Mechanism and Biological Cost of MCR-1 mediated colistin resistance in Enterobacteriaceae

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Abstract

Colistin is one of the key antibiotics to treat infections caused by multi-drug resistant (MDR) Gram-negative bacteria. However; in 2015, plasmid mediated colistin resistance, designated *mcr-1*, was first reported in China. MCR-1 was unusually found in *Escherichia coli* and conferred only low levels of resistance to colistin. Soon *mcr-1* was found worldwide and caused great concern in public health.

A small number of studies have shown that acquisition of *mcr-1* plasmid is associated with no or only a slightly decrease in bacterial fitness. In order to assess the capacity to develop high colistin resistance in *mcr-1* harbouring *E. coli* and its effect on bacterial fitness and virulence, seven wild-type *E. coli* strains (PN16, PN21, PN23, PN24, PN25, PN42, PN43) from Phitsanulok, Thailand were selected and challenged with increased concentration of colistin for 14 days. All isolates showed an increase in colistin resistance (4- to 64- fold increase in colistin MIC up to 256mg/L), and subsequently, designated high level colistin resistant mutants (HLCRMs). In all seven HLCRMs, two showed 11- and 3- fold increase in *mcr-1* expression (PN21 [showed 11-fold] and PN25). No increase in *mcr-1* copy number or mutations in the immediate genetic context of *mcr-1* was detected in all HLCRMs. Interestingly, in PN25 and PN42 HLCRMs, amino acid mutations in PmrA and PmrB were identified, respectively.

Those HLCRMs were associated with significant either fitness burden or reduction in virulence, or both. *In-vitro* fitness was measured by growth rate. Compared with wild-type isolates, HLCRMs showed slower growth in colistin-free medium (p <0.01). Competition assays showed relative fitness compared HLCRMs with parental strains which ranged for 0.4-0.7 (p=**) (except for PN16 [relative fitness 0.9]).

A Galleria pathogenicity model was used to measure the virulence of wild-type strains and mutants. In every case, the death rate of Galleria for HLCRMs was lower than that for wild-type strains. Significant difference in bacterial mortality were identified in PN16, PN21, PN23 (p=**/***).

Due to its high expression of *mcr-1* [11-fold] cellular morphology by transmission electron microscopy (TEM) was undertaken on PN21 and its HLCRM to further understand how MCR-1 has an effect on bacterial cell outer-membrane. However, no obvious difference on outer-membrane between PN21 and mutant was identified.

The study shows that HLCRMs from wild-type strains are associated with significant fitness burden and decrease in virulence. These data will contribute to our understanding of *mcr-1* and it's impact on bacterial fitness, and the emergence and management of colistin resistance.

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Abbreviations

AMR	Antimicrobial resistance
BSI	Bloodstream infection
CDC	Centers for Disease Control and Prevention
CFU	Colony-form units
CLRW	Clinical laboratory reagent water
CLSI	The clinical and Laboratory Standards Institute
CMS	Colistimethate sodium
CRE	Carbapenem-Resistant Enterobacteriaceae
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended spectrum ß-lactamase
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FDA	US Food and Drug Administration
GFP	Green fluorescent protein
HLCRMs	High-level colistin resistance mutants
hp	hypothetical protein
ICU	Intensive care unit
L-Ara4N	4-amino-4-arabinose
LPS	Lipopolysaccharide
LMICs	Low-middle income countries
MDR	Multi-drug resistant
MCRPEC	*mcr-1* positive *E. coli*
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-Resistant *S. Aureus*
NDM-1	New Delhi metallo-β-lactamase
OD	Optical density

ORF	open reading frame
PCR	Polymerase chain reaction
PDR	Pan-drug resistance
PEA	Phosphoethanolamine
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
S1-PFGE	S1 pulsed-field gel electrophoresis
SDS	Sodium dodecyl sulphate
SKG	SeaKem Gold
SNP	Single nucleotide polymorphism
TBE	Tris-boric acid/EDTA
TCS	Two-component system
TEM	Transmission electron microscopy
UTI	Urinary tract infection
VRE	Vancomycin-Resistant Enterococci
WHO	World Health Organization
WGS	Whole Genome Sequencing

Shortened name	Full name
A. baumannii	*Acinetobacter baumannii*
A. salmonicida	*Aeromonas salmonicida*
B. cepacia	*Burkholderia cepacia*
Brucella spp.	*Brucella spp.*
C. elegans	*Caenorhabditis elegans*
C. jejuni	*Campylobacter jejuni*
Campylobacter spp.	*Campylobacter spp.*
E. aerogenes	*Enterobacter aerogenes*
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
Edwardsiella spp.	*Edwardsiella spp.*
G. mellonella	*Galleria mellonella*
K. pneumoniae	*Klebsiella pneumoniae*
Klebsiella spp.	*Klebsiella spp.*
P. aeruginosa	*Pseudomonas aeruginosa*
P. mallei	*Pseudomonas mallei*
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica*
Salmonella spp.	*Salmonella spp.*
Shigella spp.	*Shigella spp.*

Chapter 1: Introduction

1.1 Overview of global antibiotic resistance

1.1.1 History of antibiotics and resistance

The discovery of the first two key antibiotics- Prontosil (the first sulphonamide), which decreased the mortality of injured soldiers by 50% during the World War I and World War II (Domagk, 1965; Gaynes, 2017), and Penicillin, which saved about 12-15 percent of soldiers' lives in the 1940's (Rao, 2016; Gaynes, 2017) - are recognised as one of the greatest discoveries in medicine. The distribution of these drugs not only saved people's lives but started a new era of antibiotics, and laid the foundation of modern pre and post surgical management (Aminov, 2010; Gaynes, 2017). During the pre-antibiotic era, most people could not live long enough to develop cancer, heart disease or other lifestyle diseases and died prematurely, in part, because of epidemics, infections by injuries/childbirth etc (Office for National Statistics[GB], 2017). This changed dramatically after antibiotics were introduced into public healthcare systems (Armstrong, Conn, & Pinner, 1999; World Health Organization, 2018). The previous deadly illness such as Pneumonia and Tuberculosis could be treated efficiently, if not completely. Mortality from surgical infections and childbirth were also dramatically reduced and common injuries would no longer take away people's life (McKenna, 2015). According to Armstrong *et al*., in the United States, mortality due to infectious diseases declined 92.4% in the 20th century, especially from 1938 to 1952. The infectious disease mortality showed a rapid decrease by 8.2% per year, with 287 deaths per 100,000 in 1937 to 75 deaths per 100,000 in 1952, respectively (Armstrong *et al*., 1999). Whilst vaccines targeted to specific pathogens, have no doubt

contributed to the lowering of mortality, the general use of antibiotics was dominant factor in these statistics (World Health Organization, available at www.who.int/publications/10-year-review/vaccines/en/).

Concurrently, bacteria were challenged and had to rapidly adapt to these environmental changes – as they have they had done for millions of years (McKenna, 2015). Bacteria create, share and spread resistance to antibiotics, and the intemperate use of antibiotics in agriculture and human medicine has been shown to accelerate this process (McKenna, 2015). Penicillin was distributed in 1943, and widespread penicillin resistance appeared by 1945. Imipenem was clinically introduced in 1985 and mobile resistance was first witnessed in 1991(Centers for Disease Control and Prevention, 2013a; Watanabe, Iyobe, Inoue, & Mitsuhashi, 1991). Daptomycin, one of the most recent antibiotics, was introduced in 2003, and resistance appeared just one year later in 2004 (Centers for Disease Control and Prevention, 2013a) (***Figure 1.1***). The pharmaceutical companies introduced many new antibiotics to solve, or at least negate, the resistance problem, but resistance soon developed and fewer new drugs have been introduced (Centers for Disease Control and Prevention, 2013a; Ventola, 2015). According to Antibiotic Research UK, only two new antibiotic classes have been clinically implemented in the last 40 years, and thus we are facing a dire situation with fewer new, let alone novel, antibiotics to fight resistant bacteria (Antibiotic Research UK, retrieved from https://www.antibioticresearch.org.uk/about-antibiotic-resistance/). Now, after many decades of curing infections, the development of multi-drug resistant (MDR) bacteria has become a realistic threat to our lives again. According to the Centers for Disease Control and Prevention

(CDC), every year more than two million people are infected by antibiotic-resistant bacteria resulting in at least 23,000 deaths in the US (Centers for Disease Control and Prevention, 2013a). In the UK, there are more than 44,000 deaths a year because of sepsis, exceeding the number of lung cancer (35,000) and bowel cancer (16,000). Among those, many deaths are due to the lack of effective antibiotic treatment (Antibiotic Research UK, retrieved from https://www.antibioticresearch.org.uk/about-antibiotic-resistance/). The Jim O'Neil report commissioned by former UK Prime Minister, David Cameron, predicted that antimicrobial resistance (AMR) will account for 10 million deaths in 2050 (O'Neill, 2014). Now we are in a lamentable situation: bacteria develop resistance so fast that the creation of new antibiotics cannot provide a sustained therapeutic window, and to make it worse, many of the large pharmaceutical companies have little interest in antibiotic research because they cannot yield any profit (McKenna, 2015; Ventola, 2015).

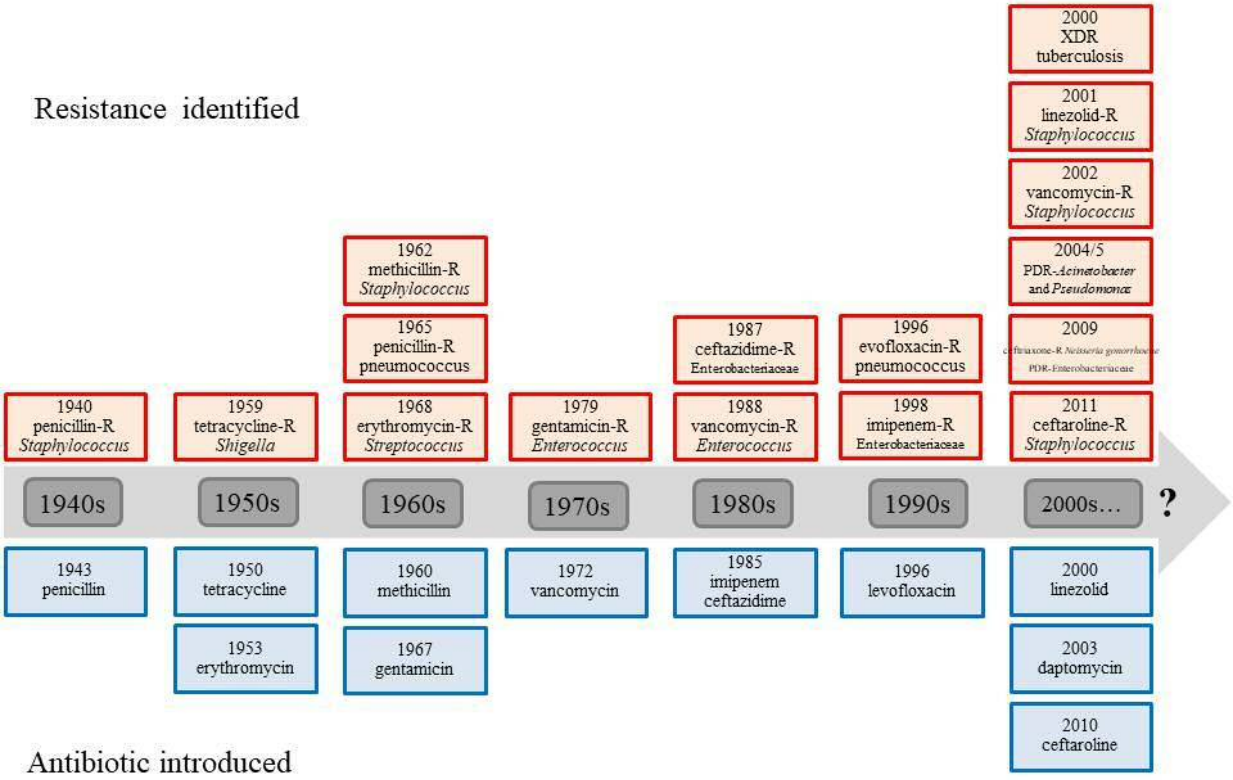


Figure 1.1 **Timeline of key events of antibiotic resistance** (Centers for Disease Control and Prevention, 2013a).

