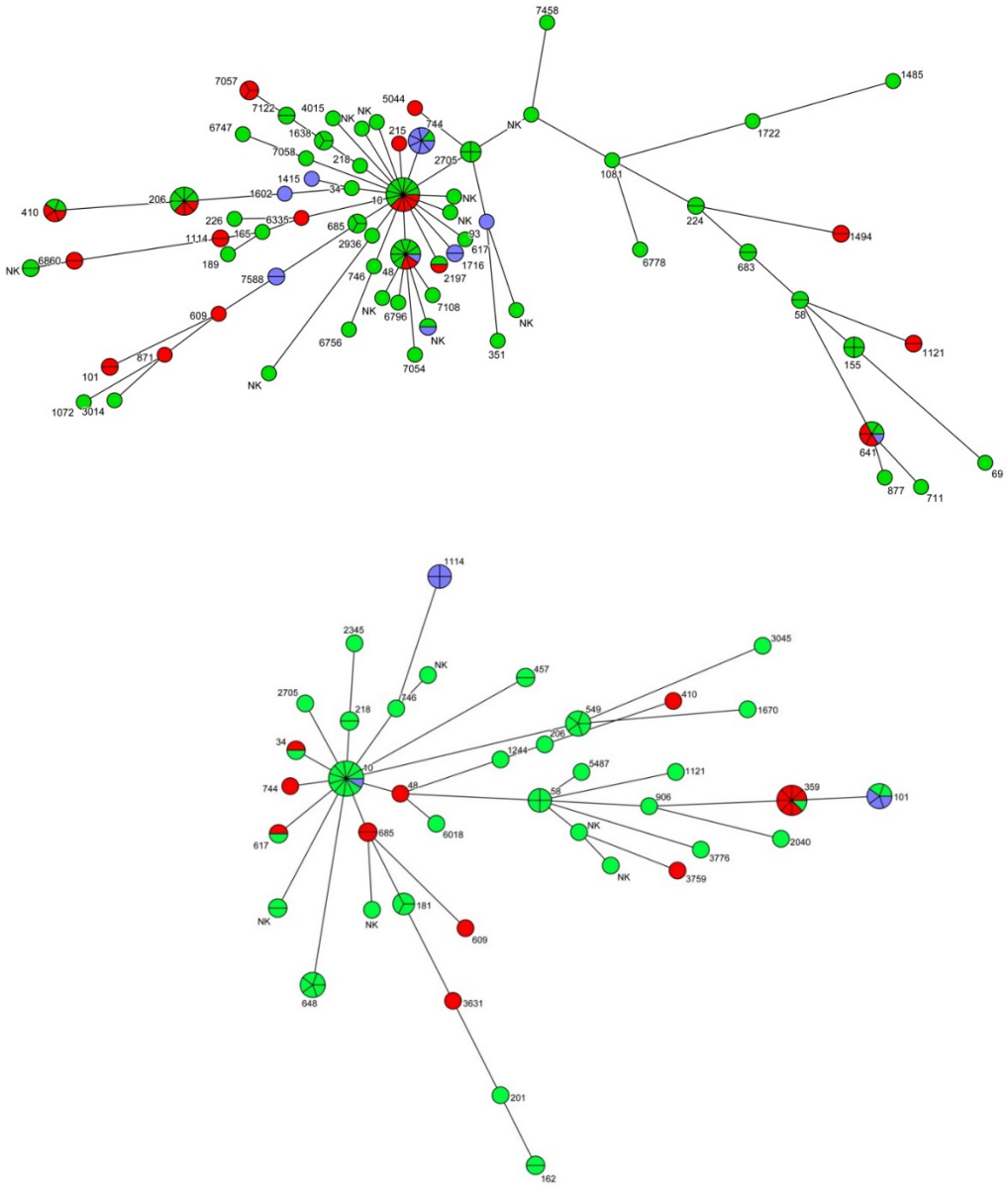


Fig. 6.2 Minimum spanning tree calculated with Bionumerics 6.6 (Applied Math, Sint-Martens-Laterm, Belgium) shows the population structure (MLST grouping) of *mcr*-positive *E. coli* (MCRPEC) strains from Vietnam (top, n=136) and Thailand (bottom, n=83), based on allele sequence combinations of seven housekeeping genes *adk, fumC, purA, recA, gyrB, icd* and *mdh*. The size of the nodes is proportional to the number of isolates. Length of branches between each node represents the number of different alleles (out of seven housekeeping genes) that differ between two linked nodes/ST. Selected nodes are labelled with corresponding ST, phylogenetic group, and number of isolates represented. MLST, multi-locus sequence typing. ST, sequence type. NK, unknown.

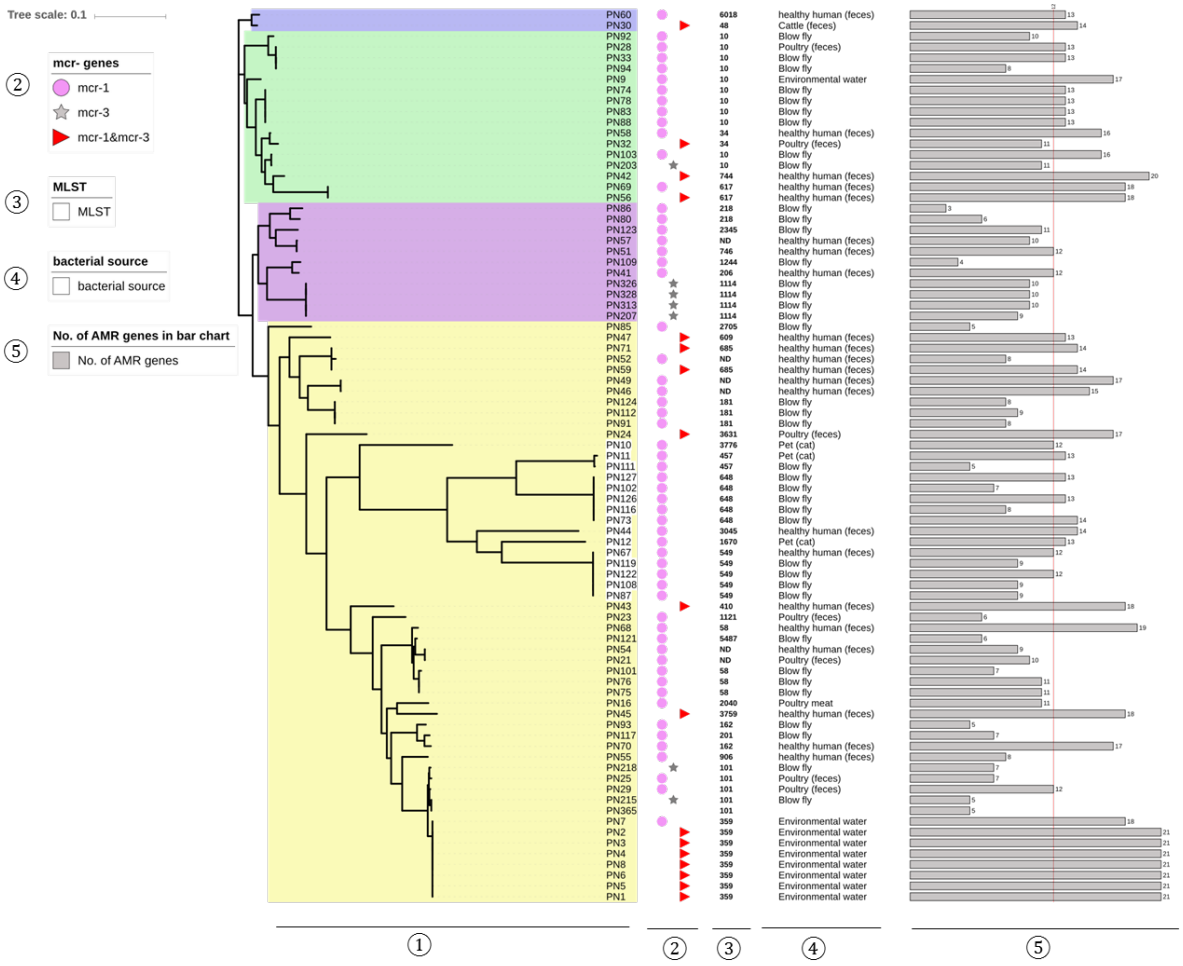


Fig.6.3 phylogenetic tree of 83 MCRPEC isolates from Thailand.① represents maximum-likelihood tree based on their core genome sequences, which was determined by Parsnp-harvest and visualized by iTOL, and four distinct lineages were indicated in different colors. The presence of *mcr*- genes ②, Bacterial MLST ③, bacteria sources ④ and the information of AMR genes ⑤, which are all analyzed by CGE website (http://www.genomicepidemiology.org/), are presented in this tree. Legends of each data set are also indicated in the left of this tree. The average number of AMR genes is indicated by a red line in ⑤.

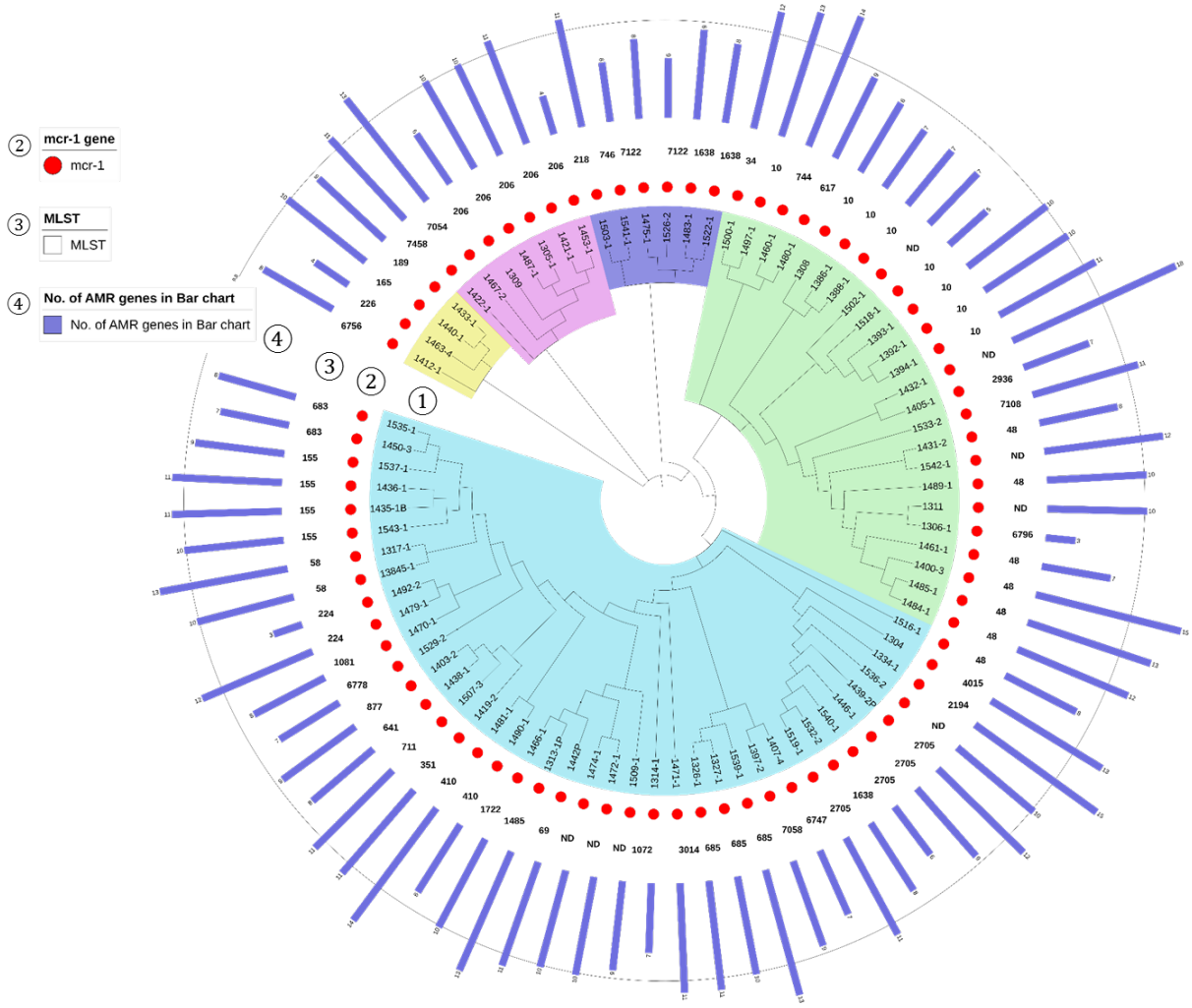


Fig. 6.4 phylogenetic tree of 81 *mcr-1*-carrying *E. coli* isolates from human stools in Vietnam. Circles from inside to outside are represented: ① maximum-likelihood tree was determined by Parsnp-harvest and visualized by iTOL using their core-genome sequences, five distinct lineages were indicated in different colors; the presence of *mcr*- genes ②, bacterial MLST ③, bacterial sources ④ and the number of AMR genes ⑤ , which are all analyzed by CGE website (http://www.genomicepidemiology.org/). Legends of each data set are also indicated in this tree. The average number of AMR genes is indicated by a grey line in ⑤.

6.2.3 Virulence factors in MCRPEC

Virulence genes were determined using the whole genomic sequence deposited in the Center for Genomic Epidemiology (**http://www.genomicepidemiology.org**) as part of their VirulenceFinder Web-based tool (**https://cge.cbs.dtu.dk/services/VirulenceFinder**). A total of 18 different virulence genes were detected in MCRPEC from Vietnam and Thailand. These genes encode *E. coli* virulence factors (VFs) including chaperone-usher fimbriae, autotransporter proteins, siderophore receptors, colicin and toxins: *gad* (glutamate decarboxylase), *iss* (increased serum survival), *lpfA* (long polar fimbriae), *astA* (heat-stable enterotoxin 1), *iroN* (enterobactin siderophore receptor protein), *mchF* (ABC transporter protein *MchF)*, *cma* (*colicin M*), *eilA* (*Salmonella HilA* homolog), *air* (enteroaggregative immunoglobulin repeat protein), *mumA* (microcin M part of colicin H), *mchB* (microcin H47 part of colicin H), *mchC* (microcin C protein), *tsh* (serine protease autotransporters of Enterobacteriaceae), *capU* (hexosyltransferase homolog), *stb* (heat-stabile enterotoxin II), *katP* (plasmid-encoded catalase peroxidase), *celb* (endonuclease colicin E2) and *aaiC* (AraC transcriptional activator). Microcin-encoding *mchABC and stb* genes that didn't found in MCRPEC from Thailand, are only detected in one and three isolates from Vietnam, respectively. *katP, celb and aaiC,* which are absent in the isolates from Vietnam, have been found in three, five and four isolates from Thailand, respectively. In general, the majority of virulence genes detected in the present study are found in MCRPEC isolates from Thailand, which exhibit a higher virulence score (mean number of virulence genes 2.93 from Thai strains vs 1.65 from Vietnamese strains). For example, approximately 46.0% and 37.8% of MCRPEC strains from Thailand contains a serum-tolerance gene (*iss*) and an enterotoxin gene (*astA*), compared with 15.2% and 23.9% Vietnam (**Fig.6.5).** Furthermore, the *gad* is strongly associated with MCRPEC in both countries Vietnam (81.2%) and Thailand (62.2%), as is *lptA* encoding long polar fimbriae (23.9% in Vietnam and 51.3% in Thailand).

Interestingly, when compared to different groups of MCRPEC, the mean numbers of virulence genes are higher in *mcr-1*- group than those in *mcr-3* groups with isolates carrying *mcr-3*-only or co-existed *mcr-1* and *mcr-3* plasmids.