1.1.2 Cause of antibiotic resistance crisis

Although AMR occurs naturally, using antibiotics inappropriately in humans and animals is accelerating the process (Shallcross & Davies, 2014). At the same time, a lack of new antibiotics gives us nowhere to run. The main reasons for the antibiotic resistance crisis today are due to overuse of antibiotics and lack of new drugs (Ventola, 2015).

1.1.2.1 Overuse of antibiotics in human medicine

Clearly, the misuse and overuse of antibiotics speed up the evolution of AMR (Ventola, 2015). The fact is that antibiotics are heavily overused not only by doctors but outpatients in the community (over the counter sales) all over the world and particularly in low-middle income countries (LMICs) (Centers for Disease Control and Prevention, 2013a; Van Boeckel *et al.*, 2014). According to the CDC, in the US nearly 50% of the antibiotics used in humans are not needed or are not effective as prescribed (Centers for Disease Control and Prevention, 2013a). Studies in the UK demonstrate that almost 50% people visit their GP because of a cough or cold hoping to be given antibiotics; and a further study in Wales shows that around 1.6 million prescriptions each year are unnecessary (Davies, 2014). Furthermore, the situation in some LMICs is worse where antibiotic use is unregulated and over the counter sales rife (Reardon, 2014). In many LMICs lacking adequate public health care, the market of antibiotics is chaotic and completely unregulated - antibiotics can be bought in pharmacies, general stores, and even in market stalls without prescription (Nepal & Bhatta, 2018). A study in Yemen, Saudi Arabia, and Uzbekistan shows that 48%-78% of the patients purchase antibiotics without prescriptions (Belkina *et al*., 2014). In addition to the uncontrolled use of antibiotics, the quality and potency of the drugs are equally worrying (Ayukekbong, Ntemgwa, & Atabe, 2017). In some developing countries, antibiotics are produced locally, sometimes illegally and can be counterfeit (Ayukekbong *et al.,* 2017; Hart & Kariuki, 1998; Mayor, 2010). According to Ozawa *et al*., in LMICs, nearly seventeen percent of antibiotics are substandard or falsified (Ozawa, Evans, Bessias, & *et al.,* 2018). All of this misuse contributes to the emergence of

AMR. Moreover, due to frequent international trading and traveling, new types of AMR can be spread globally very quickly (Bernasconi *et al.,* 2016; Cavaco & Aarestrup, 2013; D'Aoust, 1994; Fernando, Collignon, & Bell, 2010).

1.1.2.2 Irresponsible use of antibiotics in agriculture

Antibiotics are widely used in livestock to either treat infections, prevent current infections or metaphylaxis (treatment of the whole heard) or as a growth promoter (Unno, Kim, Guevarra, & Nguyen, 2015). For example, in the pig industry, antibiotics are used routinely to prevent disease or to avoid the outbreak of infections because of poor living conditions (Kempf *et al*., 2013). In modern farms, normally thousands of animals are kept together indoors with limited space and reared solely for the purpose of rapidly gaining weight – greater yield of protein and larger profits (Pappas, 2011). Famers also use antibiotics routinely as growth promoters to help animals against poor living environment (Davies, 2014; Doyle, 2001). It is estimated by European Medicines Agency that two thirds of antibiotics in European countries were used in farm animals. In the UK, the rate is about 45%, and 80% in the US (European Centre for Disease Prevention and Control, European Food Safety Authority, & European Medicines Agency, 2015; Department of Agriculture, Environment and Rural Affairs, 2007; Ventola, 2015).

The antibiotics used in farm animals is an important driver of AMR for some major infectious bacteria in humans, such as *Salmonella* and *Campylobacter* (Davies, 2014). According to the WHO, AMR in foodborne bacteria *Salmonella* and *Campylobacter*, is clearly the result of

antibiotic use in food animals, and has subsequently spread from the food-chain to humans (World Health Organization, 2011). This occurs in the following events: antibiotic use in farm animals kills susceptible bacteria and either maintains resistant bacteria or the bacteria acquire resistance (Centers for Disease Control and Prevention, 2013a). The conditions on farms, particularly in resource restricted countries also foster the rapid spread of bacteria from one animal to another (Davies, 2014).

1.1.2.3 Lack of new antibiotics

The emergence and spread of AMR can be rapid; however, the pace of discovery of new antibiotics is glacial by comparison. A decrease in novel and new antibiotics dropped sharply after the mid-1990s (***Figure 1.2***). To make matters worse, most pharmaceutical companies have little interest in antibiotic research; 15 of the 18 largest pharmaceutical companies announced their intention to leave the antibiotic field due to lack of profit (Bartlett, Gilbert, & Spellberg, 2013; Ventola, 2015). The CDC and WHO have formally announced that we are running out of drugs against Gram-negative infections, including Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter* (World Health Organization, 2017b). Although there are still 22 new antibiotics have either been launched and/or in clinical trials since 2000, they are ineffective against MDR Gram-negative infections or lack any novelty i.e. resistance is likely to develop very quickly (Butler, Blaskovich, & Cooper, 2013). However, even if novel antibiotics are developed, without widespread attitude and behaviour change, antibiotic resistance will remain a major threat (Ventola, 2015).

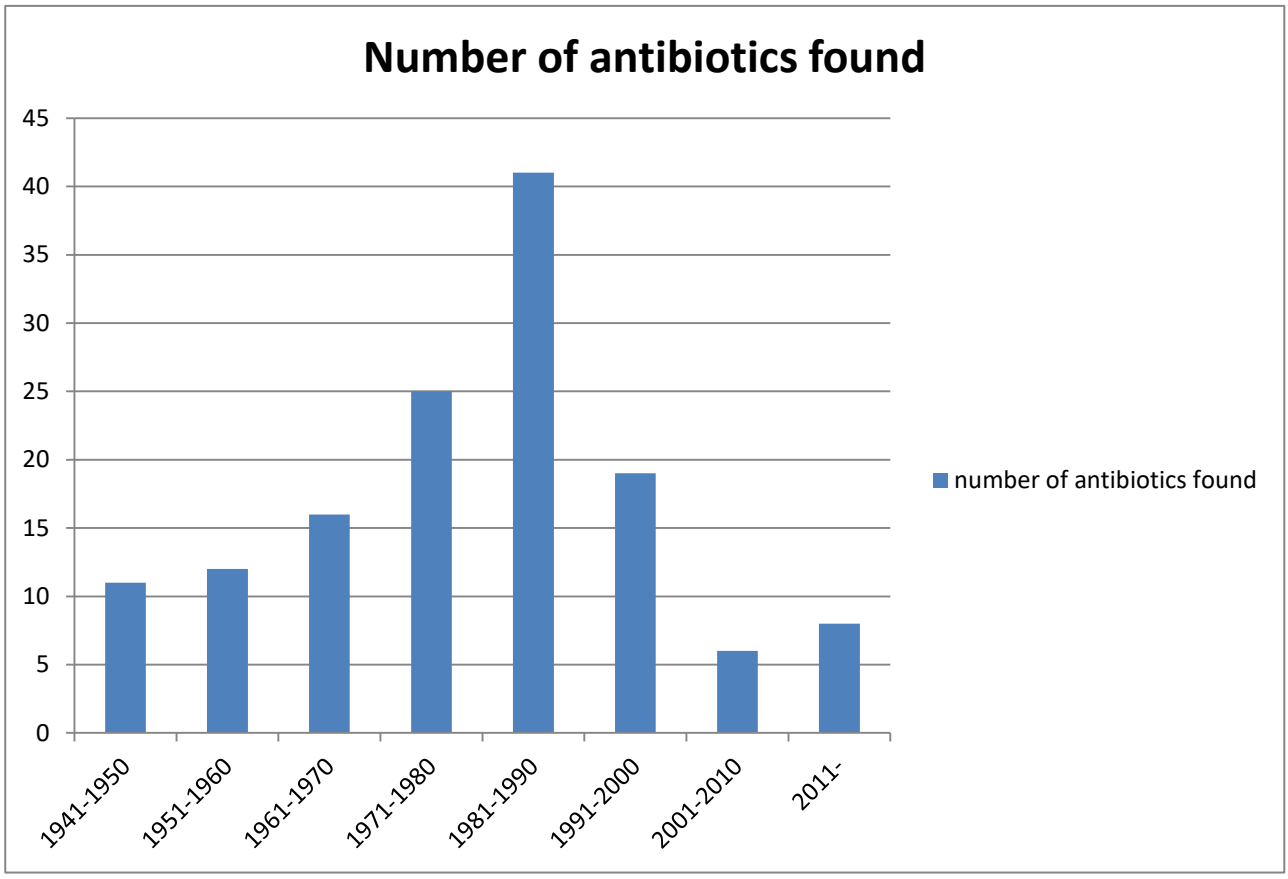


Figure 1.2 **The number of antibiotic discovered each decade** (Wikipedia, retrieved from https://en.wikipedia.org/wiki/Timeline_of_antibiotics).

1.1.3 Antibiotic-resistant infections

On global level, MDR in Gram-negative bacteria is extremely serious. According to the WHO, there is a serious lack of treatment options for MDR Gram-negative pathogens, including *Acinetobacter* and Enterobacteriaceae (World Health Organization, 2017c). The situation on AMR in Gram-positive bacteria, whilst slightly better, is also a cause for concern, among which MRSA (Methicillin-Resistant *Staphylococcus aureus*) and VRE (Vancomycin-Resistant Enterococci) are of great concern (Centers for Disease Control and Prevention, 2013a).

1.1.3.1 MDR Enterobacteriaceae

Enterobacteriaceae pathogens may cause urinary tract infections (UTI), bloodstream infections (BSI), hospital- and healthcare-associated pneumonia, and various intra-abdominal infections (Akova, 2016; Malmartel & Ghasarossian, 2016; P. Nordmann, 2014; Paterson, 2006). They can spread easily between humans by hand and by contaminated food and water, and can readily acquire additional DNA through horizontal gene transfer mediated by plasmids or transposons (Patrice Nordmann, Dortet, & Poirel, 2012; Ventola, 2015). Among these, Carbapenem-Resistant Enterobacteriaceae (CRE) and extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae are currently of greatest concern (Centers for Disease Control and Prevention, 2013a).

CRE are a group of bacteria that have become resistant to “all or nearly all” antibiotic options available, including carbapenems (Ventola, 2015). Carbapenems (imipenem, ertapenem, meropenem, and doripenem) are one of the latest developed β-lactams and possess the broadest

spectrum of activity (Patrice Nordmann *et al.,* 2012). According to the CDC, in the US, 140,000 cases of health care associated infections occurred with Enterobacteriaceae, of which 9,300 were caused by CRE (Centers for Disease Control and Prevention, 2013a). In 2010, an enzyme called New Delhi metallo-β-lactamase (NDM) was reported in India and caused great concern because it mediated resistance to almost all β-lactams and had quickly spread all around the world. (Hammerum *et al.,* 2010; Kumarasamy *et al.,* 2010; D. van Duin & Doi, 2017). Few treatment options are available for CRE and these include polymyxins, some aminoglycosides, and tigecycline (David Van Duin, Kaye, Neuner, & Bonomo, 2013).

Enterobacteriaceae producing ESBLs mediates resistance to extended-spectrum penicillins and third generation cephalosporins (Cantas, Suer, Guler, & Imir, 2015). An estimate by the CDC report that each year, nearly 26,000 (or 19%) healthcare-associated Enterobacteriaceae infections were attributed to ESBL-producing Enterobacteriaceae, resulting in 1,700 deaths (Centers for Disease Control and Prevention, 2013a). Moreover, mortality of BSI caused by ESBL-producing Enterobacteriaceae is approx. 57% higher than that of non ESBL-producing strains (Centers for Disease Control and Prevention, 2013a).

1.1.3.2 MDR *Acinetobacter*

Acinetobacter are Gram-negative bacteria that can cause nosocomial pneumonia or bloodstream infections, especially among critically ill patients. Many *Acinetobacter* have become resistant to all or nearly all antibiotics and approximately 63% of healthcare-associated *Acinetobacter* infections are caused by MDR *Acinetobacter*, accounting for 7,000 cases in the

US (Centers for Disease Control and Prevention, 2013a). Among these, 500 cases are not responsive to any antibiotics (Centers for Disease Control and Prevention, 2013a). Although MDR *Acinetobacte*r rarely cause serious infections in healthy people, it can occur in many immunosuppressed patients or in patients with other serious underlying diseases (Dijkshoorn, Nemec, & Seifert, 2007; World Health Organization, 2014). Carbapenems and polymyxins are often used to treat *Acinetobacter* infections; however, resistance to carbapenems and polymyxins are increasingly reported (Cai, Chai, Wang, Liang, & Bai, 2012; Cheng *et al.,* 2016; Dijkshoorn *et al.,* 2007; Gagnaire *et al.,* 2017; Gupta *et al.,* 2016; Ko *et al.,* 2007; López-Rojas *et al.,* 2013).

1.1.3.3 MDR *Pseudomonas aeruginosa*

P. aeruginosa is a common cause of nosocomial infections such as hospital-acquired pneumonia, complicated urinary tract infections, surgical-site infections, and bloodstream infections (Berube, Rangel, & Hauser, 2016). Approximately 8% of all hospital-associated infections (accounting for 51,000 cases) reported to CDC are caused by *P. aeruginosa*, among which 13% cases are caused by MDR *P. aeruginosa* (Centers for Disease Control and Prevention, 2013a). Each year, approximately 400 deaths in the US are attributable to infections by MDR *P. aeruginosa* (Centers for Disease Control and Prevention, 2013a). Some MDR *P. aeruginosa* strains are resistant to nearly all β-lactams, aminoglycosides, and fluoroquinolones. In such cases, colistin associated with adjunctive therapy (such as a β-lactam or rifampicin) is a potential treatment option (Chatterjee & Agrawal, 2016; Obritsch, Fish, MacLaren, & Jung, 2005; Riethmuller *et al.,* 2016).

1.2 Colistin

The rapid rise of MDR in Gram-negative bacteria has given colistin a renaissance after falling out of favour for more than 15-20 years (Biswas, Brunel, Dubus, Reynaud-Gaubert, & Rolain, 2012). As an old antibiotic, colistin was first introduced into clinical use in Japan, Europe and the US in the 1950s, but was then withdrawn due to moderate/severe adverse (toxic) effects (Biswas *et al.,* 2012; M. E. Falagas & Kasiakou, 2005; Schwarz & Johnson, 2016). In the 1990s, as lack of treatment options for MDR Gram-negative bacteria became a serious issue, colistin was introduced back into clinical use and listed as a last-resort antibiotic by WHO (M. E. Falagas & Kasiakou, 2005; World Health Organization, 2014).

1.2.1 Chemical Structure

Colistin (Polymyxin E) is a member of polymyxin family, a group of cationic polypeptide antibiotics, which contains five different derivatives (Polymyxin A, B, C, D and E) (Landman, Georgescu, Martin, & Quale, 2008). Polymyxin B and E (colistin) are available in clinical practice to treat infections by MDR Gram-negative bacteria (Schwarz & Johnson, 2016). The main difference between polymyxin B and E is that polymyxin B contains phenylalanine in position 6 while colistin contains D-leucine (Nation, Velkov, & Li, 2014). The major forms of polymyxins used worldwide are colistimethate sodium (CMS) (48.6%) and colistin sulfate （14.1%, or both forms of colistin (1.4%), while polymyxin B is rarely used (1.4%). Other forms are unknown in the study (Wertheim *et al*., 2013).

Colistin was developed from *Paenibacillus polymyxa* subsp. *Colistinus*. Its tripeptide side chain is connected to the fatty acid residue, which is identified as 6-methyl-octan-oic acid (colistin A) or 6-methyl-eptanoic acid (colistin B) (Falagas, Polymyxins, sourced from http://www.antimicrobe.org/d05.asp).

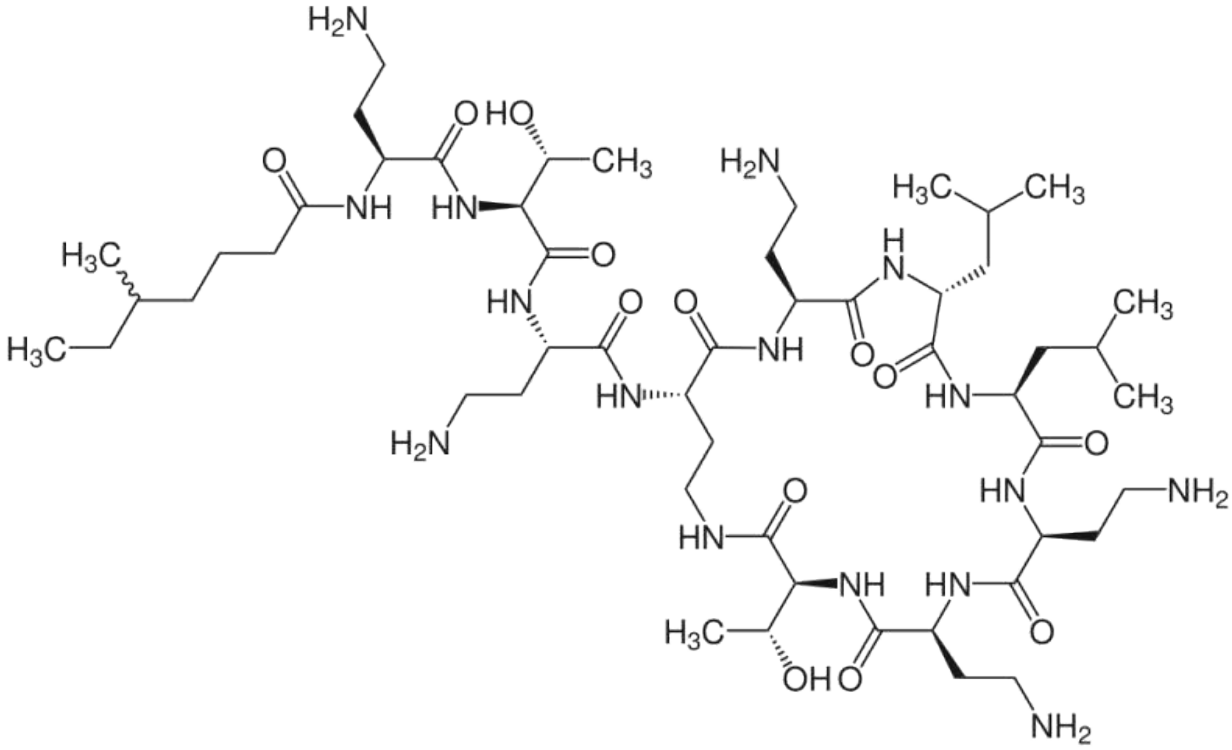


Figure 1.3 **Chemical structure of colistin.**

(Wikipedia, retrieved from https://en.wikipedia.org/wiki/Colistin.)

1.2.2 Spectrum

Most Gram-negative bacteria are susceptible to polymyxins, including *A. baumannii*, *P .aeruginosa*, and nearly all Enterobacteriaceae. However, *Pseudomonas mallei*, *Burkholderia cepacia*, *Edwardsiella spp*., *Brucella spp*. and the Proteus-group of Enterobacteriaceae are all naturally resistant to polymyxins. Furthermore, Gram-positive bacteria, fungi, and parasites are unaffected by polymyxins (Falagas, Polymyxins, sourced from http://www.antimicrobe.org/d05.asp).

The clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) provide breakpoints for colistin susceptibility for Enterobacteriaceae, *P. aeruginosa* and *A. baumannii* (**Table 1.1**). In general, an isolate with Minimum Inhibitory Concentration (MIC) ≤ 2mg/L is deemed as susceptible.

Table 1.1 Breakpoints of colistin susceptibility (MIC, mg/L) according to guidelines in United States (CLSI) and Europe (EUCAST) S, sensitive; I, intermediate; R, resistant.（M100S Performance Standards for Antimicrobial Susceptibility Testing 2018）(European committee on antimicrobial susceptibility testing 2018).