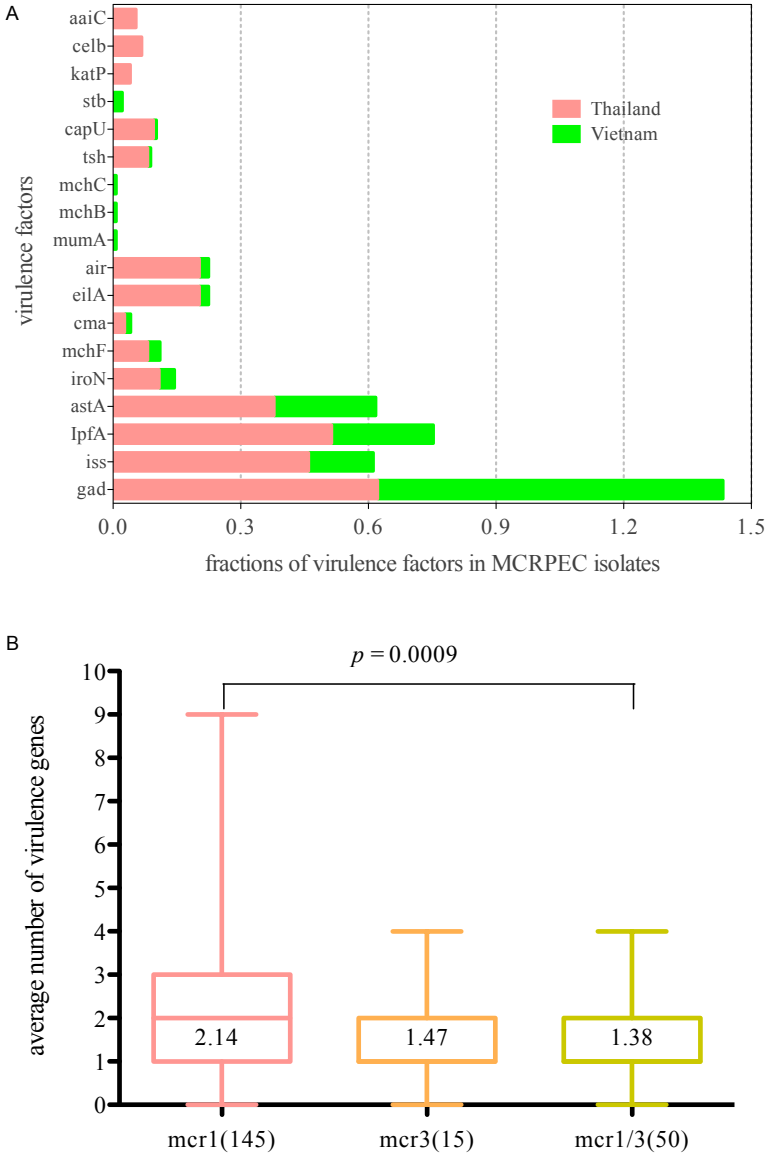


Fig. 6.5 The prevalence of virulence genes identified in MCRPEC strains from Thailand and Vietnam (A). In B, the average numbers of virulence genes were identified in each group of MCRPEC strains, *p* value was calculated using one-way ANOVA and $p < 0.05$ indicates means among group are significant different (B).

6.2.4 Antibiotic resistance genes are associated with MCRPEC isolates

In this study, there are several variations in the nucleotide sequence of *mcr-1* gene were identified, and most of them are only contain single amino acid substitution (**Fig 6.6**). Given the clinical importance of AMR genes, targeted analysis of all known AMR genes in MCRPE genomes using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) was performed. Fifteen different antibiotics classes are available in this database, which include aminoglycosides, β-lactams, fosfomycin, fluoroquinolones, glycopeptides, macrolide-lincosamide-streptogramin (MLS), phenicols, rifampicin, sulfonamides, tetracyclines and trimethoprim. As showed in **Fig.6.7**, the abundance and diversity of resistance genotypes are observed in most of MCRPEC isolates, with 34 and 36 in total of different AMR genes detected in Thailand and Vietnam, respectively. Furthermore, the genetic characteristics of MCRPEC vary between Thailand and Vietnam; for example, β-lactamase genes are significantly higher in Thai MCRPEC than those from Vietnam (mean numbers of AMR genes 4.41 vs 1.79), while aminoglycocides resistance were mainly associated with MCRPEC in Vietnam (3.15 copies/strain). These data confirm that AmpC, AmpH and CTX-M are responsible for β-lactam resistance in Thailand, while AmpC and TEM are the major causes for β-lactams resistance in Vietnam. This probably reflects that the distribution of these AMR genes in particular countries or regions but not in lineages. It is evident that MDR genotypes are commonly found in MCRPEC strains obtained from both Vietnam and Thailand with approximately 1.2 copies of resistance mechanism/isolate, the average number of AMR genes for phenicol (1.04~1.07), tetracycline (0.99~1.14), sulphonamine (0.88~1.35), animoglycocide (1.88~4.41) and β-lactams (2.28~3.15) suggesting that *mcr* is often co-existing with resistance to other antibiotic classes resulting in very limited therapeutic options. Less commonly, three plasmid-borne genes *qnrS*, *oxqA* and *oqxB*, which contribute to horizontally transmission of low-level fluoroquiolone resistance, were also found in the

MCRPEC dataset with an average number of 0.57 in Vietnam and 0.77 in Thailand. Meanwhile, approximately 53.7% and 67.6% of MCRPEC isolates from Vietnam and Thailand contain a *qnrS* gene, respectively.

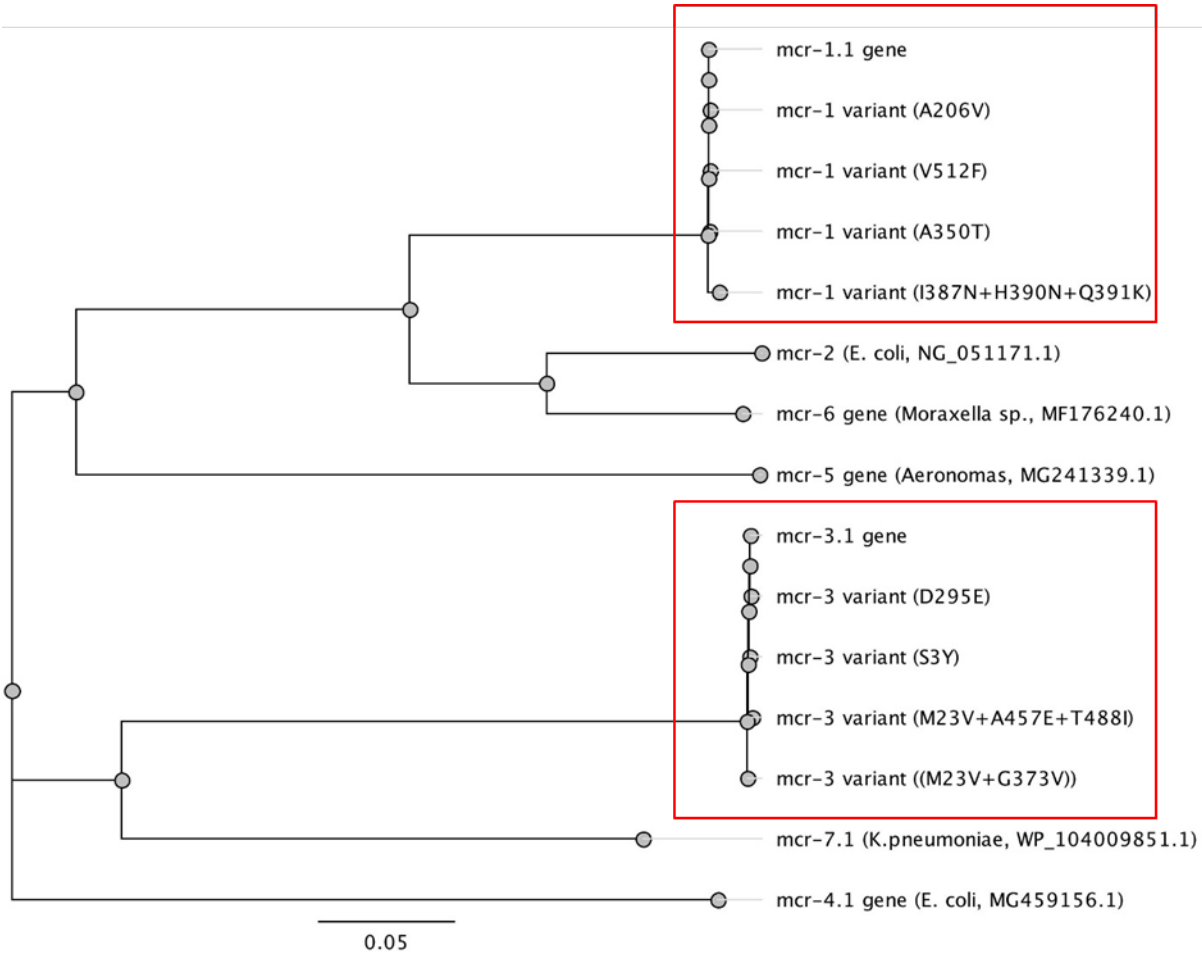


Fig.6.6 Phylogenetic trees of *mcr-like* genes. All the nucleotide sequences of *mcr*-like gene used for the phylogenetic tree were belonged to the PEA lipidA transferase family. Multiple sequence alignment was conducted using Geneious 10.0.7, and resultant output was generated with Tree View. The variants of *mcr-1* and *mcr-3* squared in red, respectively, are derived from our MCRPEC collection. *mcr-2, mcr-4, mcr-5, mcr-6* and *mcr-7* are collected from NCBI with Genbank Number indicated on the branches.

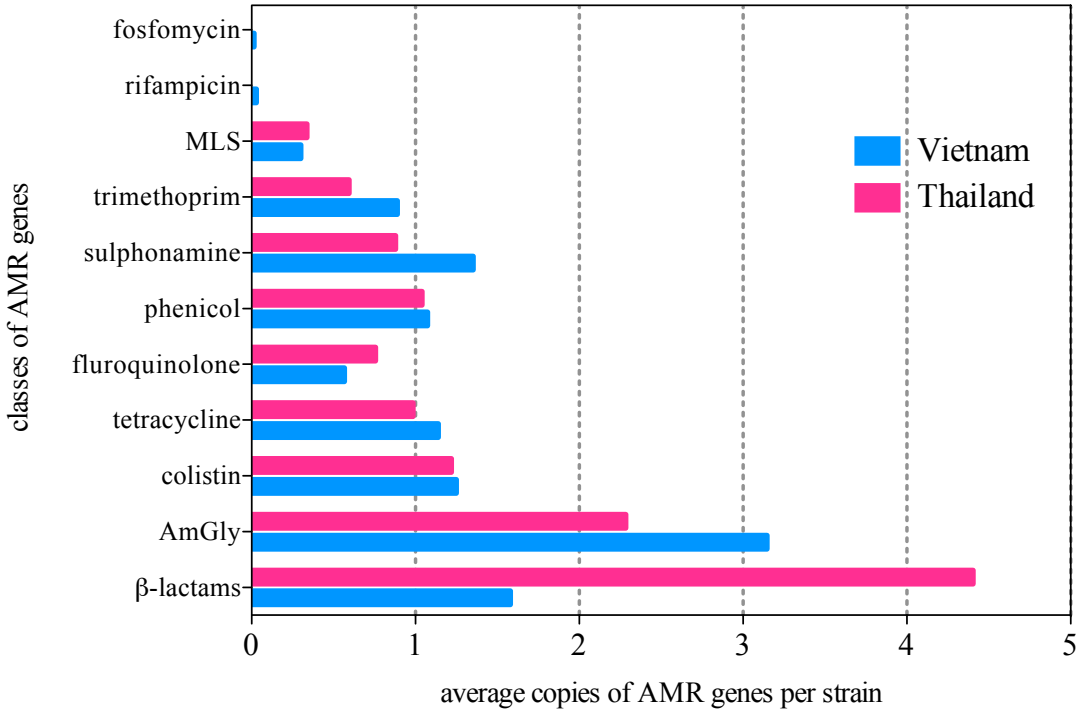


Fig. 6.7 average numbers of different classes of antimicrobial resistance (AMR) genes in our MCRPEC strains from Vietnam and Thailand. MLS indicates macrolide-lincosamide-streptogramin, and AmGly indicates aminoglycocide.

Most interestingly, the *mcr-3*-linked groups of MCRPEC from Vietnam (*mcr-3* alone or co-existence of *mcr-1* and *mcr-3* isolates), have markedly higher copies of AMR genes, compared to *mcr-1* alone group (mean 14.5~15.9 vs 10.4 copies per isolate, $p<0.0001$). Similarly, in Thailand, isolates co-harboring *mcr-1* and *mcr-3* plasmids are also strongly associated with more antibiotic resistance genes than *mcr-1*-only isolates (mean 17.88 vs 11.02, $p<0.0001$) (**Fig. 6.8**). Overall the average copy number of AMR genes in *mcr-3*-linked- and co-existed *mcr-1* and *mcr-3*- MCRPEC isolates are 15.87 and 15.51, respectively, which is significant higher than that in *mcr-1* MCRPEC isolates (mean 10.55 copies per strain, $p<0.0001$). Furthermore, significantly lower numbers of AMR genes in MCRPEC from Thailand are observed in **Fig.6.8**, with mean 8.1 copies of AMR genes per strain ($p<0.0001$), compared to *mcr-1*-only MCRPEC isolates. In combination with the results of

virulence genes described above, it indicates that *mcr-1*-linked MCRPEC strains harbor more virulence genes and less AMR genes, which is contrast to that in *mcr-3* and co-existed of *mcr-1* and *mcr-3 groups* with less virulence genes and more AMR genes.

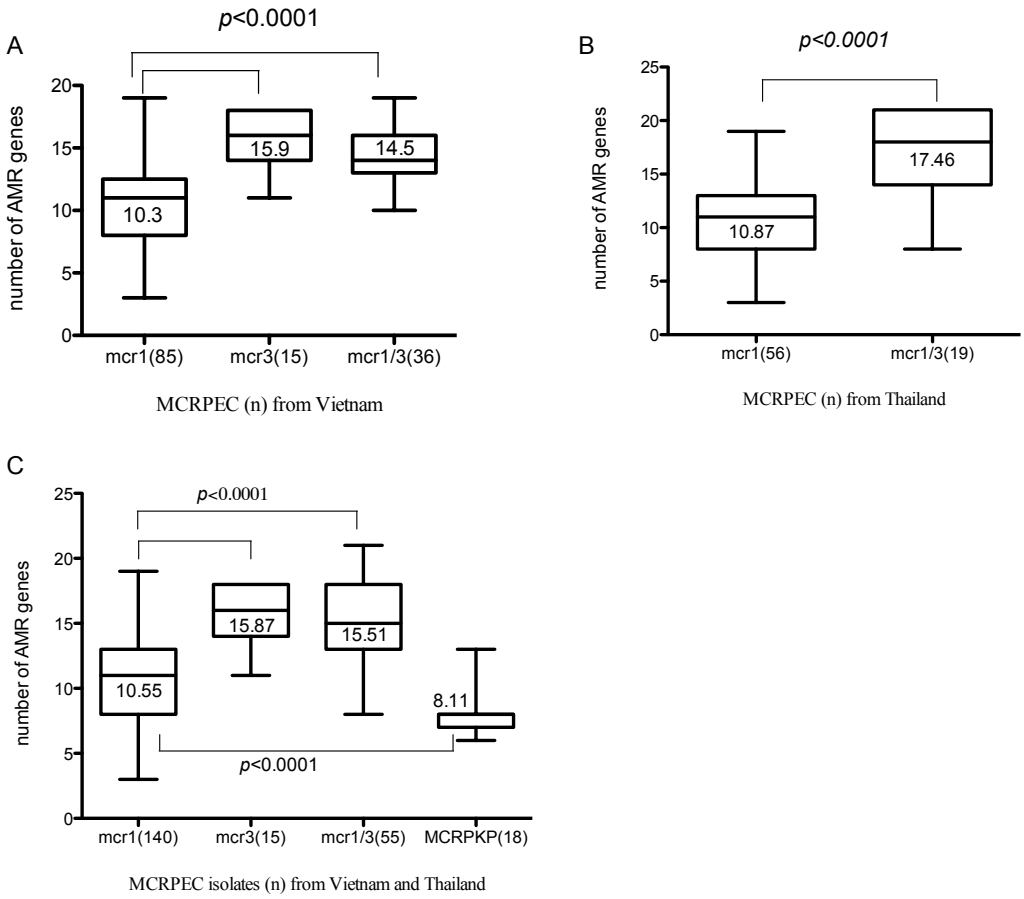


Fig. 6.8 The distribution of AMR genes in three subpopulations of MCRPEC isolates, namely, *mcr-1*-positive strains, *mcr-1*-positive strains and co-existed *mcr-1* and *mcr-3* strains. Figure A, B and C, reprent the abundance of AMR genes in MCRPEC isolates from Vietnam, Thailand and both countries, respectively. *p* values were calculated using student *t*-test. Numbers in the boxplots indicate average copies of AMR genes within each group.