	CLSI			EUCAST	
Species	S	I	R	S	R
Enterobacteriaceae	≤2	4	≥8	<2	≥2
P. aeruginosa	≤2	4	≥8	<2	≥2
A. baumannii	≤2	4	≥8	<2	≥2

1.2.3 Application of Colistin

Colistin is used in clinical practice for both adults and children as a treatment option for infections caused by MDR *Pseudomonas*, MDR *A. baumannii* and CRE (Tamma *et al*., 2013). However, the emergence of colistin resistance is rapidly rising and deeply concerning (Tamma *et al.*, 2013).

In veterinary medicine, colistin is widely used to prevent infections in livestock caused by *Escherichia coli*, such as diarrhoea, septicaemia and colibacillosis (Kempf *et al.*, 2013). High colistin use has been observed in epidemiological studies in Europe (Kempf *et al*., 2013). In countries such as China, Thailand and Vietnam, high doses of colistin have also used as feed additives for growth promotion in both large and small farms (Nguyen *et al.,* 2016; Walsh & Wu, 2016; Wongsuvan, Wuthiekanun, Hinjoy, Day, & Limmathurotsakul, 2018). China has recently banned colistin as a growth promoter in April 2017 because of the discovery of the mobile colistin resistance gene, *mcr-1* (Liu *et al.,* 2016; Walsh & Wu, 2016).

1.2.4 Mechanism of action

Colistin and Polymyxin B have a similar mechanism of activation. The structure of polymyxin contains a cyclic decapeptide bound to a fatty acid chain. The L-Dab molecules in polymyxin are positively charged, while the lipopolysaccharides (LPS) of Gram-negative bacteria are negatively charged (Velkov, Roberts, Nation, Thompson, & Li, 2013). Colistin binds to LPS in the bacterial cell outer-membrane, displace divalent cations such as Mg^{2+} and Ca^{2+} (which are

associated with stabilizing LPS molecules), and leads to increased permeability of cell outer-membrane, resulting in leakage of cell contents and cell death (Velkov *et al*., 2013).

1.2.5 Colistin Resistance

The main mechanism of colistin resistance is modification of lipid A with phosphoethanolamine（PEA）or 4-amino-4-arabinose（L-Ara4N）, resulting in lowering the affinity with polymyxins, or in rare cases the loss of LPS (Hinchliffe *et al*., 2017). In addition to these, the use of efflux pumps may also aid colistin resistance (Abiola O Olaitan, Morand, & Rolain, 2014). The following examples are key mechanisms of colistin resistance:

1.2.5.1 PmrA/B, PhoP/Q two-component system (TCS)

PmrA/B and PhoP/Q TCSs are the regulators of LPS modifications in many bacterial species. (Chen & Groisman, 2013) The activation of PmrA/B TCS leads to expression of PmrA-dependent genes resulting in resistance to polymyxins (Chen & Groisman, 2013), while the activation of PhoP/PhoQ TCS leads to polymyxin resistance by indirectly activating PmrA/PmrB TCS via PmrD (Abiola O Olaitan *et al.,* 2014). Mutations in these two systems can cause constitutive over-expression, resulting in the subsequent activation of the *arnBCADTEF* and *pmrCAB* operons and the synthesis and transfer of lipid A by L-Ara4N and lipopolysaccharides (PEA), respectively (Chen & Groisman, 2013). Modification of L-Ara4N and PEA decrease the net negative charge of LPS and reduce its avidity for colistin (Abiola O Olaitan *et al.,* 2014). In addition, inactivation of the *mgrB* gene (which encodes a

negative-feedback regulator of the PhoQ/PhoP signalling system) leads to up-activation of PhoQ/PhoP, and subsequently results in modification of LPS mediated resistance to colistin (Abiola O Olaitan *et al.,* 2014; Poirel *et al.,* 2014).

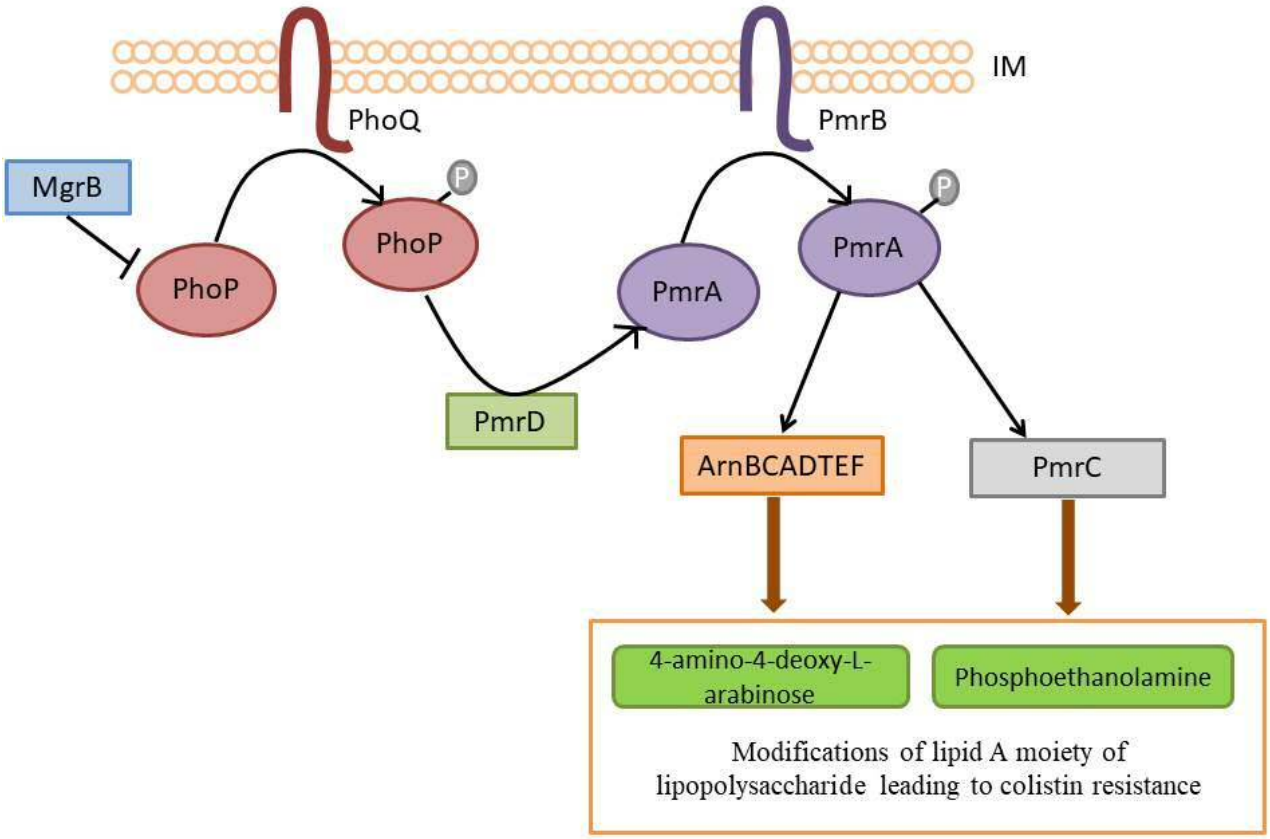


Figure 1.4 **Lipopolysaccharide-modification involved in polymyxin resistance in Gram-negative bacteria** (Chen & Groisman, 2013).

1.2.5.2 lpxA, lpxC, and lpxD

In *A. baumannii* , mutations in three lipid A biosynthesis genes- *lpxA*, *lpxC*, and *lpxD* lead to the complete loss of LPS resulting in very high resistance against colistin (MIC >128 mg/L) (Moffatt *et al.,* 2010). LPS is an important component at outer-membrane in Gram-negative bacteria, creating a permeability barrier to prevent large molecules from freely entering the cell (G. Zhang, Meredith, & Kahne, 2013). The loss of LPS leads to loss of a binding target for colistin, resulting in high resistance (Moffatt *et al.,* 2010).

1.2.5.3 Efflux pump

Efflux pump system plays an important role in AMR and has been found to be linked with the mediation of resistance to cationic antimicrobial peptides such as polymyxins (Falagas, Polymyxins, sourced from http://www.antimicrobe.org/d05.asp). For example, the Emr pump system in *A. baumannii* has been proven to be associated with the adaptation to osmotic stress and colistin resistance (Lin, Lin, & Lan, 2017); the MexAB-OprM efflux pump in *P. aeruginosa* can provide resistance to colistin (Pamp, Gjermansen, Johansen, & Tolker-Nielsen, 2008; Schweizer, 2003); and the AcrAB efflux pump can cause colistin resistance in *Klebsiella pneumoniae* and *E. coli* (Padilla *et al.,* 2010; Warner & Levy, 2010).

1.2.5.4 *mcr*-mediated colistin resistance

In 2015, a transferable resistance gene to colistin called *mcr-1* was first reported in China, which was located on a conjugative plasmid in *E. coli* (Liu *et al.,* 2016). *mcr-1* represents a

"new" machinery for colistin resistance where the modification of Gram-negative bacterial lipid A is catalyzed by the MCR-1 enzyme, showing a decreased affinity to polymyxin (Hinchliffe *et al.,* 2017). Now, *mcr-1* has been reported worldwide and is a cause of great concern (Schwarz & Johnson, 2016). Following the detection of *mcr-1*, more plasmid-mediated colistin resistant genes- *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*, have been reported (Borowiak *et al.,* 2017; Carattoli *et al.,* 2017; Xavier *et al.,* 2016; Yin *et al.,* 2017). The continuing identification of novel *mcr* genes indicates a much wider global dissemination of colistin resistance determinants in Enterobacteriaceae (Kluytmans, 2017).

1.2.6 Adverse Events

As an old antibiotic, colistin was first introduced into the clinic in the 1950s, but then was withdrawn due to its adverse side effects, including nephrotoxicity and neurotoxicity (Matthew E Falagas & Kasiakou, 2006). However, the antibiotic resistance crisis together with a lack of new or novel drugs has given colistin a renascence and it is now widely used to treat serious Gram-negative infections (Das, Sengupta, Goel, & Bhattacharya, 2017).

1.2.6.1 Nephrotoxicity

Polymyxins can cause nephrotoxicity, and colistin shows less cytotoxic compared to Polymyxin B (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). The mechanism of colistin nephrotoxicity is due to an increase in tubular epithelial cell membrane permeability, resulting in increased influx of cations, anions and water mediating cell swelling

and lysis (Javan, Shokouhi, & Sahraei, 2015). The nephrotoxicity depends on the concentration and length of exposure to polymyxins (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). Co-administration with other nephrotoxic drugs, patient-related factors (such as age, sex, other kidney diseases and severity of patient illness) are also related to colistin nephrotoxicity (Javan *et al.,* 2015).