6.2.5. Diverse plasmid patterns of *mcr*-gene acquisition

Screening against the PlasmidFinder database (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Carattoli *et al.*, 2014), 77 isolates from Vietnam were selected for plasmid replicons analysis and 32 different of known plasmid replicons were detected (**Appendix IV Table 6.2**). These include 78 from IncF-related plasmids, i.e., 26 IncFIA (HI1), 3 IncFIA, 26 IncFIB (K), 11 IncFIB (AP001918), 2 IncFIC (FII) and 3 IncFII (pSEI1). The number of plasmids found in per MCRPEC isolate are ranging from 1 to 9, further providing evidence of the genetic plasticity of these MCRPEC isolates. However, linking *mcr* genes to specific plasmids was not determined as whole genomic assemblies were generated using Illumina short-reads and the repetition of mobile elements complicates assembly of full-length plasmid sequences. Fortunately, some contigs containing both plasmid replicon sequences and *mcr* were found, thus incompatibility groups for some *mcr*-bearing plasmid could be determined (**Table 6.1** and **Appendix Table 6.1**). In this study, Inc types could definitively be determined for a total of 78 MCRPEC but not for the rest of MCRPEC isolates. At least seven different Inc groups of *mcr*-bearing plasmids were found, including IncX4, IncX1, IncP1, p0111, IncFII, IncHIB and IncHIA. Plasmids belonging to IncX4 group are strongly associated with *mcr-1*-gene and detected in 33 MCRPEC. Moreover, *mcr-1*-IncHIB (R27) and *mcr-1*-IncHIA plasmids have also been identified in 5 and 2 MCRPEC strains from Thailand, respectively. In Vietnam, where all MCRPEC strains are collected from patient faeces, more diverse *mcr*-linked plasmid types have been found. In Hanoi, *mcr*-identified plasmids belong to five incompatibility groups: IncP1 plasmids associated with both *mcr-1* (n=10) and *mcr-3* genes (n=9); 7 IncX plasmids (6 IncX4 plasmids with *mcr-1* gene and one IncX1 with *mcr-3* gene); 2 IncFII plasmids related to *mcr-3* gene; 2 IncHI1 plasmids with *mcr-1* gene only (IncHI1A and IncHI1B) and one p0111 plasmid with *mcr-1* gene.

Table 6.1 Incompatibility types of *mcr*- carrying plasmids

Plasmid types		IncX4	IncX1	IncFII	IncHIB	IncHIA	IncHIA /HIB	IncP1	repB (p0111)	colE
Vietnam* (n=28)	*mcr-1*	5	0	0	1	1	0	10	1	0
	mcr-3	0	1	2	0	0	0	9	0	0
Thailand* (n=51)	*mcr-1*	33	0	0	4	2	1	0	0	0
	mcr-3	0	0	6	0	0	0	1	0	1
Total No.	78	38	1	8	5	3	1	20	1	1

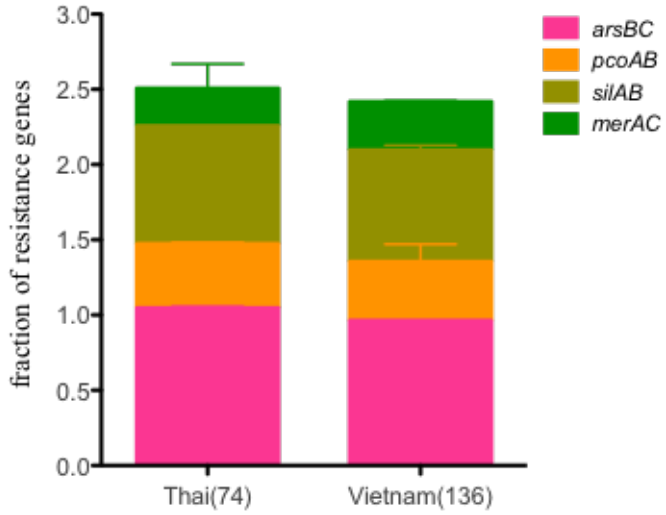
*only list the isolates where *mcr*-carrying contigs also contain plasmid replicon sequences.

6.2.6 Accessory functions associated with MCRPE isolates

6.2.6.1 Coexistence of heavy metal resistance genes

Metal compounds such as mercury, arsenic, copper and silver also have been used in veterinary and clinical settings as therapeutic purposes for decades. The prevalence and linkage of metal resistance genes to other antibiotic resistance genes was examined; in particular, the genetic operons bearing metal resistance genes by whole genomic sequencing analysis. Firstly, I created a database for four most commonly detected operons responsible to metal resistances and searched in MCRPEC strains using megaBLAST in Geneious 10.0.7; namely, i) mercuric resistance operon, *mer*, commonly consisting of six genes *merRTPCAD*, where *merA* is mercuric reductase, reducing mercuric ions Hg^{2+} to non-toxic Hg^{0} in the cytoplasm. ii) plasmid-mediated copper resistance system, Pco, containing four membrane protein PcoABCD. PcoA is a copper oxidase, contributing to oxidize Cu (I) to less toxic Cu (II). iii) silver efflux system, *sil*, originally found on plasmid pMG101. The *sil* system forms a silCBA complex, attributing to efflux Ag^{+} directly to the outside of the cell. iv) arsenic resistance operon, *ars*, which consists of several genes *arsA*, *arsB*, *arsC* and *arsD*. It has been

proposed that ArsA, and ArsB can form an ATP-energized effluxer, conferring to arsenic resistance (Hobman & Crossman, 2015). As showed in **Fig.6.9,** there are similar genotypic characteristics of metal resistance in MCRPEC strains from Thailand and Vietnam. Most of MCRPEC strains contain a number of genes associated with arsenic resistance (0.96.3-105.41%) and silver resistance system (71.85-78.38%). Less commonly, approximately 43% of MCRPEC strains carriy genes encoding copper resistance such as *pcoA* and *pcoB*. Of note, mercuric resistance genes, *merA* and *merC,* were also found 31.85-36.49% and 13.51-32.59% MCRPEC strains, respectively.



% of resistance genes	arsenic resistance		copper resistance		sillver resistance		mercuric resistance	
	arsB	*arsC*	*pcoA*	*pcoB*	*silAB*	*silAB*	*merA*	*merC*
Thailand (74)	105.41%	104.05%	43.24%	41.89%	78.38%	78.38%	36.49%	13.51%
Vietnam (136)	96.30%	96.30%	47.41%	31.11%	76.30%	71.85%	31.85%	32.59%

Fig. 6.9 The prevalence of most commonly heavy metal resistance genes in MCRPEC

6.2.6.2. The abundance of TAs on MCRPEC and non-MCRPEC isolates

To determine the MCRPE genome for toxin-antitoxin systems (TAs), I have generated a TAs database containing 22 pairs of TAs belonged to type I-V groups, then searched in

MCRPE strains using megaBLAST in Geneious 10.0.7. As showed in **Fig. 6.11**, aside *parDE*, the other 21 TAs present in MCRPEC strains had frequencies ranging from 0.36 to 3.79. Multiple copies of toxin YeeV were observed in most of the isolates, with a mean number of 2.1 in Vietnam and 3.79 in Thailand, which are usually higher than in cognate yeeU (mean copies are 1.2 and 2.1 in Vietnam and Thailand, respectively). Significantly high prevalence (94.2-100.0%) of TAs *ghoST*, *hipAB, hicAB, tisAB* and *mazEF* were found in our collection, followed by *yafQ-dinJ*, *higAB* and *yefM-yoeB* ranging from 72.2% to 86.9%. As showed in the hierarchical bar chart (**Fig. 6.11**), detection rates (3.6%-28.0%) for *vapB, pemKI*, *ccdAB*, *hok*, *relBE* and *yacAB*, are relatively low. The average number of TAs detected in MCRPEC from Thailand is 17.24, which is higher than in Vietnamese MCRPEC (p<0.0001, average number 14.39 ranging from 7 to 29). Moreover, there is no significant difference the average number of TAs in *mcr-1* only, *mcr-3* only or co-harboring *mcr-1* and *mcr-3* isolates, with mean 15.52, 14.62 and 15.41 per strain, respectively.

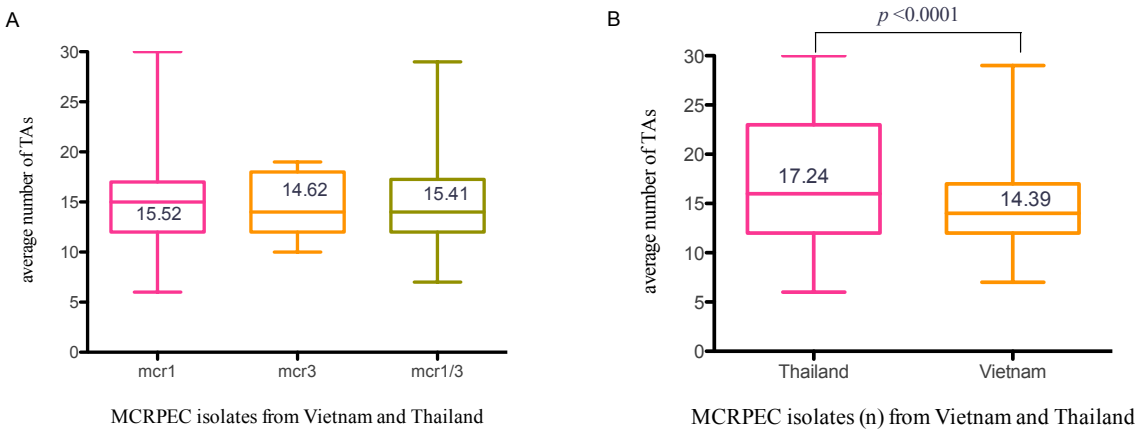


Fig. 6.10 Prevalence of TAs systems in MCRPEC isolates from Thailand (A) and Vietnam (B)

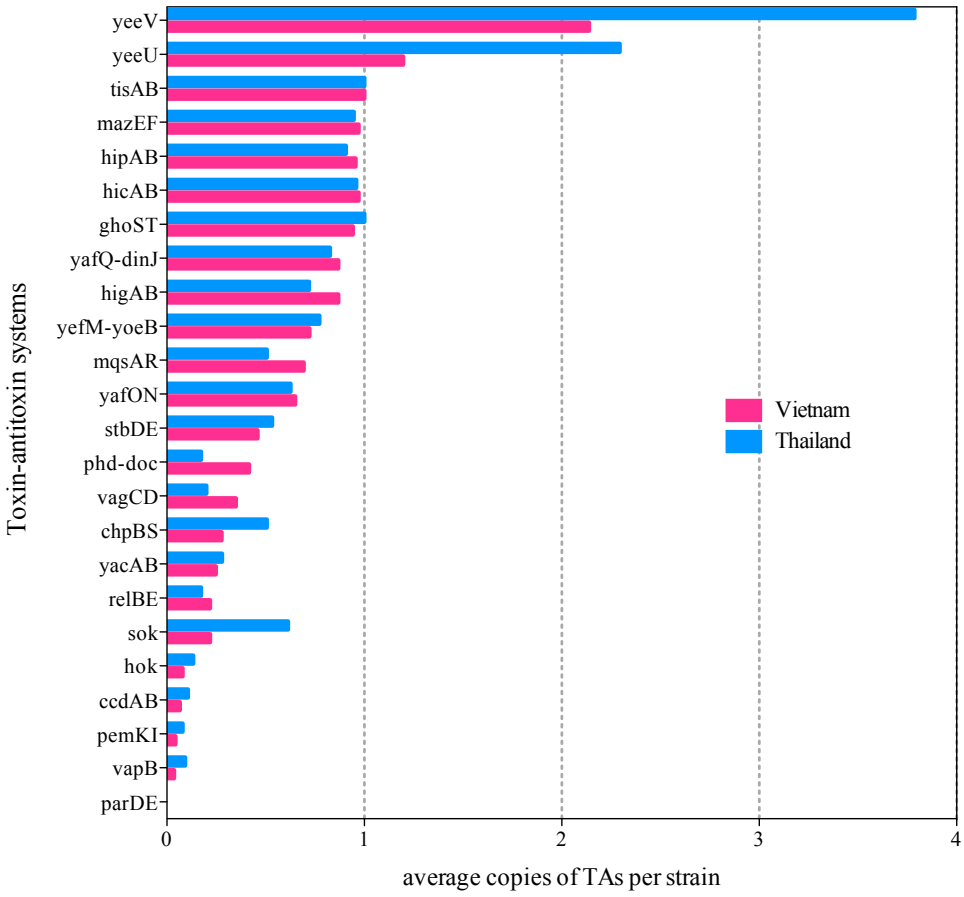


Fig. 6.11 Distribution of 22 TAs groups in *mcr-3*-positive isolates (heat-map)

Less frequent linkages between *mcr* and TAs identified in this study:

(i) *yeeU-yeeV* is the most popular TAs in all groups of isolates, and multiple copies of *yeeU* and *yeeV* are identified in the majority bacteria, especially in those from Thailand isolates (see **Fig. 6.11**). *yeeUV* belonging to type IV TAs, in which the protein antitoxin YeeU directly binds to both MreB and FtsZ, rather than forming a complex with the toxin YeeV (previously designated as CbtA) *via* direct toxin-antitoxin binding (Masuda *et al.*, 2012). YeeV is the first toxin of the TA systems that affects cellular morphology (Tan *et al.*, 2011). YeeV binds and inhibits the polymerization of bacterial cytoskeletal proteins, MreB and FtsZ, which are responsible for maintaining the cell shape and cell homeostasis. The

antitoxin, YeeU, suppresses the YeeV toxicity by promoting the activity of MreB and FtsZ, which are inhibited by YeeV (Masuda *et al.*, 2012).

(ii) As shown in **Fig. 6.11**, the genes for five TAs are particular popular with high percentage in MCRPEC strains; namely, *mazEF* (86.7-96.2%), *ghost* (85-96.2%), *hicAB* (86.7-98.1%), *hipAB* (83.3-96.2%) *and tisAB* (88.9-100%). Except *tisAB* belonging to type I TAs and *ghoST* belonging to type V TAs, the resting TAs are all belonged to type II TAs. These TAs are involved in a variety of essential cellular processes, for instance, TisB, produces clusters of narrow anion-selective pores in lipid bilayers that significantly disturbs the cytoplasmic membrane (Wagner & Unoson, 2012). GhoT is a membrane-damaging protein, and its production can lyse the cell membrane and change its morphologies. Ultimately, this causes the formation of ghost cells, a group of dead or dying cells in which cell outline is still visible but the cytoplasmic area are transparent (Wang *et al.*, 2012).