Clinical studies show that the frequency of nephrotoxicity of polymyxins varies from as low as 0% to as high as 55% (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). In an early study (1965) by Tallgren *et al.,* 36% patients with acute or chronic renal disease showed an increase in plasma creatinine levels (TALLGREN, LIEWENDAHL, & KUHLBÄCK, 1965). After the re-introduction of colistin into clinical treatment for MDR Gram-negative infections, the data did not support the high incidence of polymyxin nephrotoxicity previously reported (Matthew E Falagas & Kasiakou, 2006). Two studies in intensive care units (ICUs) showed that 14.3% (Markou *et al.,* 2003) and 18.6% (Michalopoulos, Tsiodras, Rellos, Mentzelopoulos, & Falagas, 2005) of patients had demonstrable deterioration in renal function during colistin therapy. To reduce the potential damage of colistin to the kidney, it is important to stop polymyxin treatment as soon as primary signs of renal dysfunction are recognised (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp).

1.2.6.2 Neurotoxicity

Compared with nephrotoxicity, the incidence of colistin related neurotoxicity is substantially

less (Wadia & Tran, 2014). The reported neurological toxicity of colistin is associated with dizziness, generalized muscle weakness, facial and peripheral paresthesia, partial deafness, visual disturbances, vertigo, confusion, hallucinations, seizures, ataxia, and neuromuscular blockade (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). Clinical studies showed that the most frequently neurological adverse effects were paresthesias, and patients with impaired renal function or myasthenia gravis have higher risk of neurological adverse effects (Nigam, Kumari, Jain, & Batra, 2015).

1.2.6.3 Other adverse events

Other adverse events such as pruritus, dermatoses, drug fever and gastrointestinal disturbances may also occur during colistin therapy. Leukopenia and granulocytopenia may also be associated with colistin treatment (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). Furthermore, people with an allergy to bacitracin are at higher risk of hypersensitivity reactions with the use of polymyxins (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp).

1.2.7 Dosage

Two forms of colistin are clinically available - colistin sulfate and colistimethate sodium (CMS) (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). Colistin sulfate is usually administered orally for bowel decontamination or is used as treatment of skin infections caused by bacteria. CMS is often used for parenteral therapy and is less toxic with fewer

adverse events (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp).

The recommended dose of CMS in adult patients is different in countries. Generally, 1mg of CMS equals 12,500 IU. In the European countries except for France, CMS is recommended at 4-6mg/kg or 50,000-70,000 IU/kg daily in 2-3 divided doses (Falagas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). In France, 75,000-150,000 IU/kg CMS is recommended daily, divided in 1-3 doses. The maximum dosage one day is no more than 12 million IU (Theuretzbacher, 2014). In the US, the recommended dose of CMS is 2.5-5 mg/kg in 2-4 doses daily. (Falagas and Vardakas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp) For Children, the adult dosing of colistin is recommended (Falagas and Vardakas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp). However, current data in children is limited and most cases are for the treatment of *P. aeruginosa* in cystic fibrosis patients where dosing schemes are empirical. Therefore, the dose for children has not been defined (Falagas and Vardakas, Polymyxins, retrieved from http://www.antimicrobe.org/d05.asp).

Furthermore, colistin dosage must be reduced for patients with impaired renal function, and for obese patients, the dosage should depend on daily weight (Coly-Mycin, 2005). Although colistin is listed in the FDA (US Food and Drug Administration) pregnancy category, it should be only be used when the potential benefit is greater than risk (Kazy, Puhó, & Czeizel, 2005).

1.3 Transferable colistin resistant gene- *mcr-1*

1.3.1 Discovery of *mcr-1*

In 2015, Liu *et al.,* first reported transferable colistin resistance mediated by a plasmid from food animals in China. The colistin resistance gene, *mcr-1*, was located on an IncI2-type plasmid and conferred a colistin MIC at 8 mg/L (Liu *et al.,* 2016). Successful transfer of *mcr-1* carrying plasmid to *E. coli* C600 by conjugation indicated the potential spread of colistin resistance under natural conditions. This first report showed that *mcr-1* carriage in *E. coli* was also found in meat, chickens, pigs and humans (Liu *et al.,* 2016). The report was significant not only because it described a new mechanism of colistin resistance, but indicated that MCR-1 positive *E. coli* (MCRPEC) are widespread (Schwarz & Johnson, 2016).

1.3.2 Global Spread of *mcr-1*

Following this first report, incidences of *mcr-1* were soon reported worldwide: Asia (China, Japan, South Korea, Malaysia, Thailand, Cambodia, Laos and Vietnam), Europe (Denmark, France, Germany, UK, Poland, Spain, Portugal, Switzerland, Italy, Belgium and The Netherlands), Africa (Egypt, Algeria, Nigeria and South Africa), South and North America (Argentina, Canada and the USA) (M. S. Arcilla *et al.,* 2016; Coetzee *et al.,* 2016; Falgenhauer, Waezsada, Yao, *et al.,* 2016; Figueiredo *et al.,* 2016; Hasman *et al.,* 2015; Hu, Liu, Lin, Gao, & Zhu, 2016; Izdebski *et al.,* 2016; Khalifa *et al.,* 2016; Kluytmans–van den Bergh *et al.,* 2016; Kusumoto *et al.,* 2016; Lim *et al.,* 2016; Liu *et al.,* 2016; Malhotra-Kumar, Xavier, Das,

Lammens, Butaye, *et al.,* 2016; Malhotra-Kumar, Xavier, Das, Lammens, Hoang, *et al.,* 2016; McGann *et al.,* 2016; Mulvey *et al.,* 2016; A. O. Olaitan, Chabou, Okdah, Morand, & Rolain, 2016; A. Quesada *et al.,* 2016; Rapoport *et al.,* 2016; Schwarz & Johnson, 2016; Stoesser, Mathers, Moore, Day, & Crook, 2016; Webb *et al.,* 2016; Zurfuh *et al.,* 2016). The bacterial host of *mcr-1* are various, including *E. coli*, *Salmonella spp*., *K. pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae* (Doumith *et al.,* 2016; Du, Chen, Tang, & Kreiswirth, 2016; Liu *et al.,* 2016; Webb *et al.,* 2016; Zeng, Doi, Patil, Huang, & Tian, 2016), from a wide range of sources, including animals (livestock, pet animals and several wild-birds), food (meat and vegetables), water, patients and healthy people (Maris S Arcilla *et al.,* 2016; Liakopoulos, Mevius, Olsen, & Bonnedahl, 2016; Liu *et al.,* 2016; Mendes *et al.,* 2018; Schwarz & Johnson, 2016; J. Wang *et al.,* 2017; X. F. Zhang *et al.,* 2016; Zurfuh *et al.,* 2016).

Liu *et al.,* analysed 2649 *E. coli* isolates (523 samples of raw meat, 804 of animals and 1322 of inpatients) collected during 2011-2014 in China, which showed MCRPEC rates of 15%, 21% and 1%, respectively (Liu *et al.,* 2016). Suzuki *et al.,* reported that five of 671 isolates collected from patients, animals and environment from Japan during 2000-2014 were MCRPEC. Interestingly, all of the five *mcr-1* positive isolates were from animal samples (Suzuki, Ohnishi, Kawanishi, Akiba, & Kuroda, 2016). However, Kusumoto *et al.,* found that 45% of *E. coli* isolates collected from diseased swine in Japan during 1991–2014 showed resistance to colistin, among which 29% were MCRPEC (Kusumoto *et al.,* 2016). A report from France during 2007-2014 showed that prevalence of MCRPEC was 5.9% in turkeys, 1.8% in broilers and 0.5% in pigs (Perrin-Guyomard *et al.,* 2016). Colistin resistance in Spain was detected in 0.5% *E.coli*

in clinical isolates from 2012–2015, among which 15 of 50 available colistin resistant isolates were MCRPEC (Prim *et al.,* 2016). A report from the UK identified 15 *mcr-1*-positive isolates (13 *Salmonella enterica* and 3 *E. coli* isolates from patients, 2 *S. enterica* isolates from poultry meat) from ~24,000 Enterobacteriaceae (including *S. enterica*, *E. coli*, *Klebsiella spp.*, *Enterobacter spp.*, *Campylobacter spp.* and *Shigella spp*.) isolated between 2012 and 2015 (Doumith *et al.,* 2016). These global reports support the concern that *mcr-1* has been spread worldwide. Additionally, with air travel and trade exchanges between countries, no country can possibly avoid the acquisition of mcr-mediated colistin resistance (Rhouma, Beaudry, & Letellier, 2016).

Even though the emergence of *mcr-1* was first reported in 2015, it was also detected in isolates dating back to 1980s (1987) from China and predates the discovery of all mobile carbapenemase genes (Shen, Wang, Shen, Shen, & Wu, 2016). Thus, transferable colistin resistance existed in the gut flora of food animals for more than 25 years without being detected (Schwarz & Johnson, 2016; Shen *et al.,* 2016). The outbreak of colistin resistance is, in part, due to the increasing use of colistin in agriculture and aquaculture, particularly since 2007-8 (Rhouma, Beaudry, & Letellier, 2016). According to Shen *et al.,* the presence of colistin resistance and *mcr-1* positive rates were at a minimal level which increased sharply during 2009 to 2014 in China. During this period, the annual use of colistin increased from 2470 to 2875 metric tons in food animals and therefore is likely to have contributed to the rapid spread of *mcr-1* in China *(*Shen *et al.,* 2016).

1.3.3 Analysis of molecular characterization

1.3.3.1 *mcr-1* carrying plasmid

mcr-1 is normally located on stable plasmids, while rarely on the chromosome (Matamoros *et al.,* 2017). Matamoros *et al.,* analysed the population structure of *E. coli* and the mobile genetic elements carrying the *mcr-1* gene that had been reported worldwide. Up to 2017, 13 plasmid incompatibility types had been reported carrying *mcr-1* (Refer to ***Figure 1.5***). Among these, 90.4% of the identified plasmids belonged to IncX4 (35.2%), IncI2 (34.7%), IncHI2 (20.5%) plasmid types. Interestingly, 65.8% of the IncI2 plasmids carrying *mcr-1* were reported from Asia, while the major plasmid type carrying *mcr-1* in Europe was IncI2 plasmids (73.3%) (Matamoros *et al.,* 2017). The distribution of these 3 plasmid types from animal ($p = 0.24$), human ($p = 0.88$) and environmental sources ($p = 0.38$) was not significantly different (Matamoros *et al.,* 2017).

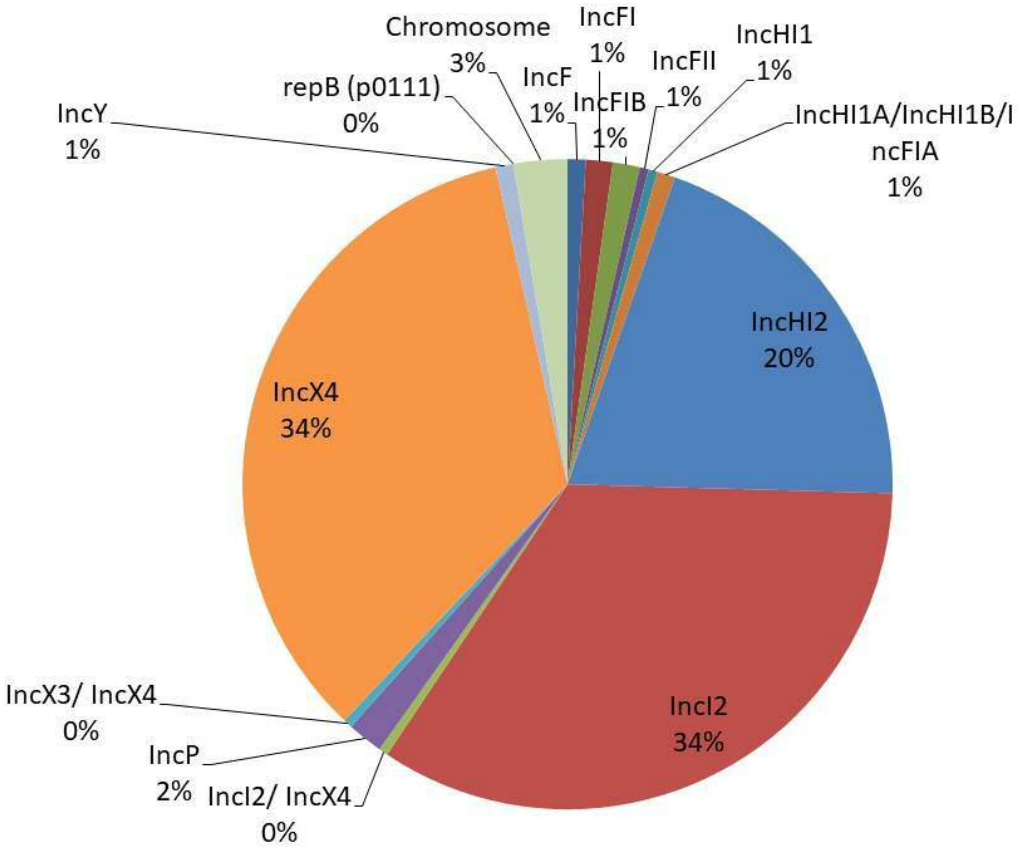


Figure 1.5 **Global phylogenetic analysis of plasmids carrying the *mcr-1* gene** (Matamoros *et al.,* 2017)

Interestingly, the co-existence of IncX4 with other replicons such as IncHI2 and IncI2 were detected in *mcr-1* positive bacteria (J. Sun *et al.,* 2017). A study by Sun *et al.,* displayed the existence of two copies of *mcr-1* located separately on two inc types from the same *E. coli* strain. Furthermore, they also found multiple copies of *mcr-1* located on both plasmid and chromosome in the same strain (J. Sun *et al.,* 2017).

1.3.3.2 Population structure

Multi-locus sequence typing (MLST) is an accurate procedure for characterising isolates of many bacterial species. According to Matamoros *et al.,* among 410 Enterobacteriaceae in 215 studies, MCRPEC showed a high overall diversity of in its population structure. The 410 strains represents 112 sequence types, among which ST10 was the most prevalent (12.8%) (Matamoros *et al.,* 2017). Reported ST types of *mcr-1* harbouring isolates from different origins worldwide were shown in ***Figure 1.6***.

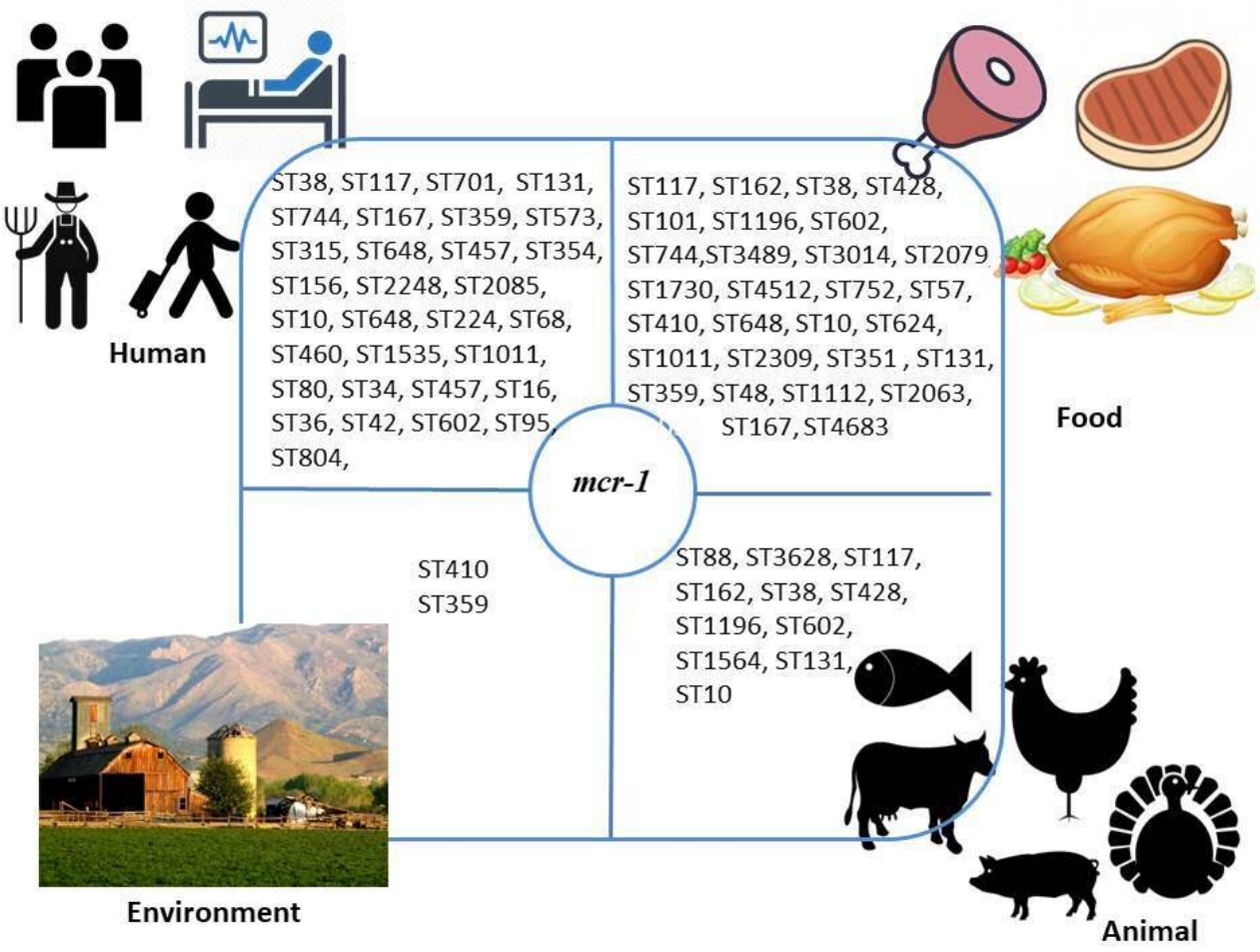


Figure 1.6 **ST types of *mcr-1* isolates from different origins** (Y. Wang *et al.,* 2017)**.**

1.3.3.3 Mobile genetic elements

In *mcr-1* carrying isolates, an *ISApl1* transposon element is often found upstream of *mcr-1* on IncHI2 or IncI2 plasmid (Matamoros *et al.,* 2017). In the study by Matamoros *et al.,* 77.8% *mcr-1* on IncHI2 plasmids is flanked by *ISApl1* upstream, while only 37.9% of IncI2 plasmids. No Insertion element has been reported upstream of *mcr-1* in IncX4 plasmids (Matamoros *et al.,* 2017).

ISApl1 encodes a putative transposase belongs to IS*30* family (J. Liu *et al.,* 2008) and is a key

component in the mobilization of *mcr-1* (Snesrud *et al.,* 2016). Its movement is independent of *mcr-1* (Snesrud *et al.,* 2017). Studies have shown that under certain condition when *ISApl1* is highly active, its movement may be detrimental to the host cell (Snesrud *et al.,* 2017).

1.3.3.4 Multiple resistant genes on *mcr-1* harbouring plasmid

In the first report of *mcr-1*, the *mcr-1* carrying plasmid did not carry other resistance genes (Liu *et al.,* 2016). However, in later reports, *mcr-1* plasmids often harbour other resistance genes such as ESBL and carbapenemase genes (McGann *et al.,* 2016; R. Wang *et al.,* 2018; H. Zhang, Seward, Wu, Ye, & Feng, 2016). These plasmids not only support the co-transfer of resistance genes, but also the existence of *mcr-1* under selective antibiotic pressure even without colistin (Schwarz & Johnson, 2016).

1.3.4 Mechanism of MCR-1

The mechanism of colistin resistance by MCR-1 is due to the modification of LPS on bacterial cell outer-membrane (Liu *et al.,* 2016). Briefly, *mcr-1* encodes a transferase enzyme belonging to the PEA transferase enzyme family which catalyzes the addition of PEA to lipid A moiety of LPS through which reduces the negative charge resulting in reduction of colistin avidity, and therefore colistin resistance (Hinchliffe *et al.,* 2017).

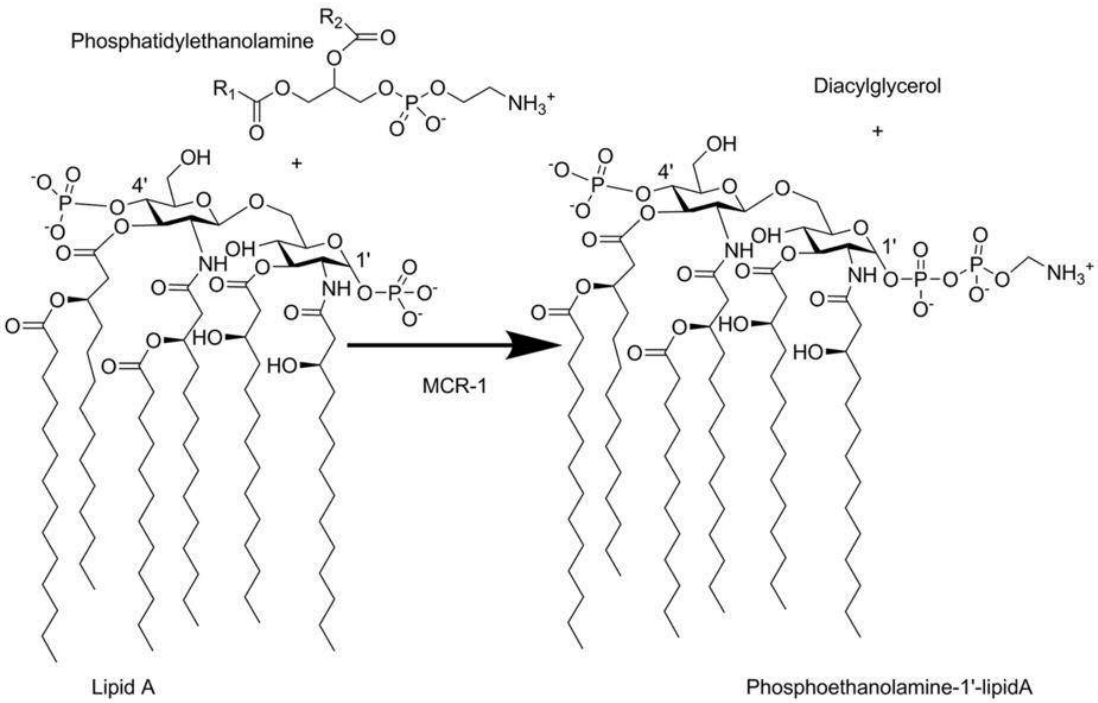


Figure 1.7 **PEA transfer reaction catalysed by MCR-1** (Hinchliffe *et al.,* 2017).