(iii) Interestingly, several TAs (*mqsAR, stbDE, vagCD* and *relBE*), seem to be linked with *mcr-3*-positive isolates with clearly high detection rate than the other two groups. Specifically, *mqsAR* is the first TAs shown to be involved in biofilm formation was *mqsAR* (motility quorum sensing regulator), a typical type II TAs in which the toxicity of protein MqsR is neutralized by its conjugate antitoxin *MqsA* (Gonzalez Barrios *et al.*, 2005, Brown *et al.*, 2009, Wang & Wood, 2011). Andres et al. demonstrated that toxin MqsR is significantly stimulated by biofilm formation and enhanced cell motility (Gonzalez Barrios *et al.*, 2005).

(iv) Lastly, one of SOS response inducer, TisB, is commonly found in MCRPEC strains. It has been proposed that once activated, the toxin TisB gradually accumulates and rapidly binds to the cytoplasmic membrane, leading to membrane damage and the proton motive force (pmf) and ATP levels are decreased. This causes the rates of DNA, RNA and protein synthesis to decrease, and the intake of drugs to the cells are blocked. As a result, growth

slows down and a multidrug resistant persister is formed (Vogel *et al.*, 2004, Darfeuille *et al.*, 2007, Unoson & Wagner, 2008, Dorr *et al.*, 2010).

6.3. Discussion

It has been reported that MCRPEC isolates from China are genetically and phenotypically diverse (Wang *et al.*, 2017). Here I show a detailed genomic framework for MCRPEC isolates based on WGS of over 210 isolates from Thailand and Vietnam. My results show the existence of highly diverse MCRPEC clones that have access to a diverse pool of AMR/metal resistance genes and plasmid maintenance systems (TAs), suggests that there is potential for the emergence of *mcr*-linked extended drug-resistance (XDR) clones in hospitals setting. ST10 and ST48 are prominent among MCRPEC isolates. It has been reported that *E. coli* ST10 is associated with carbapenem resistance and an XDR nosocomial outbreak (Zhang *et al.*, 2017). ST10-MCRPEC clone has been reported globally including China, South America (Monte *et al.*, 2017), Belgium (Xavier *et al.*, 2016), Netherlands (Veldman *et al.*, 2016), Switzerland (Bernasconi *et al.*, 2016), France and Italy (El Garch *et al.*, 2017). However, given the diversity of ST groups and unknown epidemic clones detected in all MCRPEC strains, it suggests that the acquisition of *mcr*-bearing plasmids is not attributed to the *E. coli* genetic background, making the emergence of MCPREC complicated and unpredictable. A wider population framework of MCRPE need to be undertaken before determining any *mcr*-linked clonal background and identifying new and emerging clones.

mcr-linked plasmids have been found in at least 10 different Inc groups including IncX4, IncX3, IncX1, IncP1, IncFII, IncHI2, IncI2, IncHIB and IncFIB (Li *et al.*, 2017, Matamoros *et al.*, 2017). Among which, the major type of *mcr-1*-bearing plasmids is IncX4 plasmids, which belongs to the subgroup IncX family consisting of six subgroups, IncX1-X6 (Lo et al.,

2014). IncX is a self-transmissible plasmid with high frequencies (10^{-1} ~ 10^{-4}), which is significantly higher than the epidemic IncFII plasmids (by 10^{2} ~ 10^{5} fold) (Lo *et al.*, 2014). IncX plasmids are proposed to be responsible for promoting worldwide-spread of antibiotic resistance genes including genes encoding ESBLs, i.e. $bla_{\text{CTX-M}}$ (Lo *et al.*, 2014, Dobiasova & Dolejska, 2016), plasmid-mediated quinololone resistance genes (i.e., *oqxAB* and *qnrS1*) (Dobiasova & Dolejska, 2016) and carbapenemase genes ($bla_{\text{NDM-5}}$) (Li *et al.*, 2018). *mcr-1*-linked IncX4 plasmids have globally spread to various species including *E. coli*, *K. pneumoniae* and *Salmonella* spp. from human and animals from Denmark (Hasman *et al.*, 2015), Germany (Falgenhauer *et al.*, 2016), China (Li *et al.*, 2017), South American (Monte *et al.*, 2017), South Africa (Li *et al.*, 2017) and United Kingdom (Doumith *et al.*, 2016). In this study, short-read sequence contigs did not fully facilitate the identification of the genetic location of all *mcr* genes. Fortunately, *mcr-1*-IncX plasmids were definitively determined from 40 isolates, consisting of two subgroups, 39 IncX4 and 1 IncX1, also linked to *mcr*-genes. Of interest is the co-existence of IncX3-$bla_{\text{NDM-5}}$ and IncX4-*mcr-1* plasmids (Mediavilla *et al.*, 2016, Sun *et al.*, 2016). Collectively, these findings highlight that IncX plasmids play a quintessential role in promoting the spread of *mcr*-medicated colistin resistance. The second major *mcr*-carrying plasmid type is IncP, mainly found from our Vietnam samples, 10 linked to *mcr-1* gene and 9 linked to *mcr-3* gene. Unlike IncX plasmids that are normally narrow-spectrum plasmids, IncP1 is a broad host range plasmid, which can spread genetic information across taxonomic barriers (Schluter *et al.*, 2007). Therefore, the link between *mcr*-gene and IncP1 plasmids has drawn considerable attention, as IncP1 will, theoretically, facilitate the rapid spread of *mcr* genes across various bacterial populations in natural and clinical environments. It is known that one of mobile genetic elements, IS family, including IS3, IS30, IS110, IS26 and IS*CR* type elements, play an important role in resistance gene translocations. IS*Apl1* is a type of IS surrounded by a pair of left and right inverted

repeats (IRL and IRR), which was first identified in *Actinobacillus pleuropneumoniae*. The fact that the *mcr-1* gene is consistently associated with IS*Apl1* and that the IS*Apl1-mcr-1* cassette may be inserted into different genetic loci in different plasmids.

WGS analysis identified well-established antibiotic resistance-conferring genes (**Fig.6.5**). The presence of co-resistance to clinical importance antibiotics, such as β-lactams and fluoroquiolones in MCRPE isolates is worrying, because our data shows that *mcr*-depended colistin resistance is strongly associated with diverse β-lactamases, thus eventually limiting therapeutic options to treat infections. These include ESBLs (CTX-M groups); AmpC β-lactamases (AmpC and CMY-2) hydrolyzing broad-spectrum cephalosporins, i.e., cefotaxime, ceftazidime and ceftriaxone (Jacoby, 2009). When comparing *mcr-1*-positive isolates that are associated with other AMR genes (mean number 10.4 per isolate), *mcr-3*-related *E.coli* are MDR and have a greater number of AMR genes (approx. 15.7 per isolate). These findings suggest that the accumulation of AMR genes in *mcr-3*-linked MCRPEC strains is the consequence of the successful spread of such clone with better fitness than those in *mcr-1*-only MCRPEC as described in Chapter 4. It is noteworthy that, in addition to emerging multiple patterns of antibiotic resistance, heavy metals represent another major sources of environmental and clinical contamination that may, fortuitously, select for antibiotic resistance (Silver & Phung, 1996, Gullberg *et al.*, 2014). Heavy metal compounds are use in animal growth feeds and therapeutic treatment, including commonly used metal compounds such as mercury, silver, arsenic and cooper. These compounds are common place in pig and poultry production and unlike antibiotic food additives, can accumulate in soil, water, aquaculture and marine antifouling treatments or industrial effluent (Wales & Davies, 2015). It has been proposed that antibiotic-resistant bacteria are enriched at locations contaminated with metals, and genes conferring co-selection to heavy metal and antibiotic are often found together in many clinical isolates (Dhakephalkar & Chopade, 1994, Baker-Austin *et al.*, 2006,

Fard *et al.*, 2011, Ji *et al.*, 2012, Seiler & Berendonk, 2012). Furthermore, genes conferring heavy metal tolerance may coexist on the same genetic element (e.g. plasmid), which could further promote co-dissemination and resistance (Akinbowale *et al.*, 2007, Seiler & Berendonk, 2012). In this study, genomic analysis show that metal resistance operons are widely identified in MCRPEC strains, with high detection rates 13.51-105.41%. Most interesting, a mercuric resistance operon consisting of eight ORFs, *merRTPCADE ,* and a gene cluster *terABCDEW* contributing to tellurium resistance, were found on a *mcr-1*-IncP plasmid pKH457-3-BE isolated from bovine strain in Belgium (Malhotra-Kumar *et al.*, 2016).

Another interesting genetic feature is the abundance of TAs in MCRPEC strains. Because of their ubiquity and crucial intracellular targets, the study of bacterial TAs will help us to better understand their role in the dissemination and evolution of bacterial antibiotic resistance. TAs, originally linked to plasmid maintenance systems (Ogura & Hiraga, 1983), exert important activities in the context of bacterial resistance (Wen *et al.*, 2014, Harms *et al.*, 2016, Patel, 2016). TAs are small modules consisting of a stable toxin and its unstable cognate antitoxin. Antitoxins are more labile than toxins and readily degraded under stress conditions, this allow the toxins to exert their detrimental effects, promoting plasmid maintenance, slow growth and dormancy, which is rather linked with chromosomally encoded TAs (Page & Peti, 2016). Besides, TAs like *hok-sok* and *ccdAB*, are responsible for the plasmids stabilization, thus TAs also have been viewed as "addiction modules"(Engelberg-Kulka *et al.*, 2006). Beside plasmids, TAs appear to play a stabilizing role in genomic islands, for instance, SXT, an integrative and conjugative element (ICE) that mediates tolerance to multiple antibiotics in *Vibrio cholera* (Wozniak & Waldor, 2009). One novel TA pair (designated *mosAT*) within SXT has been identified to promote SXT stability. Ectopic expression of *mosT* causes growth inhibition and MosA can neutralize the toxic effect of overexpressed MosT. Similar to plasmid-borne toxins, when SXT is vulnerable to

loss, MosT expression is activated to minimize the SXT-free cells. Therefore, the activity of *mosAT* may contribute to the maintenance of SXT in bacterial populations (Wozniak & Waldor, 2009). In this study, high prevalence of *hicAB* (96.0-97.1%) and *higAB* (72.0-86.9%) were identified in MCRPEC strains, in addition, they are associated with *mcr*-carrying plasmids: *hicAB* found in most of *mcr-1*-linked IncX4 plasmid described in **Fig.5.5** in **Chapter 4**; and *higAB* identified in a *mcr-3*-harboring IncP1 plasmid shown in **Fig.4.2** in **Chapter 5**. However, their physiological roles connected to *mcr*-plasmids have not yet been elucidated. Besides regulated by its cognate antitoxin HigA, it has been reported that HigB toxin is also regulated by the DNA damage (SOS response) inhibitor LexA, thereby promoting bacterial growth arrest when bacteria is under amino acid starvation (Christensen-Dalsgaard & Gerdes, 2006, Kirkpatrick *et al.*, 2016). More importantly, both *higAB* loci can significantly enhance the stability of the test plasmid, up to 600-fold increase in genetic stability (Christensen-Dalsgaard & Gerdes, 2006). The effect of toxin HicA seems to be bacteriostatic and its overexpression cause *E. coli* growth inhibitation (Li *et al.*, 2016). Therefore, it would be interesting to further study the genetic roles of these TA loci in the maintenance and dissemination of *mcr*-harboring plasmids.

Chapter 7

General Discussion

7.1 Multifaceted aspects of *mcr*-like genes conferring colistin resistance

Today, antibiotic resistance has become a global crisis, evidenced by the increasing conflict between rapid emergence and dissemination of antibiotic resistance genes and the marked decline in the discovery and pipeline development of novel antibiotic agents. The global dissemination of CREs capable of lysing nearly all β-lactams, are also frequently associated with multiple resistance to aminoglycosides and fluoroquinolones- historically, both have been effective treatment options for Gram-negative infections. XDR or pan-drug resistant phenotypes leave very few therapeutic options and force clinicians to re-consider older antibiotics, such as colistin, discovered in 1947. For the past decade, colistin has been considered as the last-line antibiotics against CRE pathogens. The discovery of the plasmid-borne *mcr-1* gene conferring colistin resistance in the late 2015 is therefore deeply disturbing. Plasmid-mediated horizontal transmission of *mcr*-genes exacerbates this predicament of global colistin resistance and makes it more unpredictable and complex. There are several features of *mcr*-mediated colistin resistance in my study summarized as follow:

(i) Multiple reservoirs of mcr-conferring colistin resistance, a concerned threat in both human and animal health. An established but increasingly challenged paradigm, is the antibiotic resistance is associated with the volume of antibiotic use. Colistin has been used in food-producing animals since 1950s, either as a growth promoter, metaphylaxis or treatment of individual animals or flocks/herds and thereby can facilitate the emergence and spread of colistin resistance. It is therefore not surprise that the rate of *mcr-1*-mediated resistance is

much higher in the veterinary sector than in clinical settings (Skov & Monnet, 2016). In this thesis, over 219 MCRPEC strains originating from human, animals and environmental samples from Vietnam and Thailand, were characterized using WGS. A vast diversity of MCRPEC clones were observed, reflected by the variety in sequencing types (ST) and distinct sets of accessory genes including TAs, heavy metal resistance determinants, virulence factors and other antibiotic resistance genes. Worryingly, the ST10-like MCRPEC were identified from all sources including patients, food-producing animals, blowflies and environmental sectors (e.g. water and soil), indicating widespread distribution and therefore, the possibility of further transmission of MCRPEC strains. There is an increasing perception that flies can spread antibiotic resistant bacteria from the environment to humans, and *vice versa*. In Chapter 5, the high prevalence of *mcr-1*-carrying isolates (16%, 48/300) were recovered from blowflies trapped from Northern Thailand, consisting of 17 MCRPKP and 31 MCRPEC. The clonal relationship among 17 MCRPKP strains was affirmed by all isolates belonging to ST43, indistinguishable PFGE patterns and an identical phylogenetic tree. The presence of numerous virulence factors and virulence potential in the *Galleria mellonella* model, also suggests that the ST43 MCRPKP clone is hypervirulent. WGS-based analysis of MCRPE isolates from blow flies show that *mcr-1* is predominantly located on IncX4 plasmids (n=29/48), which are similar to our previously described IncX4-*mcr-1* plasmids obtained from human and animals. This suggests that the IncX4-*mcr-1* plasmid has been circulated in various environments in Thailand, particularly in the clonal and virulent MCRPKP isolates, which would cause a serious threat for public health.

(ii) The expanding groups of mcr-like PEtN transferases family. To date, a total of eight *mcr*-like genes (*mcr-1*, *-2*, *-3*, *-4*, *-5*, *-6*, *-7* and *mcr-8*) have been identified. These *mcr*-like genes share similar functions - modifying the bacterial LPS with PEtN additions, and share 35-78% nucleotide identity. *mcr-2*, *mcr-4*, *mcr-5* and *mcr-6* have been identified from animal

origins in Europe (AbuOun *et al.*, 2017, Borowiak *et al.*, 2017, Carattoli *et al.*, 2017); in contrast, *mcr-1*-gene has been globally disseminated and based on my dataset from South Asia, *mcr-3* appears to exist be noticeably present in both humans and animals. A large dataset of *mcr-1*- and/or *mcr-3*-positve Enterobacteriaceae (MCRPE) were studied in this thesis. In Chapter 4, co-occurrence of *mcr-1* and *mcr-3* genes are identified in 34 out of 52 isolates (61.5%) from humans in Vietnam. Two variants of MCR-3 with three amino acid substitutions, have been further studied. A number of *mcr*-harboring plasmids were characterized and plasmid IncX4 was strongly associated with *mcr-1* and *mcr-3* genes, and the broad-host rang plasmid, IncP1 was also found to carry *mcr-1* and *mcr-3*. These plasmids are transferable at high frequency (up to 6 $x10^{-3}$). These data provide an important milestone in our understanding of *mcr* movement and dissemination.