MCR-1 has 41% and 40% identity to the PEA transferases LptA and EptC (Liu *et al.,* 2016), and the fold of the MCR-1 catalytic domain is similar to that of the LptA and EptC transferases (Stojanoski *et al.,* 2016). LptA is usually being found in *Neisseria* and specifically transfers PEA to only lipid A phosphoryl groups which then confers colistin resistance, while EptC (in *Campylobacter jejuni*) displays a broader substrate tolerance (Liu *et al.,* 2016). Phylogenetic analyses showed that MCR-1 is highly homologous to the PEA lipid A transferase in *Paenibacili*, a producer of polymyxins (Gao *et al.,* 2016).

The recent released MCR-1 crystal structure infers that the catalytic domain of membrane-bound MCR-1 is a zinc metallo-protein with three disulphide bonds (see ***Figure 1.8***)

(Hinchliffe *et al.,* 2017). Zinc, disulphide bonds and conserved active site residues (including zinc ligands, the acceptor Tr285 and additional positions adjacent to the metal centre) are all vital to MCR-1 function in *E. coli* (Hinchliffe *et al.,* 2017).

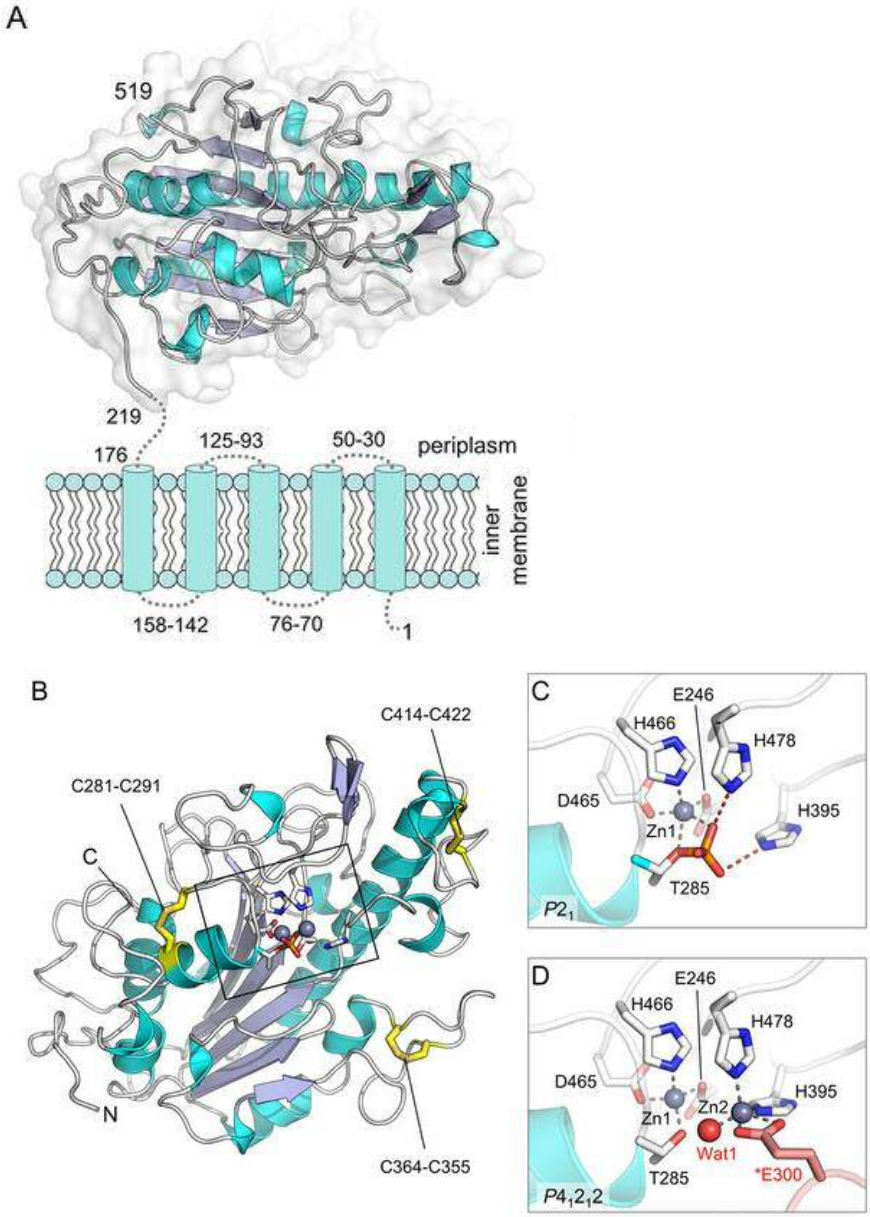


Figure 1.8 **Crystal Structure of MCR-1 catalytic domain**. A.B. Overall fold of MCR-1 catalytic domain. The crystal structures reveal an overall α-β-α fold and contain 3 intramolecular disulphide bonds (labelled). Active site is boxed and formed in C and D; C.D. Active site of MCR-1 enzyme (Hinchliffe *et al.,* 2017).

1.3.5 Continuous discovery of new transferable colistin-resistant genes

Following the discovery of *mcr-1* in 2015, other plasmid carrying genes encoding colistin resistance were reported; namely, *mcr-2*, *mcr-3* etc (Xavier *et al.,* 2016; Yin *et al.,* 2017). The latest mobile colistin resistance gene reported is *mcr-8* (Wang *et al*., 2018).

The *mcr-2* gene is a 1,617 bp long phosphoethanolamine transferase gene harboured on an IncX4 plasmid and shows 76.75% nucleotide identity to *mcr-1* (Xavier *et al.,* 2016). *mcr-2* showshigher prevalence (20%) in colistin-resistant *E. coli* in Belgium than *mcr-1* (13%) (Xavier *et al.,* 2016). *mcr-3* shows 45.0% and 47.0% nucleotide sequence identity to *mcr-1* and *mcr-2*, respectively (Yin *et al.,* 2017). Interestingly, MCR-3 shows 75.6%-94.8% identity to phosphoethanolamine transferases found in *Aeromonas* species (Yin *et al.,* 2017). Moreover, a transposon element, named Tn*As2*, which has only been characterized in *Aeromonas salmonicida*, is identified upstream of *mcr-3*. The ΔTn*As2-mcr-3* element found from genome sequencing and reported from many countries suggests the likelihood of *mcr-3* global dissemination (Yin *et al.,* 2017).

Now, *mcr-4* and *mcr-5* genes have been identified indicating a much wider global dissemination of colistin resistance determinants in Enterobacteriaceae (Borowiak *et al.,* 2017; Carattoli *et al.,* 2017).

1.4 Fitness cost of antimicrobial resistance

Antibiotic resistance occurs under the selective pressure of antibiotics (Xiong, Sun, Ding, Wang, & Zeng, 2015). The continuing existence of antibiotics helps AMR bacteria survive and successfully compete against susceptible phenotype (Tech, 2017). Today, the extensive resistance to antibiotics and lack of pipeline drugs is a cause of great concern; therefore, the biological cost (such as growth and survival rates inside/outside a host, transmission rates and pathogenesis protential) seems to be a strand of potential hope of making it possible to reverse antibiotic resistance (D. I. Andersson, 2003; Hernando-Amado, Sanz-Garcia, Blanco, & Martinez, 2017).

The hypothesis that antibiotic resistance may bring a biological cost to bacterial hosts comes from the analysis of antibiotic resistant mechanisms. Bacteria acquire resistance by two different genetic events: recurrent mutation (including recombination) and horizontal genes transfer (Sommer, Munck, Toft-Kehler, & Andersson, 2017). The impact of antibiotic resistance varies according to different events. In mutation-driven resistance, mutations usually happen in genes encoding antibiotic targets or transporter systems (e.g. porins), which are intrinsically linked to cellular physiology (Hernando-Amado *et al.,* 2017). Therefore, their mutations indicate a less proficient function and a lowering of competitiveness compared to sensitive wild-type isolates (Björkman & Andersson, 2000; Melnyk, Wong, & Kassen, 2015). In the case where resistance is acquired via horizontal transfer, a physiological burden is expected as a consequence of the resources required for replication, transcription and translation of mobile genetic elements (Hernando-Amado *et al.,* 2017). Concurrently, the

synthesized products may interfere with cellular physiology (Hernando-Amado *et al.,* 2017; Lenski, 1998).

Therefore, the analysis of biological cost of antibiotic resistance is important if we hope to predict resistance development or evaluate the possibility of reducing antibiotic resistance, as real-time studies both in clinical or laboratory show the effect of antibiotic resistance on bacterial fitness and how mutations can influence the trajectory of adaptive evolution under selective pressure (D. I. Andersson & Hughes, 2011; Sommer *et al.,* 2017). Several theoretical and experimental studies have demonstrated that the cost of antibiotic resistance is a key factor to determine the rate or maintenance of resistance under certain antibiotic pressures or without the presence of antibiotics (Melnyk *et al.,* 2015). For example, Levin and colleagues used a mathematical model to understand the relationship between antibiotic treatment and the frequency of resistant genotypes (Levin *et al.,* 1997). According to that model, as long as hosts are treated with an antibiotic, a residual population of resistant bacteria would be found. Moreover, a decrease in the frequency of AMR bacteria would be seen under a reduction of antibiotic treatment (Levin *et al.,* 1997). Thus it seems possible that healthier sensitive genotypes could outcompete their MDR counterparts and therefore, displace resistant populations in the absence of antibiotic selection over time (dan i Andersson & hughes, 2010). In other words, temporarily ceasing antibiotic use can help eliminate resistant bacteria by allowing sensitive genotypes to out-compete the resistant population (Melnyk *et al.,* 2015).