(iii) The expression of mcr-1 and mcr-3 gene causes bacterial fitness burden. Fitness is an important factor to predict the spread and development of antibiotic resistance. In Chapter 3, our data shows that over-producing MCR-1 in *E.coli* imposes a substantial fitness loss, observed by slow growth rates, impaired membrane integrity, reduced bacterial survival and decreased competitiveness (Yang *et al.*, 2017). The fitness burden imposed by *mcr-3* seems to be lower than that of *mcr-1*, and *mcr-3* is more stable and competitive, compared to *mcr-1*-expressing isolates. Further comparisons between *mcr-1* and *mcr-3* have also been explored in Chapter 4 and discussed below.

(iv) The impact of MCRPE isolates on bacterial virulence. The activity of MCR-enzyme is to catalyze the transfer of PEtN to the phosphate groups of lipid A, the endotoxin portion in GNB outer-membranes. It has been proposed that modified lipid A provides bacteria with protection from innate immune systems in host tissues. In chapter 3, *mcr-1*-modified lipid A decreased the production of pro-inflammatory cytokines TNF-α and IL-6. Furthermore, after the acquisition of a *mcr-1*-producing natural plasmid, the bacteria become less virulent and

with reduced Galleria mortality. These data suggest that the activity of MCR-1 may aid bacteria in evading host immune systems.

7.2 The links between fitness and the future of colistin resistance: would the *mcr*-conferring fitness cost lead to the reversion of colistin resistance in *E. coli*?

The emergence and development of antibiotic resistance is a complex biological process driven by many factors, including the volume of antibiotic use, the resistance-conferred fitness burden and the frequency of compensatory mutations (Andersson & Hughes, 2010, Hughes & Andersson, 2015). Factors effecting fitness cost of antibiotic resistance have been reviewed in my introduction. Briefly, antibiotic resistances conferred by chromosomal mutations and plasmid-carrying genes are initially associated with a fitness loss evidenced by low growth rates and less competitiveness. Fitness cost conferred by colistin resistance plays a role in limiting the emergence and development of resistance to colistin; for example, in *A. baumannii*. According to SENTRY Antimicrobial Surveillance Program (2006-2009), analyzing a large number of *A.baumannii* (4686 isolates), the reported rates of resistance was 0.8% for polymyxin B and 0.9% for colistin, and most of MDR *A. baumannii* were susceptible to colistin (Gales *et al.*, 2011). In another recent large population study, the colistin resistance of *A. baumannii* was also very low level with resistant rates of 2.1% (9/401) from Turkey (Aydin *et al.*, 2017). These data suggest that colistin is still active against *A. baumannii*. This phenomenon is probably due to the fitness costs imposed by non-silent mutations in the LPS synthesis genes (*lpxACD*) and *pmrAB* systems (Adams *et al.*, 2009, Beceiro *et al.*, 2014, Pournaras *et al.*, 2014). It has also been reported that the *in vitro* growth rates of *lpx*-mutants were dramatically diminished; furthermore, both *in vitro* and *in vivo* competition experiments revealed a significant reduction of fitness - as low as less than 0.01 (Beceiro *et al.*, 2014). Similarly, *pmrB*-mutants also failed to reach the same growth rate as

their parental strains, and *pmrB*-mediated colistin resistance is associated with a fitness loss *in vivo*. Both colistin- resistance mechanisms have been shown to cause a noticeable fitness cost, with a greater reduction associated with mutations in *lpxACD* resulting in complete loss of LPS, than with *pmrB* mutants mediating PEtN addition to lipid A (Beceiro *et al.*, 2014). To date, no *mcr*-gene has been identified in *A. baumannii.*

Given the above, an obvious question to ask is: does plasmid-mediated colistin resistance, *mcr*-genes, firstly discovered in late 2015, have a fitness cost too? Apparently, fitness costs of resistance alone are not a good predictor of subsequent clinical resistance development. The dissemination of plasmid-encoded colistin resistance also depends on the following three factors: *First*, efficiency of transfer versus genetic barriers; *second*, the genetic contexts/background of the resistance genes, and *third*, the ecological opportunity for plasmid-encoded resistance genes to enter a pathogen (Hughes & Andersson, 2015). All of these factors are key to understanding and predicting the development of this novel colistin resistance mechanism, but currently, we have very limited data of these factors. The main host of *mcr-1* and *mcr-3* genes is *E. coli*, and *mcr*-genes, located on transferable plasmids, confer low-moderate resistance to colistin (MICs 2-8 mg/l) (Liu *et al.*, 2015), when compared to chromosomal-mediated resistance (MICs 8-256 mg/l). In Chapter 3, the biological costs on the expression of *mcr-1*, including *in vitro* growth rate, competiveness, cell morphological changes and cell viability, have been determined. The decrease in growth rates of *mcr-1*-producing *E .coli* induced by different concentrations of L-arabinose, were clearly evident. In the competition assay, over-expression of *mcr-1* causes a high fitness loss, suggesting that the level of *mcr-1* expression in *E. coli* is responsible for bacterial fitness cost; similar observations have been made in *K. pneumoniae* (Tietgen *et al.*, 2017). More importantly, pleiotropic effects of *mcr-1* expression on *E. coli* physiology also have been observed. Firstly, MCR-1 is an outer membrane protein consisting of five-helix hydrophobic domain and a

soluble form, the expression of *mcr-1* has profound effects on the outer membrane topology, the multi-layer cell envelope has been significantly compromised. Furthermore, the over-production of MCR-1 is toxic to the bacterial cell, observed by a decreasing cell populations during bacterial growth cycles and a significantly higher ratio between dead- and alive- cells. These data may explain why expression of *mcr-1* has been tightly controlled resulting in only low-moderate colistin resistance (Yang *et al.*, 2017). However, the carriage of a natural occurring plasmid encoding *mcr-1* also causes only negligible effects on growth rates of clinical isolates, which is consistent with Tietgen's results (Tietgen *et al.*, 2017, Yang *et al.*, 2017). Moreover, bacterial architecture is intact and cell viability are not affected after the acquisition of a *mcr-1* positive plasmid (Yang *et al.*, 2017). This is possible because these clinical isolates can moderate the acquisition of *mcr-1* by compensatory adaptations, facilitating the expression of *mcr-1.* Tietgen et al. examined the *mcr-1*-imposed fitness costs by a competition growth experiment in *K. pneumoniae* and *E. coli* and showed that the acquisition of a natural *mcr-1*-positive plasmid impaired fitness in *K. pneumoniae* but not in *E.coli,* this may, in part, explain the high prevalence of *mcr-1* in *E.coli*, but less present in other species like *K. pneumoniae* (Tietgen et al., 2017). Most recently, a deactivated *mcr-1* gene by the insertion of an IS*1294b* element has been found in an uropathogenic *E.coli* ECO3357 (Zhou *et al.*, 2018), this inactive form of *mcr-1* gene can be induced under colistin pressure by the removal of IS*1294b* element. Furthermore, the acquiring of this inducible *mcr-1*-plasmid did not impair bacterial fitness. This finding provides a novel insight into the fluidity and plasticity of genetic elements augmenting gene expression in response to external stimuli such as colistin challenge.

7.3 Are compensatory mutations in *mcr-1* responsible for the amelioration of bacterial fitness loss?

It maybe be too naïve to imagine that the high fitness cost imposed by *mcr-1* expression, will limit the spread and maintenance of *mcr*-mediated colistin resistance in pathogen population. It has been identified that some clinical strains carrying acquired-resistance genes can compensate the fitness cost, thereby producing a selective advantage and raising the potential spread of antibiotic resistance (MacLean *et al.*, 2010). The amelioration of the high cost imposed by antibiotic resistance plasmid provides a good illustrative example of how high fitness costs can be negated by compensatory mutations, which is crucial for the maintenance and dissemination of antibiotic resistance in clinical and natural environments (Loftie-Eaton *et al.*, 2017). Plasmids R1 and RP4 both carrying multidrug resistance genes imposing an initial fitness burden on bacteria (Dahlberg & Chao, 2003), but the cost was reduced through changes in both the plasmids and the bacteria. The altered plasmids no longer impose a cost on the hosts when transferred to a plasmid-free recipient; additionally, the recipient showed enhanced plasmid maintenance, thereby enhancing plasmid persistence (Loftie-Eaton *et al.*, 2017). The evolution trajectories of costly multi-drug resistance are more interesting. There are several factors influencing the trajectory of compensatory mutations associated with fitness cost: **i)** selective pressure by antibiotics; **ii)** level of resistance; **iii)** frequency of mutation rates; **iv)** impact of each mutation on fitness burden (Toprak *et al.*, 2011, Lesho *et al.*, 2013, Hughes & Andersson, 2017, Wong, 2017). It has been reported that mutations in *pmrB* can be induced by high concentration of colistin, leading to an increase in resistance to colistin and imposing a fitness cost (Phan *et al.*, 2017). Due to the global spread of *mcr-1* mediating moderate levels of colistin resistance, in Chapter 3, I developed a selective passage that continuously monitor bacterial growth, colistin sensitivity and fitness cost. The data shows that wild-type MCRPEC (4-8 mg/l) also can be readily induced to high-

level of colistin resistance (16-256 mg/l) by multi-step passage in the increasing concentrations of colistin (Yang *et al.*, 2017). Most of these HLCRMs initially confer huge fitness costs with slow growth rates comparing their respectively parental strains, except PN16. However, their fitness costs can be subsequently compensated by serial passages in the absence of colistin, reflected by their increasing growth rates (**Fig.7.1**). This observation supports the fact that clinical strains with high levels of colistin resistance, leading to large fitness costs, possibly evolve alternative pathways for rapid compensatory mutations, thereby potentially stabilizing the costly HLCRMs in colistin-free condition. However, little is known about the genetic adaptations of these HLCRMs, deeper sequencing is currently being determined to better understand the evolution of HLCRMs.

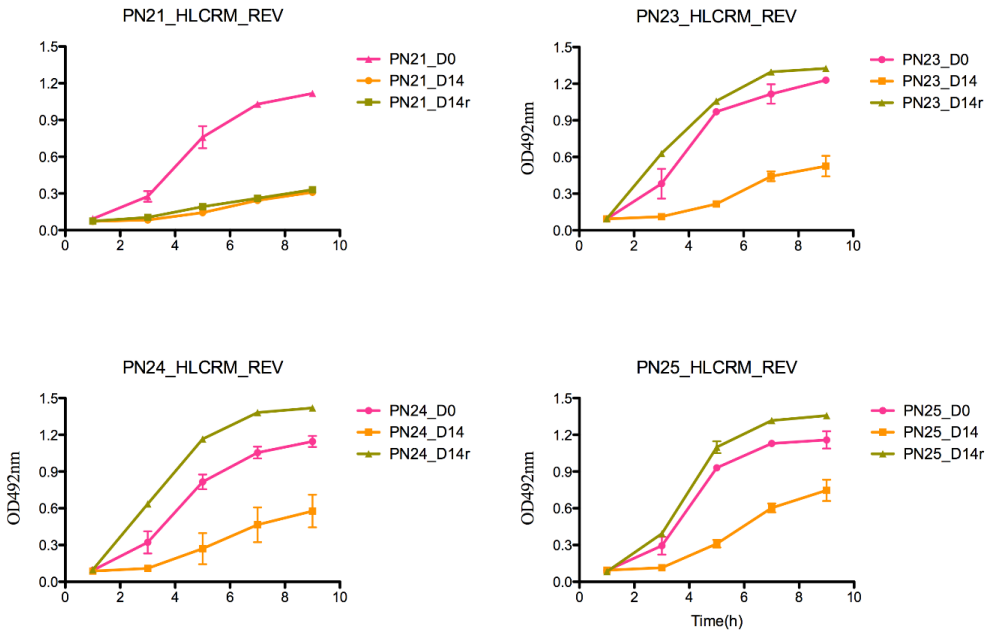


Fig.7.1. Growth curves of ancestral MCRPEC strains (referred as _D0) and their respective HLCRMs (referred as _D14) and revertants (referred as _D14r). The means of three independent replicates were shown and the error bars represent the S.D (n=3).

The fitness measurements of *mcr-1* expression have also been employed in *mcr-3*-carrying strains. Interestingly, in comparison with the high fitness burden imposed by *mcr-1* expression, *mcr-3* gene seems to confer colistin resistance with low fitness costs, evidenced by higher growth rates, less cell toxicity and higher fitness (described in **Chapter 4**). Most importantly, the abundance and stability of *mcr-3*-plasmids is higher that of *mcr-1*-plasmid in both competition models in laboratory strains J53 and wild-type clinical strains containing both *mcr-1* and *mcr-3* plasmids (**Fig.4.9** and **Fig.4.10** in **Chapter 4**). Based on these observations the expression of *mcr-3* is less toxic to the cell, and bacteria carrying *mcr-3* seems to be fitter and more stable even in the colistin-free environments, when compared to that of *mcr-1*-carrying strains. Although it is prudent to be circumspect, *mcr-3* gene confers colistin resistance with a lower fitness and thus it is tempting to predict that *mcr-3* may become globally dominant. However, our results also indicate the limitations of strategies predicting the development of colistin resistance based only on growth competition measurement *in vitro*. If laboratory assays of relative growth together with epidemiological studies on the prevalence of targeted resistance genes in clinical isolates, the fitness measures made *in-vitro* can have better clinical relevance (Hughes & Andersson, 2015). Thus, it is necessary to conduct an international systematic surveillance of *mcr* carried with biological cost studies to better predict which genotypes may globally dominate.

7.4 What is the impact of *mcr*-mediated lipid A modification on the interations between bacteria and its hosts?

All mammals possess innate immune mechanisms which are induced during the invasion of pathogens into the host. The immune responses involves the synthesis of cytokine and chemokine, killing microbes with membrane-bound vacuoles, or secretion of molecules with antimicrobial activities (Ernst *et al.*, 2001). Currently, one of the best characterized stimuli of

the mammalian immune system is lipid A, the endotoxic component of LPS from GNB. Lipid A comprises of fatty acid chains and two-phosphate substitutents bound to 1'- and 4-position of a glucosamine dimer. In response to lipid A, host cells synthesize and secrete a number of cytokines and adhesion molecules that result in initiating a wide range of immune responses, including enhanced killing by phagocytes, activation of potent mediators (such as IL-1β and TNF-α) of inflammation, thereby eventually eliminating the invaded bacteria (Ernst *et al.*, 2001, Raetz *et al.*, 2007). Such immune responses can clear bacterial infections but can over-produce inflammatory factors resulting in sepsis, for example, damaging of blood vessels and causing septic shock.

In mammalian cells, at least ten members of toll-like receptors (TLRs) are responsible for the recognition of microbes. TLR4 complexed with an accessary protein MD-2, is primarily responsible for the response to bacterial lipid A (Beutler, 2000, Scott *et al.*, 2017). The special structural features of lipid A, particularly its two phosphate groups, are essential to activate TLR4/MD2 in human cells (Beutler, 2000, Raetz *et al.*, 2007). The LPS response pathway involves a series of interactions with several signaling molecules, contributing to the early detection and engagement of bacteria after they invade the host (Beutler, 2000, Lu *et al.*, 2008, Rosadini & Kagan, 2017). Briefly, LPS initially binds to the LPS-binding protein, LBP, which in turn facilitates its interaction with CD14 on the surface of mononuclear cells. Protein CD14 acts as a co-receptor together with TLR4 and transfers LPS to a TLR4/MD2 complex on the cell surface; thereby, stimulating macrophages to produce pro-inflammatory cytokines including interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF-α). As a result, LPS-TLR4/MD2 activated innate immune systems can effectively eliminate invading microbes; however, high-level of LPS may trigger the overproduction of cytokines, which can drive the immune system into "overload" resulting in clinical presentations such as septic shock (Ernst *et al.*, 2001).

According to above description, alterations of the Gram-negative outer-membrane; particularly LPS, may present an evasive mechanism by Gram-negative bacteria disguising them from host immune surveillance and defense systems (Ernst *et al.*, 2001, Raetz *et al.*, 2007, Needham & Trent, 2013). It has been widely studied that the chromosomal two-component systems, *pmrAB* and *phoPQ*, control expression of LPS modification genes in GNB, including *E. coli*, *Salmonella Typhimurium*, *K. pneumoniae* and *A. baumannii*. In addition to promoting resistance to polymyxins, PmrA- or PhoP- modified lipid A has been shown to reduce host inflammatory response and confer resistance to serum, thereby resulting in the persistence of colistin-resistance bacteria in host tissues. Because lipid A is the key signal for host recognition of invading microbes, and the modified lipid A may result in reduced TLR4/MD2 and LPS interactions, in addition to reduced innate immune responses, persistence may facilitate survival and dissemination. The following is the brief descriptions of pathogenic impacts of colistin resistance-mediated LPS modifications mediating on two commonest GNB, *Salmonella* spp and *K. pneumoniae*, where *mcr* genes have been found.

(i) *Salmonella* spp.: This phenomenon in *Salmonellh* spp. has been extensively reviewed (Guo *et al.*, 1997, Ernst *et al.*, 2001, Gunn, 2008, Kawasaki, 2012, Dalebroux & Miller, 2014). It also has been found that the protein PmrA-dependent anti-virulence of *S. Thyphimurium* is associated with down-regulation of the type III secretion system Spi/Ssa, which is necessary for bacterial survival within macrophages by delivering effectors into the phagosomal surface (Choi & Groisman, 2013). In *S. Thyphimurium*, *phoP/phoQ* system regulates a number of unique genes including *pagL*, *pagQ* and *pagP*, of these contributing to the specific structural changes in lipid A, thereby promoting survival and persistence in host tissues (Ernst *et al.*, 2001, Kawasaki *et al.*, 2004). Additionally, PhoP-dependent modifications of lipid A has been shown to affect host innate inflammatory response (Loftie-Eaton *et al.*, 2017). LPS extracted from PhoP-null bacteria induces higher level of E-selection

expression from human endothelial cells and higher TNF-α expression from human monocyte-derived macrophage, comparing to wild-type LPS (Guo *et al.*, 1997). This probably because the inability of LPS modifications in *phoP*-null bacteria, and complete structural LPS enhance the host recognition of invading bacteria, thereby promoting the activation of inflammatory system for clearance of bacteria (Gunn, 2008). The altered inflammatory responses to environmentally modified lipid A suggest that *Salmonella* can gain survival advantage within host tissues. Unsurprisingly, in the mouse model, virulence of *Salmonella* strains with *pmrA*-activated Ara4N addition to lipid A are highly attenuated when the strains were administered orally, but not by intraperitoneally (Guo *et al.*, 1997, Gunn *et al.*, 2000). This result includes that when mice orally inoculated with the same dose of bacteria, the survival rates of mice with *pmrF::Tn10d* mutants has seven-fold higher (63% vs 9%) than that with wild-type strains. Additionally, the extended survivals with more than 20 days was observed in some mice infected with the *pmrF::Tn10d* mutants, while the mice inoculated with wild-type *Salmonella* strains only have the average 12 days survival (Gunn *et al.*, 2000). However, in some studies, PEtN modification mediated by *pmrC* and *cptA* shown a modest virulence defect in the mouse model (Tamayo *et al.*, 2005).

(ii) *K. pneumoniae*: the inactivation of MgrB, the repressor of PhoQ/PhoP signal system, is a common mechanism of colistin resistance in *K.pneumoniae*, via up-regulation of *phoP* and *pmrK* genes responsible for LPS decorations (Cannatelli *et al.*, 2014, Giani *et al.*, 2015). However, in contrast to the above findings that colistin resistance is commonly associated with fitness loss and impaired virulence, *mgrB* mutation not only confers colistin resistance by inducing phoP-activated multiple lipid A remodeling, but also enhances *K.pneumoniae* virulence in a *G.mellonella* model (Kidd *et al.*, 2017). Furthermore, infection with *mgrB* mutant was not attenuated in a C57BL/6 mice model with similar bacterial load in murine lung and spleen to those of wild type *K. pneumoniae*. However, the *mgrB* mutation also

results in reduced the secretion of proinflammatory cytokine TNF-α in macrophage upon infection. This scenario in *K. pneumoniae* whereby *mgrB*-dependent colistin resistance mechanism enhances bacterial virulence, may provide some insight into the high rate of clinical mortality associated with colistin resistance *K. pneumoniae* (Kidd *et al.*, 2017). comparing to their isogenic strains, the bacterial loads of *pagP* and *pmrF* mutants were lower in both trachea and lung (Llobet *et al.*, 2011), suggesting *pagP*- and *pmrF*-activated lipid A modifications in *K.pneumoniae* play a role in immune-evasion strategies in the mouse model.

In conclusion, the variety of modifications of GNB lipid A have multifaceted effects on innate immune responses. However, whilst much is known of PhoQ/PhoP in, for example, Klebsiella and Pseudomonas, there are no similar studies in *E. coli*. Therefore, we sought to determine the impact of MCR-1 modification(s) *mcr-1*) on lipid A and the subsequent response of the human immune system. Results described in Chapter 3 showed that *mcr-1*-dependent PEA lipid A modification has an effect on the production of TNF-α and IL-6 in human macrophage differentiated by human monocytic cell lines (THP-1 cells). LPS extracted from MCR-1-producing bacteria produces lower levels of TNF-α and IL-6 expression from human macrophage, relative to wild-type LPS (**Fig.3.10** in **Chapter 3**). Thus, *mcr-1*-mediated PEA modification of lipid A in *E. coli* plays a dual role in protecting bacteria from colistin activity and enhancing survival advantages against host innate defenses. Additionally, MCRPEC also reduces virulence in the *G. mellonella* model, compared to non-MCRPEC. *In vitro* growth rate is not significantly different between the *mcr*-harboring transconjugants and their respective recipients, demonstrating that the killing rates or pathogenic potential is not due to a growth deficiency. These data are further supported by two recent studies examining the fitness of *E. coli* harboring *mcr-1* plasmid (Tietgen *et al.*, 2017, Zhang *et al.*, 2017). Both studies (Zhang et al., 2017 and Tietgen et al., 2017) demonstrated that acquisition of a natural *mcr-1* plasmid had no significant fitness cost in *E.*

coli. In the Zhang et al. study, it also shown that *E. coli* carrying both *mcr-1* and $bla_{\text{NDM-5}}$ plasmids possess a slower growth rate and reduced fitness than that with the $bla_{\text{NDM-5}}$ plasmid alone (Zhang *et al.*, 2017). Impaired virulence was observed in *E. coli* harboring both *mcr-1* and $bla_{\text{NDM-5}}$ plasmids: the time to murine death was over 14 days, while mice injected with E. coli carrying the $bla_{\text{NDM-5}}$ plasmid alone died within one day (Zhang *et al.*, 2017). Tietgen and colleagues also examined the impact of *mcr-1* cytotoxicity in A549 lung epithelial cells and virulence in the *G. mellonella* model, and shown that it has no significant impact in both models (Tietgen *et al.*, 2017).

7.5 WGS analysis of MCRPEC strains: what factors facilitate the global spread of *mcr* genes?

Since its discovery in November 2015, the global spread of *mcr* genes have been extensively reported. Aside from mediating resistance to colistin, MCRPE are frequently associated with phenotypic and genotypic resistance to multiple classes of antibiotics including carbapenems, fluoroquinolones and aminoglycosides. MCRPE isolates are genetically diverse and previous efforts to identify specific features that can distinguish human clinical isolates from animal or environmental isolates have yet to yield meaningful data. (Matamoros *et al.*, 2017, Wang *et al.*, 2018). According to a recent study that included over 400 MCRPEC isolates from the literature, their genetic relatedness based on highly diverse MLST profiles suggest that no specific host-linkage is responsible for its global transmission. Among them, a higher prevalence of *mcr-1*-carring *E.coli* ST10 has been identified (40/312, 12.8%) from Africa, Asia, Europe and South-America, compared to other STs (Wang *et al.*, 2018). So far, at least 14 incompatibility groups of *mcr-1*-linked plasmids have been found, including IncF, IncFI, IncFII, IncFIB, IncHI1, IncHI1A (FIA), IncHI1B, IncHI2, IncI2, IncP, IncX4, IncX3/X4, IncY, repB (p0111) (Matamoros *et al.*, 2017).

Unlike *mcr-1* that has been widely studied, *mcr-3*, which was first reported in late 2017, are still relatively unknown. To better understand the dynamics of *mcr* genes, especially *mcr-1* and *mcr-3*, in this study, I have combined the power of WGS with a genome-data-base analysis, encompassing a total of 236 clinical, animal and environmental MCRPE isolates from Thailand and Vietnam, consisting of 219 MCRPEC (see the detail in **Chapter 6**) and 17 MCRPKP (see the detail in **Chapter 5**). A variety of MLST profiles was found in 219 MCRPEC isolates, consisting of 54 STs in 136 isolates from Vietnam and 31 STs in 83 isolates from Thailand. In comparison with other STs, a higher prevalence of ST10 has been observed in MCRPEC strains (23/219, 10.50%), including 18 ST10 carrying *mcr-1* only and 5 ST10 carrying both *mcr-1* and *mcr-3* plasmids from human, environmental and animal isolates. *E. coli* ST10 is also linked to *mcr-2* identified in porcine isolates from Belgium (Xavier *et al.*, 2016). Therefore, we hypothesize that several *E. coli* strains, notably those related to ST10, may play a crucial role as a reservoir of *mcr* genes. Interestingly, ST43 *K. pneumoniae* has been identified in 17 MCRPKP isolates obtained from blowflies from difference places in Thailand. Many possess the same size *mcr-1*-plasmid belonging to IncX4 isolated from blowflies, companion animals, food-producing animals and human faeces (described in **Chapter 5**). These findings suggest that ST43 *K. pneumoniae* from blow flies may act as an efficient vector for disseminating *mcr-1* genes between environments and humans. IncX4 plasmid is the most prevalent *mcr*-linked plasmid type, which have found in human feces, animals, environmental water and blow flies in MCRPE collection. In addition, 26 of IncX4 plasmids share an identical plasmid background, further suggesting widespread dissemination of IncX4 in the Thai environment (**Fig.5.5** in **Chapter 5**). Furthermore, broad-host range plasmid IncP, the second biggest group of *mcr*-associated plasmids, are mainly found in clinical isolates from Vietnam (n=19), in which nine IncP plasmids contain *mcr-3*, and ten with *mcr-1* (**Table 6.1** in **Chapter 6**).

7.6 Future Perspectives

Unlike the high detection rates of MCRPE in animals, with *mcr-1* gene in particular, in human population and environmental factors have been reported to be substantially low (0.2-1.7%) (described in **1.3.2.1** in **Chapter 1**). Based on 'one health' perspective, what roles have animal-originated Enterobacteriaceae played in the subsequent spread of *mcr*-genes? In order to better understand the possible interspecies transmission of *mcr*- genes, the most obvious follow-up work is to do a comprehensive sampling and testing of animal-, environmental- and human-pathogenic bacteria for the presence of *mcr*-like genes. This project is collaborated with our current collaborators in Thailand, Vietnam, Combodia and China.

Besides epidemiological surveillance, there is an urgent need to develop novel tools for predicting and preventing the evolution of *mcr*-conferring colistin resistance. Whole-genome sequence data has highlighted the significant genetic diversity of MCRPE isolates, but the impact of this diversity on the evolution and dissemination of *mcr*-linked colistin resistance remains poorly understood. Deeper WGS-based analysis and comprehensive epidemiological data, combined with the 'game' between acquiring and loss of *mcr*-like genes will provide insights for mathematical modellings, to scale up *mcr*-fitness landscape, and predict the global spread trend of MCRPE isolates. This project is on-going.

Another follow-up research interest is to ascertain the exact mechanisms behind the HLCRMs. Phenotypic characteristics of these HLCRMs have been determined, such as high-level MICs of colistin (32-256 mg/L), reduced fitness and highly attenuated virulence in a Galleria mellonella model (Yang *et al.*, 2017). However, there is a missing link between genetic alterations and phenotypic changes. Therefore, in the future research woek, I am interested in understanding how genomic background change the rate and evolution of

colistin resistance in these HLCRMs, using combination of high-quality genomic and functional analysis.

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Appendice

Appendix I Culture media and Stock solutions

Appendix 1.1 list of culture media

(1) Luria Bertani (LB) Agar, Miller (Thermo Fisher Scientific, Waltham, USA): Supplemented with specific antimicrobials for subculture and passage experiments.

(2) LB Broth, Miller (Thermo Fisher Scientific, Waltham, USA): Used for subculture, conjugation and passage experiments.

(3) Mueller Hinton (MH) Agar (E&O Laboratories Ltd): Used for susceptibility testing of antimicrobials.

(4) BrillianceTM UTI Clarity Agar (Oxoid Ltd, Basingstoke, UK): Used for selective isolation in mating experiments by adding different antibiotics.

Appendix 1.2 list of stock solutions for PFGE and Southern blotting

Cell suspension buffer

1M Tris (pH8.0)	100 ml
0.5 Na_2EDTA(pH8.0)	200 ml

Dilute to 1000 ml with sterial distilled water.

Cell lysis buffer

1 M Tris (pH8.0)	50 ml
0.5 M Na_2EDTA(pH8.0)	100 ml
10% Sarcosyl	100 ml

Make up to 1000 ml with sterial distilled water, 10% sarcosyl was added after autoclaved.

1X TE buffer

1 M Tris-HCl (pH8.0)	10 ml
0.5 M Na_2EDTA (pH8.0)	2 ml

Dilute to 1000 ml with sterile water.

10X S1 buffer

Sodium acetate (Fisher)	12.3 g
Zinc acetate (Fisher)	0.92 g

Dissolve both in 200 ml of water, followed by adding 250 ml of glycerol and adjust pH to 4.6. Then add sterile distilled water to 500 ml. Keep 10X S1 buffer at -20 ^{o}C.

10X TBE buffer

Tris base (Fisher)	108 g
boric acid (Sigma)	55 g
0.5 M EDTA (pH 8)	40 ml

Adjust pH to 8 with concentrated HCl and make up to 1000 ml with sterile distilled water.

Denaturing solution

NaCl	87.66 g
NaOH	20 g

Dissolve them in 1000 ml of sterile distilled water.

Neutralising solution

Tris base (Fisher)	78.5 g
NaCl	87.6 g

Dissolve both in 800 ml of sterile water, adjust pH to 7.5 with concentrated HCl, and then make up to 1000 ml with sterile distilled water.

Pre-hybridisation stock solutions

5% Polyvinylpyrrolidone (Sigma)	0.4 ml
5% ficoll (Sigma)	0.4 ml
10% SDS	1 ml
20X SSC	6ml
full cream UHT milk	1 ml
10mg/ml herring testes DNA (Sigma)	0.3 ml

Make up to 20 ml with Sterile distilled water.

20X Saline-sodium citrate solution (SSC)

Nacl (Fisher)	17.5 g
Na_3citrate.H_2O(Fisher)	8.8 g
Concentrated HCl	adjust pH to 7.0

Make up to 100 ml with sterile distilled water.

Note: all solutions need to be autoclaved before use.

Appendix II List of studied isolates from Thailand and Vietnam

Appendix Table 2.1 list of 100 MCRPE isolates from Thailand used in the study

Isolate	species	Sources	*mcr*-genes	Year
PN101	*E.coli*	Blow fly	*mcr-1*	2015
PN102	*E.coli*	Blow fly	*mcr-1*	2015
PN103	*E.coli*	Blow fly	*mcr-1*	2015
PN108	*E.coli*	Blow fly	*mcr-1*	2015
PN109	*E.coli*	Blow fly	*mcr-1*	2015
PN111	*E.coli*	Blow fly	*mcr-1*	2015
PN112	*E.coli*	Blow fly	*mcr-1*	2015
PN116	*E.coli*	Blow fly	*mcr-1*	2015
PN117	*E.coli*	Blow fly	*mcr-1*	2015
PN119	*E.coli*	Blow fly	*mcr-1*	2015
PN121	*E.coli*	Blow fly	*mcr-1*	2015
PN122	*E.coli*	Blow fly	*mcr-1*	2015
PN123	*E.coli*	Blow fly	*mcr-1*	2015
PN124	*E.coli*	Blow fly	*mcr-1*	2015
PN126	*E.coli*	Blow fly	*mcr-1*	2015
PN127	*E.coli*	Blow fly	*mcr-1*	2015
PN33	*E.coli*	Blow fly	*mcr-1*	2015
PN73	*E.coli*	Blow fly	*mcr-1*	2015
PN74	*E.coli*	Blow fly	*mcr-1*	2015
PN75	*E.coli*	Blow fly	*mcr-1*	2015
PN76	*E.coli*	Blow fly	*mcr-1*	2015
PN78	*E.coli*	Blow fly	*mcr-1*	2015
PN80	*E.coli*	Blow fly	*mcr-1*	2015
PN83	*E.coli*	Blow fly	*mcr-1*	2015
PN85	*E.coli*	Blow fly	*mcr-1*	2015
PN86	*E.coli*	Blow fly	*mcr-1*	2015
PN87	*E.coli*	Blow fly	*mcr-1*	2015
PN88	*E.coli*	Blow fly	*mcr-1*	2015
PN91	*E.coli*	Blow fly	*mcr-1*	2015
PN92	*E.coli*	Blow fly	*mcr-1*	2015
PN93	*E.coli*	Blow fly	*mcr-1*	2015
PN94	*E.coli*	Blow fly	*mcr-1*	2015
PN203	*E.coli*	Blow fly	*mcr-3*	2015
PN207	*E.coli*	Blow fly	*mcr-3*	2015
PB215	*E.coli*	Blow fly	*mcr-3*	2015
PN218	*E.coli*	Blow fly	*mcr-3*	2015

Isolate	species	Sources	*mcr*-genes	Year
PN313	*E.coli*	Blow fly	*mcr-3*	2015
PN326	*E.coli*	Blow fly	*mcr-3*	2015
PN328	*E.coli*	Blow fly	*mcr-3*	2015
PN107	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN114	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN118	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN120	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN77	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN79	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN81	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN84	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN95	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN96	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN97	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN98	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN104	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN105	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN106	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN110	*K. pneumoniae*	Blow fly	*mcr-1*	2015
PN100	*K. pneumoniae*	Blow fly	*mcr-1*	2015
Tha30	*E.coli*	Cattle (feces)	*mcr-1,mcr-3*	2015
PN1	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN2	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN3	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN4	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN5	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN6	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN7	*E.coli*	Environmental water	*mcr-1*	2015
PN8	*E.coli*	Environmental water	*mcr-1,mcr-3*	2015
PN9	*E.coli*	Environmental water	*mcr-1*	2015
PN41	*E.coli*	feces from healthy human	*mcr-1,mcr-3*	2015
PN42	*E.coli*	feces from healthy human	*mcr-1,mcr-3*	2015
PN43	*E.coli*	feces from healthy human	*mcr-1*	2015
PN44	*E.coli*	feces from healthy human	*mcr-1*	2015
PN45	*E.coli*	feces from healthy human	*mcr-1, mcr-3*	2015
PN46	*E.coli*	feces from healthy human	*mcr-1*	2015
PN47	*E.coli*	feces from healthy human	*mcr-1,mcr-3*	2015
PN49	*E.coli*	feces from healthy human	*mcr-1*	2015
PN51	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN52	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN54	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN55	*E.coli*	rectal swab from patient	*mcr-1*	2015

Isolate	**species**	**Sources**	***mcr*-genes**	**Year**
PN56	*E.coli*	rectal swab from patient	*mcr-1,mcr-3*	2015
PN57	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN58	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN59	*E.coli*	rectal swab from patient	*mcr-1,mcr-3*	2015
PN60	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN67	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN68	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN69	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN70	*E.coli*	rectal swab from patient	*mcr-1*	2015
PN71	*E.coli*	rectal swab from patient	*mcr-1,mcr-3*	2015
PN38	*K. pneumoniae*	rectal swab from patient	*mcr-1*	2015
PN10	*E.coli*	Pet (cat)	*mcr-1*	2015
PN11	*E.coli*	Pet (cat)	*mcr-1*	2015
PN12	*E.coli*	Pet (cat)	*mcr-1*	2015
PN21	*E.coli*	Poultry (feces)	*mcr-1*	2015
PN23	*E.coli*	Poultry (feces)	*mcr-1*	2015
PN24	*E.coli*	Poultry (feces)	*mcr-1,mcr-3*	2015
PN25	*E.coli*	Poultry (feces)	*mcr-1*	2015
PN28	*E.coli*	Poultry (feces)	*mcr-1*	2015
PN29	*E.coli*	Poultry (feces)	*mcr-1*	2015
PN32	*E.coli*	Poultry (feces)	*mcr-1,mcr-3*	2015
PN16	*E.coli*	Poultry meat	*mcr-1*	2015

Appendix Table 2.2 list of 136 MCPREC isolates from Vietnam used in this study

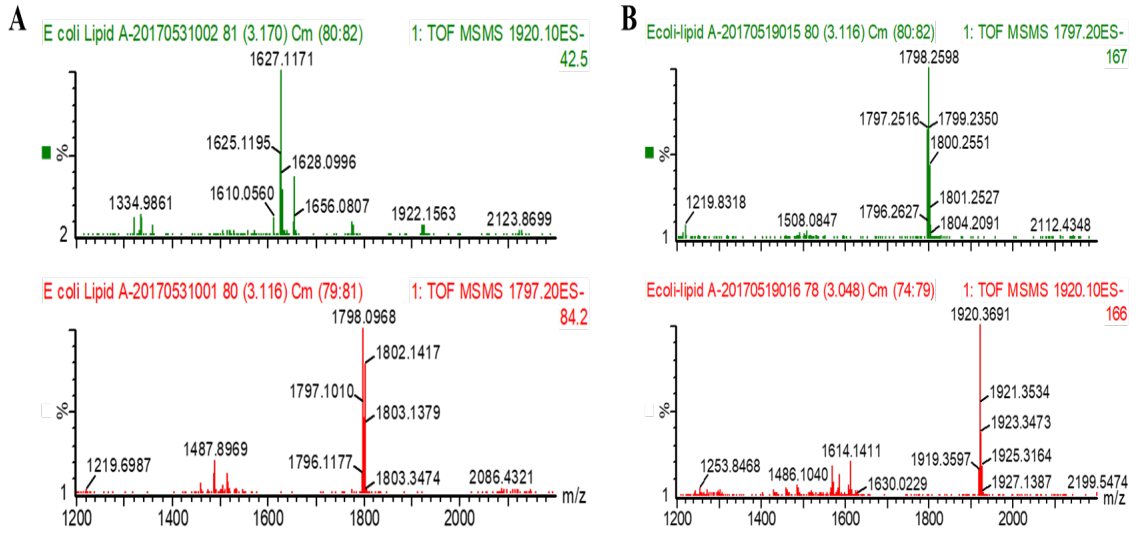
Isolate	*Species*	Sources	Geno	Year
1407.4	*E.coli*	Human Stool	*mcr-1*	2015
1460.1	*E.coli*	Human Stool	*mcr-1*	2015
1440.1	*E.coli*	Human Stool	*mcr-1*	2015
1439.2P	*E.coli*	Human Stool	*mcr-1*	2015
1509.1	*E.coli*	Human Stool	*mcr-1*	2015
1516.1	*E.coli*	Human Stool	*mcr-1*	2015
1519.1	*E.coli*	Human Stool	*mcr-1*	2015
1438.1	*E.coli*	Human Stool	*mcr-1*	2015
1467.2	*E.coli*	Human Stool	*mcr-1*	2015
1470.1	*E.coli*	Human Stool	*mcr-1*	2015
1529.2	*E.coli*	Human Stool	*mcr-1*	2015
1526.2	*E.coli*	Human Stool	*mcr-1*	2015
1450.3	*E.coli*	Human Stool	*mcr-1*	2015
1463.4	*E.coli*	Human Stool	*mcr-1*	2015
1461.1	*E.coli*	Human Stool	*mcr-1*	2015
1474.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1394.1	*E.coli*	Human Stool	*mcr-1*	2015
1481.1	*E.coli*	Human Stool	*mcr-1*	2015
1497.1	*E.coli*	Human Stool	*mcr-1*	2015
1405.1	*E.coli*	Human Stool	*mcr-1*	2015
1400.3	*E.coli*	Human Stool	*mcr-1*	2015
1484.1	*E.coli*	Human Stool	*mcr-1*	2015
1492.2	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1490.1	*E.coli*	Human Stool	*mcr-1*	2015
1392.1	*E.coli*	Human Stool	*mcr-1*	2015
1482.1	*E.coli*	Human Stool	*mcr-1*	2015
1483.1	*E.coli*	Human Stool	*mcr-1*	2015
1386.1	*E.coli*	Human Stool	*mcr-1*	2015
13845.1	*E.coli*	Human Stool	*mcr-1*	2015
1475.1	*E.coli*	Human Stool	*mcr-1*	2015
1487.1	*E.coli*	Human Stool	*mcr-1*	2015
1489.1	*E.coli*	Human Stool	*mcr-1*	2015
1432.1B	*E.coli*	Human Stool	*mcr-1*	2015
1412.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1388.1	*E.coli*	Human Stool	*mcr-1*	2015
1393.1	*E.coli*	Human Stool	*mcr-1*	2015
1442P	*E.coli*	Human Stool	*mcr-1*	2015
1446.1	*E.coli*	Human Stool	*mcr-1*	2015
1472.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1471.1	*E.coli*	Human Stool	*mcr-1*	2015

Isolate	***Species***	**Sources**	**Geno**	**Year**
1500.1	*E.coli*	Human Stool	*mcr-1*	2015
1540.1	*E.coli*	Human Stool	*mcr-1*	2015
1541.1	*E.coli*	Human Stool	*mcr-1*	2015
1422.1	*E.coli*	Human Stool	*mcr-1*	2015
1421.1	*E.coli*	Human Stool	*mcr-1*	2015
1485.1	*E.coli*	Human Stool	*mcr-1*	2015
1435.1B	*E.coli*	Human Stool	*mcr-1*	2015
1543.1	*E.coli*	Human Stool	*mcr-1*	2015
1542.1	*E.coli*	Human Stool	*mcr-1*	2015
1502.1	*E.coli*	Human Stool	*mcr-1*	2015
1503.1	*E.coli*	Human Stool	*mcr-1*	2015
1397.2	*E.coli*	Human Stool	*mcr-1*	2015
1436.1	*E.coli*	Human Stool	*mcr-1*	2015
1433.1	*E.coli*	Human Stool	*mcr-1*	2015
1432.1	*E.coli*	Human Stool	*mcr-1*	2015
1431.2	*E.coli*	Human Stool	*mcr-1*	2015
1419.2	*E.coli*	Human Stool	*mcr-1*	2015
1480.1	*E.coli*	Human Stool	*mcr-1*	2015
1479.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1435.1	*E.coli*	Human Stool	*mcr-1*	2015
1518.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1507.3	*E.coli*	Human Stool	*mcr-1*	2015
1466.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1533.2	*E.coli*	Human Stool	*mcr-1*	2015
1535.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1532.2	*E.coli*	Human Stool	*mcr-1*	2015
1539.1	*E.coli*	Human Stool	*mcr-1*	2015
1537.1	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1536.2	*E.coli*	Human Stool	*mcr-1*	2015
1453.1	*E.coli*	Human Stool	*mcr-1*	2015
1403.2	*E.coli*	Human Stool	*mcr-1*	2015
1404.2	*E.coli*	Human Stool	*mcr-1,mcr-3*	2015
1522.1	*E.coli*	Human Stool	*mcr-1*	2015
1309	*E.coli*	Human Stool	*mcr-1*	2015
1310	*E.coli*	Human Stool	*mcr-1*	2015
1311	*E.coli*	Human Stool	*mcr-1*	2015
1304	*E.coli*	Human Stool	*mcr-1*	2015
1306.1	*E.coli*	Human Stool	*mcr-1*	2015
1308	*E.coli*	Human Stool	*mcr-1*	2015
1334.1	*E.coli*	Human Stool	*mcr-1*	2015
1313.1P	*E.coli*	Human Stool	*mcr-1*	2015

Isolate	***Species***	**Sources**	**Geno**	**Year**
1314.1	*E.coli*	Human Stool	*mcr-1*	2015
1317.1	*E.coli*	Human Stool	*mcr-1*	2015
1327.1	*E.coli*	Human Stool	*mcr-1*	2015
1326.1	*E.coli*	Human Stool	*mcr-1*	2015
HN1402.1	*E.coli*	Human Stool	*mcr-3*	2015
HN1272.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1271.3	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1271.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1456.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1409.2	*E.coli*	Human Stool	*mcr-3*	2015
HN1404.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1050.3	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1398.5	*E.coli*	Human Stool	*mcr-3*	2015
HN964.4	*E.coli*	Human Stool	*mcr-3*	2015
HN961.3a	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN961.3b	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN763.3	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN943.3	*E.coli*	Human Stool	*mcr-3*	2015
HN514.1	*E.coli*	Human Stool	*mcr-3*	2015
HN596.5	*E.coli*	Human Stool	*mcr-3*	2015
HN657.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN657.4	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN657.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN621.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1142.2a	*E.coli*	Human Stool	*mcr-3*	2015
HN1142.2b	*E.coli*	Human Stool	*mcr-3*	2015
HN1142.5	*E.coli*	Human Stool	*mcr-3*	2015
HN1142.4	*E.coli*	Human Stool	*mcr-3*	2015
HN1142.3a	*E.coli*	Human Stool	*mcr-3*	2015
HN1142.3b	*E.coli*	Human Stool	*mcr-3*	2015
HN1380.4	*E.coli*	Human Stool	*mcr-3*	2015
HN1364.4	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1545.1	*E.coli*	Human Stool	*mcr-3*	2015
HN1335.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1335.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN40.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN75.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN75.3	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN2.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN367.4	*E.coli*	Human Stool	*mcr-3*	2015
HN367.2	*E.coli*	Human Stool	*mcr-3*	2015

Isolate	***Species***	**Sources**	**Geno**	**Year**
HN1410.4	*E.coli*	Human Stool	*mcr-3*	2015
HN225.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN225.4	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN225.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN225.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN83.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN83.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN269.2	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN269.3	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1270.5	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
HN1466.5	*E.coli*	Human Stool	*mcr-3*	2015
HN1466.4	*E.coli*	Human Stool	*mcr-3*	2015
HN1570.3a	*E.coli*	Human Stool	*mcr-3*	2015
HN1570.3b	*E.coli*	Human Stool	*mcr-3*	2015
HN1499.1	*E.coli*	Human Stool	*mcr-3*	2015
HN1272.5	*E.coli*	Human Stool	*mcr-3*	2015
AV106.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
AV28.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015
1305.1	*E.coli*	Human Stool	*mcr-3,mcr-1*	2015

Appendix III supplementary data in Chapter 3



Appendix Fig.3.1 ESI-QTOF/MS revealed the PEA modification of bacterial lipid A mediated by *MCR-1* gene.

A, ESI-MS/MS spectrum of the negative ion of the lipid A extracted from the constructed control strains *E. coli* W3110+ pUC19. The lipid A has a prominent peak at m/z 1797.10 and no PEA modification existed at m/z 1920. B, ESI-MS/MS spectrum visualization of the negative ion of the lipid A extracted from the constructed positive strains *E. coli* W3110 +pUC19-*mcr-1*. A PEA (123u) is added into the bis-phosphorylated hexaacylated lipid A (m/z 1920).

Note: In this study, the bacterial lipid A was analyzed using Waters Synapt HR mass spectrometry under two different ion channels (m/z 1797 and m/z 1920). TOF MSMS 1920.10ES-: Selected ion monitoring of the product of lipid A modification at m/z 1920.10 with negative ion scan. TOF MSMS 1797.20ES-: Selected ion monitoring of the bacterial lipid A at m/z 1797.20 with negative ion scan

(This figure is contributed by co-authors from China Agriculture University)(Yang *et al.*, 2017)

Appendix Table 3.1 Laboratory strains and plasmids used in this study

Plasmid name	Host strain	Plasmid (size)	Colistin MIC (mg/L)	Reference
mcr-1/pBAD	*E.coli* TOP10	~6000bp	2	This study
pBAD alone	*E.coli* TOP10	~4100bp	0.25	Thermo Fisher, UK
E.coli TOP10	-	-	0.25	Thermo Fisher, UK
mcr-1/pSU18	*E.coli* TOP10	~4000bp	4	(Hinchliffe *et al.*, 2017)
GFP-pHT315	DH5 *alpha*	-	0.5	(Daou *et al.*, 2009)
bla_{TEM-1b}/pBAD	*E.coli* TOP10	~5000bp	0.25	This study
E246A/pBAD	*E.coli* TOP10	~6000bp	0.25	This study
mcr-1 soluble domain /pBAD	*E.coli* TOP10	~5400bp	0.25	This study

Appendix Table 3.2 Relative Fitness (RF) using Flow cytometer

RF(compared to the *mcr-1*/pBAD D0 Strain)	repeats	mean	SD	propagated errors	*p*-value
E.coli TOP10	6	1.0960	0.0129	0.0171	0.132
E.coli TOP10 pBAD	6	1.0955	0.0054	0.0134	0.0649
E.coli TOP10 *mcr-1*/pBAD D3	6	1.0080	0.0062	0.0140	0.041*
E.coli TOP10 *mcr-1*/pBAD D4	6	1.0132	0.0257	0.0283	0.3095
E.coli TOP10 *mcr-1*/pBAD D8	6	0.8258	0.0088	0.0165	0.0022**
E.coli TOP10 *mcr-1*/pBAD D14	6	0.7922	0.0084	0.0164	0.0022**
RF(compared to *mcr-1*/pBAD uninduced strain – 0%[#])					
E.coli TOP10	6	1.0000	0.0147	0.0177	0.8182
E.coli TOP10 *mcr-1*/pBAD +0.0002%	6	0.9992	0.0088	0.0132	0.6991
E.coli TOP10 *mcr-1*/pBAD +0.002%	6	0.9991	0.0153	0.0182	1
E.coli TOP10 *mcr-1*/pBAD +0.02%	6	0.7278	0.0031	0.0108	0.0022**
E.coli TOP10 *mcr-1*/pBAD +0.2%	6	0.4272	0.0074	0.0262	0.0022**

[#] indicates the concentration of L-arabinose (w/v). * indicates $0.01 < p$ value < 0.05 and ** indicates p value < 0.01

Appendix Table 3.3 MICs of colistin for *mcr-1*/pBAD after 14-day serial passage

Passage	MICs of Colistin (mg/L)				
days	***mcr-1*(1)***	***mcr-1*(2)***	**pBAD-1**	**pBAD-2**	**TOP10**
1	0.5	0.5	0.125	0.125	0.125
2	2	1	0.125	0.125	0.25
3	4	2	0.125	0.125	0.25
4	4	4	0.125	0.125	0.25
5	4	4	0.125	0.125	0.25
6	8	4	0.125	0.125	0.25
7	8	4	0.125	0.125	0.25
8	8	16	0.125	0.125	0.25
9	16	16	0.125	0.125	0.25
10	16	16	0.125	0.125	0.25
11	32	16	0.06	0.06	0.125
12	32	16	0.06	0.06	0.125
13	32	16	0.06	0.06	0.25
14	32	32	0.06	0.125	0.25

* indicate duplicates were performed.

Appendix IV supplementary data in Chapter 6

Appendix Table 6.1 Inc types of *mcr*-linked plasmids

Isolate ID	*mcr-3*-linked Inc types				*mcr-1*-linked Inc types					
	P1	FII	X1	colE	X4	P0111	P1	HI1A	HI1B (R27)	HI1A/HI1B (R27)
1335-1	1									
1456-5	1									
657-5	1									
AV106-1	1									
AV28-1	1									
1272-2	1									
269-2	1									
1364-4	1									
1398-5	1									
83-5			1							
943-3		1								
1410-4		1								
1309							1			
1422.1							1			
1460.1					1					
1471.1					1					
1479.1							1			
1475.1							1			
1481.1								1		
1482.1									1	
1497.1						1				
1509.1							1			
1516.1							1			
1529.2					1					
1535.1							1			
1537.1							1			
1540							1			
1543.1					1					
1435.1							1			
1442p					1					
PN47	1									
PN111					1					
PN112					1					
PN116					1					
PN117					1					
PN119					1					
PN12					1					
PN121					1					
PN122					1					

Isolate ID	*mcr-3*-linked Inc types				*mcr-1*-linked Inc types					
	P1	FII	X1	colE	X4	P0111	P1	HI1A	HI1B (R27)	HI1A/HI1B (R27)
PN123					1					
PN124					1					
PN126					1					
PN127					1					
PN16					1					
PN21					1					
PN23					1					
PN25					1					
PN28					1					
PN29					1					
PN33					1					
PN41					1					
PN43					1					
PN44					1					
PN46					1					
PN49					1					
PN51					1					
PN52					1					
PN54					1					
PN55					1					
PN57					1					
PN58					1					
PN60					1					
PN67					1					
PN68					1					
PN7								1		
PN78								1		
PN80									1	
PN83									1	
PN87									1	
PN88									1	
PN9										1
F203		1								
F207		1								
F215				1						
F218		1								
F326		1								
F328		1								
F313		1								
Total No.	10	8	1	1	39	1	10	3	5	1

Appendix Table 6.2 plasmid types found in randomly selected 77 MCRPEC strains from Vietnam

strains	I1	I2	FIA[1]	FIA	FIA[2]	FII[1]	FII$_K$	FIC	FIB[3]	FIV[4]	FII[5]	FIB[6]	FII	FIC	FIB$_K$	HI1A	HI1B[7]	HI2	HI2A	P1	Q1	X1	X2	X4	N	R	Y	Col[8]	ColE10	ColpVC	p0111	Total No.
1386-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
1388-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
1393-1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	5
1394-1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	9
1397-2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	4
1403-2	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	6
1404-2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	4
1407-4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
1419-2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	3
1431-2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	5
1432-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	4
1432-1-b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	4
1439-2B	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	4
1442	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	4

strains	I 1	I 2	FI A[1]	FI A	FI A[2]	FI I[1]	FI I_K	FI C	FI B[3]	FI V[4]	FI I[5]	FI B[6]	F II	FI C	FI B_K	HI 1A	HI 1B[7]	H I2	HI 2A	P 1	Q 1	X 1	X 2	X 4	N	R	Y	C ol[8]	Col E10	Colp VC	p01 11	Total No.
B																																
1442 P	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	7
1446-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1450-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1453-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	4
1460-1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	5
1463-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	3
1467-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
1470-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	2
1472-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	4
1475-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
1479-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	2
1481-1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
1483-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	4
1484-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	3

strains	I 1	I 2	FI A[1]	FI A	FI A[2]	FI I[1]	FI I_K	FI C	FI B[3]	FI V[4]	FI I[5]	FI B[6]	F II	FI C	FI B_K	HI 1A	HI 1B[7]	H I2	HI 2A	P 1	Q 1	X 1	X 2	X 4	N	R	Y	C ol[8]	Col E10	Colp VC	p01 11	Total No.
1489-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1490-1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
1492-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2
1497-1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2
1500-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	4
1507-3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	4
1519-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1532-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2
1533-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	1	5
1535-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
1537-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2
1540-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2
1542-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	3
1543-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	5
1392-	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	5

strains	I 1	I 2	FI A[1]	FI A	FI A[2]	FI I[1]	FI I_K	FI C	FI B[3]	FI V[4]	FI I[5]	FI B[6]	F II	FI C	FI B_K	HI 1A	HI 1B[7]	H I2	HI 2A	P 1	Q 1	X 1	X 2	X 4	N	R	Y	C ol[8]	Col E10	Colp VC	p01 11	Total No.
1																																
1400-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	4
1405-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	2
1412-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2
1421-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1422-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	4
1433-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	4
1435-1B	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	8
1435-1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	8
1436-1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	8
1438-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
1439-2P	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4
1502-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2
1440-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	2
1466-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2

strains	I 1	I 2	FI A[1]	FI A	FI A[2]	FI I[1]	FI I_K	FI C	FI B[3]	FI V[4]	FI I[5]	FI B[6]	F II	FI C	FI B_K	HI 1A	HI 1B[7]	H I2	HI 2A	P 1	Q 1	X 1	X 2	X 4	N	R	Y	C ol[8]	Col E10	Colp VC	p01 11	Total No.
1471-1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2
1461-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	4
1474-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	4
1480-1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
1482-1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	7
1485-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	3
1485-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	3
1487-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	4
1503-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
1509-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
1516-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
1518-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
1522-1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2
1526-2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
1529-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1

strains	I 1	I 2	FI A[1]	FI A	FI A[2]	FI I[1]	FI I_K	FI C	FI B[3]	FI V[4]	FI I[5]	FI B[6]	F II	FI C	FI B_K	HI 1A	HI 1B[7]	H I2	HI 2A	P 1	Q 1	X 1	X 2	X 4	N	R	Y	C ol[8]	Col E10	Colp VC	p01 11	Total No.
2																																
1536-2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
1539-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
1539-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
1541-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2
13845-1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	4
Total No.	**2**	**2**	**23**	**3**	**2**	**1**	**1**	**2**	**2**	**1**	**3**	**11**	**2**	**1**	**26**	**10**	**7**	**12**	**12**	**16**	**1**	**25**	**1**	**8**	**19**	**4**	**4**	**3**	**4**	**1**	**42**	

* The *ori* variants in from FIA[1](HI1), FIA[2] (pBK30683); FII[3] (pHN7A8), FIV[4] (pB171), FII[5] (pSEI1); FIB[6](pB171), HI1B[7](R27), Col[8] (BS512)

Appendix V copies of published papers

Journal page of paper I

nature COMMUNICATIONS

ARTICLE

DOI: 10.1038/s41467-017-02149-0 OPEN

Balancing *mcr-1* expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms

Qiue Yang[1], Mei Li[1], Owen B. Spiller[1], Diego O. Andrey[1,2], Philip Hinchliffe[3], Hui Li[4], Craig MacLean[5], Pannika Niumsup[6], Lydia Powell[7], Manon Pritchard[7], Andrei Papkou[5], Yingbo Shen[8], Edward Portal[1], Kirsty Sands[1], James Spencer[3], Uttapoln Tansawai[6], David Thomas[7], Shaolin Wang[8], Yang Wang[8], Jianzhong Shen[8] & Timothy Walsh[1]

MCR-1 is a lipid A modifying enzyme that confers resistance to the antibiotic colistin. Here, we analyse the impact of MCR-1 expression on *E. coli* morphology, fitness, competitiveness, immune stimulation and virulence. Increased expression of *mcr-1* results in decreased growth rate, cell viability, competitive ability and significant degradation in cell membrane and cytoplasmic structures, compared to expression of catalytically inactive MCR-1 (E246A) or MCR-1 soluble component. Lipopolysaccharide (LPS) extracted from *mcr-1* strains induces lower production of IL-6 and TNF, when compared to control LPS. Compared to their parent strains, high-level colistin resistance mutants (HLCRMs) show reduced fitness (relative fitness is 0.41–0.78) and highly attenuated virulence in a *Galleria mellonella* infection model. Furthermore, HLCRMs are more susceptible to most antibiotics than their respective parent strains. Our results show that the bacterium is challenged to find a delicate equilibrium between expression of MCR-1-mediated colistin resistance and minimalizing toxicity and thus ensuring cell survival.

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Journal page of paper II

Toxin–antitoxin systems and their role in disseminating and maintaining antimicrobial resistance

Article *in* FEMS microbiology reviews · March 2017

DOI: 10.1093/femsre/fux006

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MECHANISMS OF RESISTANCE

Heavy Metal Resistance Genes Are Associated with bla_{NDM-1}- and $bla_{CTX-M-15}$-Carrying *Enterobacteriaceae*

Qiu E. Yang,[a] Siham Rajab Agouri,[a] Jonathan Mark Tyrrell,[a] Timothy Rutland Walsh[a]

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ABSTRACT The occurrence of heavy metal resistance genes in multiresistant *Enterobacteriaceae* possessing bla_{NDM-1} or $bla_{CTX-M-15}$ genes was examined by PCR and pulsed-field gel electrophoresis with S1 nuclease. Compared with clinical susceptible isolates (10.0% to 30.0%), the *pcoA*, *merA*, *silC*, and *arsA* genes occurred with higher frequencies in bla_{NDM-1}-positive (48.8% to 71.8%) and $bla_{CTX-M-15}$-positive (19.4% to 52.8%) isolates, and they were mostly located on plasmids. Given the high association of metal resistance genes with multidrug-resistant *Enterobacteriaceae*, increased vigilance needs to be taken with the use of heavy metals in hospitals and the environment.

KEYWORDS heavy metal resistance, bla_{NDM-1}, $bla_{CTX-M-15}$, plasmids, coresistance

The increasing spread of multidrug-resistant superbugs in clinical environments has prompted worldwide concern, because antibiotic resistance genes, such as bla_{NDM-1} and $bla_{CTX-M-15}$, limit treatment options to combat bacterial infections (1–4). Note that in addition to emerging antibiotic resistance, heavy metals represent another major source of environmental contamination that may select for antibiotic resistance (5). Heavy metal compounds for growth promotion and therapeutic treatment, like zinc and copper, have been used in pig and poultry production; and unlike antibiotic food additives, metals can accumulate in soil, water, aquacultural and marine antifouling treatments, and industrial effluent (6). It has been proposed that antibiotic-resistant bacteria are enriched at locations contaminated with metals, and genes conferring coselection to heavy metals and antibiotics are often found together in many clinical isolates (7–11). Furthermore, genes conferring heavy metal tolerance may coexist on the same genetic element (e.g., plasmid), which may further promote codissemination and resistance (10, 12). Here, we characterize the phenotype and genotype of heavy metal resistance in a collection of clinical Gram-negative isolates, including *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*, and *Providencia stuanti*, isolated from the United Kingdom and India.

A total of 95 nonduplicate isolates were tested in this study (Table 1): 39 bla_{NDM-1}-positive isolates originated from human lower respiratory and urinary tract samples from the United Kingdom and Chennai and Haryana, India, as previously described (13); 36 $bla_{CTX-M-15}$-carrying isolates originated from patients with burns, bacteremia, and urinary tract infections (UTIs) from various Indian hospitals (Haryana, Mumbai, Kolkata, Kerala, Delhi, and Vellore); and 20 control *E. coli* and *K. pneumoniae* isolates susceptible to all known antibiotic classes as control samples were provided by Specialist Antimicrobial Chemotherapy Unit (SACU), Public Health Wales. MICs of four heavy metal ions, i.e., $CuSO_4.5H_2O$ for copper (Cu^{2+}), $HgCl_2$ for mercury (Hg^{2+}), $AgNO_3$ for silver (Ag^+), and $AsNaO_2$ for arsenic (As^{3+}), were measured by agar dilution using Mueller-Hinton agar (Becton Dickinson, USA). *E. coli* (ATCC 25922) was used as a negative control. MIC levels of ≥10 mM for Cu^{2+}, ≥2 mM for As^{3+}, ≥32 μM for Hg^{2+}, and ≥ for 128 μM Ag^+

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