Fitness deficit is variable depending on the mutation, the organism, and the model used to determine the cost (Pope, McHugh, & Gillespie, 2010). The biological cost of resistance can be

measured in essentially three ways. Firstly, fitness can be evaluated by the growth parameters. The simplest way to determine fitness is to grow both resistant and susceptible isolates *in vitro* and to measure the density of each culture over time (Melnyk *et al.,* 2015). Secondly, competition assays between susceptible "parents" and resistant mutants (daughter cells) can be created to evaluate the physiological vigour of the isolates. Fitness is shown as the ratio between susceptible and resistant strains after serial passaging the mixture under antibiotic-free conditions (Melnyk *et al.,* 2015). Thirdly, *in vivo* assays are useful to measure fitness cost and are more relevant to clinical conditions. Several animal models are recommended, such as mice, chickens, *Galleria mellonella*, *Caenorhabditis elegans*, and cell cultures. Animal models can also be applied on competition experiments between two culture by measure the modification of ratio after several days (Pope *et al.,* 2010).

Fitness cost of colistin resistance has been reported in some species, especially in *A. baumannii* and *K.pneumoniae* (Beceiro *et al.,* 2014; Choi & Ko, 2015; Da Silva & Domingues, 2017). Two mechanisms have been described in *Acinetobacters*, one is mentioned PmrA/B mutation resulting in modification of LPS, the other is complete loss of LPS by *lpxA*, *lpxC*, or *lpxD* mutation (Adams *et al.,* 2009; Moffatt *et al.,* 2010; W. Zhang *et al.,* 2017). Beceiro and colleagues compared fitness cost and impact on virulence of both mechanisms and showed that complete loss of LPS gave very high colistin resistance (MIC >128 mg/L), while PmrA/B mutations showed MIC increases from 2-8 mg/L to 16-64 mg/L. Both mechanisms caused a significant decrease in bacterial fitness; however, no significant decrease in virulence was detected in PmrA/B mutants (Beceiro *et al.,* 2014). Also, López-Rojas *et al.,* published studies

about fitness burden and decrease in virulence by *pmrA* and *pmrB* mutations, respectively (López-Rojas *et al.,* 2011; López-Rojas *et al.,* 2013). Recently, Mu *et al.,* compared serum resistance from both LPS-loss and LPS-modified mutants and showed that both LPS-loss and LPS-modified mutants decreased resistance to serum (Mu *et al.,* 2016). However, in some other studies, colistin resistance by PmrA/B mutations were not associated with reduction of fitness or virulence. According to Durante-Mangoni, colistin resistance caused by a P233S substitution in the PmrB sensor kinase did not associate with reduction in bacterial fitness or virulence (Durante-Mangoni *et al.,* 2015). These results might suggest that the cost in fitness or virulence caused by PmrA/B mutations was variable due to location, type, and number of mutations (Beceiro *et al.,* 2014).

In *K. pneumoniae*, loss of fitness and virulence was normally associated with colistin resistance. In addition to the PmrA/PmrB TCS, mutations in the PhoP/PhoQ two-component regulatory system can also lead to colistin resistance (Chen & Groisman, 2013). Additionally, mutations in *mgrB* are related to colistin resistance, as its product MgrB conveys feedback between PmrA/PmrB and PhoP/PhoQ TCSs (Poirel *et al.,* 2014; Wright *et al.,* 2015). Cannatelli *et al.,* compared the fitness of two KPC-producing *K. pneumoniae* isolated obtained from same patient after low dosage of colistin treatment. Acquisition of colistin resistance after colistin treatment was associated with *pmrB* mutations but showed no reduction in fitness (Cannatelli *et al.,* 2014). Furthermore, they also reported no significant biological cost or reduction in virulence (Arena *et al.,* 2016; Cannatelli, Santos-Lopez, Giani, Gonzalez-Zorn, & Rossolini, 2015). Finally, Kidd *et al.,* found enhanced virulence in a *mgrB* mutant, which mediated a

128-fold increase in colistin MIC but showed no change in bacterial growth or biofilm formation (Kidd *et al.,* 2016). As *K. pneumoniae* can cause a wide range of infections, the lack of fitness cost of colistin resistance may lead to higher dissemination of resistance and undermine the treatment of *K. pneumoniae* infections (Paczosa & Mecsas, 2016) .

1.5 Aims of the Project

Resistance mutations are expected to have an effect on fitness because their targets have important functions on bacterial physiology (Melnyk *et al.,* 2015). Colistin resistance is always due to the modification or loss of LPS of bacterial cell outer-membrane, resulting in reduction of fitness and virulence (Abiola O Olaitan *et al.,* 2014). Previous studies have shown that colistin resistance caused by chromosomal mutation can be associated with reduction of bacterial fitness in *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and *S. enterica* (Choi & Ko, 2015; Da Silva & Domingues, 2017; Lee, Park, Chung, Na, & Ko, 2016; S. Sun, Negrea, Rhen, & Andersson, 2009). However, this biological cost is variable among different mutations, some of these point mutations are proved to have less fitness cost or even no fitness cost (Beceiro *et al.,* 2014).

Hitherto, little has been known about fitness and virulence in *mcr-1* harbouring isolates. Zhang *et al.,* showed that no reduction in fitness was found between *mcr-1* positive transconjugates and *E .coli* J53 (Y. Zhang *et al.,* 2017). This may indicate that acquisition of *mcr-1* carrying plasmid is cost free (Hernando-Amado *et al.,* 2017). As the production of *mcr-1* normally gives relatively low resistance in *E. coli* (4-16 mg/L colistin MIC) (Liu *et al.,* 2016) compared to chromosomally mediated mechanisms, it is of interest to explore the effects of increasing colistin resistance in *mcr-1* positive isolates. Furthermore, as colistin resistance disseminates with the prevalence of *mcr-1* (Shen *et al.,* 2016), it is important to determine whether there is fitness cost with *mcr-1* and consequently, provide a limitation to colistin resistance.

Thus, the aims of this study are to acquire *mcr-1* positive high-level colistin resistant mutants, and to measure the change in bacterial fitness and virulence and to assess their stability. Whole genome sequencing (WGS) on the isogenic sets analysis will also be undertaken.

Chapter 2: Methods and Materials

2.1 strains used in this study

Table 2.1 Strains used in this study

No.	Identification	Date of isolation	Location	Source
PN16	*E.coli*	27 October 2013	Phitsanulok, Thailand	chicken meat
PN21	*E.coli*	12 February 2014	Phitsanulok, Thailand	chicken faeces
PN23	*E.coli*	22 February 2014	Phitsanulok, Thailand	duck faeces
PN24	*E.coli*	22 February 2014	Phitsanulok, Thailand	duck faeces
PN25	*E.coli*	22 February 2014	Phitsanulok, Thailand	duck faeces
PN42	*E.coli*	15 November 2013	Phitsanulok, Thailand	faeces from healthy human
PN43	*E.coli*	15 November 2013	Phitsanulok, Thailand	faeces from healthy human

2.2 Antibiotics, chemicals and regents

Colistin sulfate used in this study was obtained from Alfa Aesar, USA.

2.3 Growth medium

2.3.1 Urinary tract infection (UTI) agar (Sigma-Aldrich, USA)

Ingredients per litre

Peptic digest of animal tissue 18.0 g

Casein enzymic hydrolysate 4.0 g

Beef extract 4.0 g

Chromogenic mixture 12.44 g

Agar15.0 g

Final pH (at 25) 7.2+/–0.3

Suspend 55.4 g medium in 1 litre distilled water. Sterilize at 121 for 15minutes.

Table 2.2 Cultural characteristics of UTI agar

Organisms (ATCC)	Growth	Colour of colony
E. coli (25922)	+++	Pink–red
Proteus mirabilis (10975)	+++	Light brown
K. pneumonia (13883)	+++	Blue to purple (mucoid)
P. aeruginosa (27853)	+++	colourless
S. aureus (25923)	+++	Golden yellow
Enterococcus faecalis (29212)	+++	Blue (small)

2.3.2 LB agar (Fisher Scientific, UK)

Ingredients per litre

Tryptone 10.0 g

Yeastn extract 5.0 g

Sodium Chloride 10.0 g

Agar 5.0 g

Suspend 40 g in 1 litre of purified water.

2.3.3 LB broth (Fisher Scientific, UK)

Ingredients per litre

Tryptone 10.0 g

Yeastn extract 5.0 g

Sodium Chloride 5.0 g

Suspend 20 g powder in 1 litre of purified water.

2.3.4 BBL™ Mueller Hinton (MH) II broth (Cation-Adjusted) (BD Biosciences, USA)

Ingredients per litre

Beef extract 3.0 g

Acid hydrolysate of casein 17.5 g

Starch 1.5 g

Final pH 7.3+/–0.1

Suspend 22 g of the powder in 1 litre of purified water. Autoclave at 116–121 for 10 minutes.

2.3.5 Mueller Hinton (MH) II agar (BD Biosciences, USA)

Ingredients per litre

Beef extract 2.0 g

Acid hydrolysate of casein 17.5 g

Starch 1.5 g

Agar 17.0 g

Final pH 7.3+/–0.1

Suspend 38 g of the powder in 1 litre of purified water. Autoclave at 121 for 15 minutes.

2.3.6 M9 medium (Sigma-Aldrich, UK)

Ingredients per litre

Ammonium chloride 1 g

Disodium hydrogen phosphate 6 g

Potassium dihydrogen phosphate 3 g

Sodium chloride 0.5 g

Final pH 7.4+/–0.2

2.4 Generation of HLCRMs

The optical density (OD) of overnight culture was adjusted to 0.08– 1.0 at 600 nm, and bacterial sample was further diluted 20 X before being grown in MH broth with 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 mg/L colistin overnight at 37 . After that the last well with bacterial growth and first well without growth were mixed together and incubated for 6 hours at 37 . The OD_{600nm} of mixture was adjusted to 0.08–1.0 and diluted for 20 more times before being grown with colistin. The same steps were repeated for 14 days

to get HLCRMs.

2.5 Minimum inhibitory concentration (MIC)

1) Preparation of antibiotic stock solution and dilution range

Colistin was diluted serially with MH broth in 96-well plate with a final concentration from 128 mg/L to 0.25 mg/L (100 μL each well), the first row was left as a colistin zero (Table 2.3).

2) Preparation of bacterial inoculum

OD of overnight culture was adjusted to 0.08-0.1 at 600nm, before further diluted to 1 in 10 with MH broth. ATCC25922 was used as quality control.

3) Sample (100 μL each well) and antibiotic were mixed and grown for 16–20 hours at 37 .

4) After incubation， the concentration of colistin for the first row without bacterial growth was identified as MIC.

Table 2.3 MIC template in 96-well plate for colistin

	Colistin(mg/L) 0	0.25	0.5	1	2	4	8	16	32	64	128	
Sample A												Clean LB
Sample A												Clean LB
Sample B												LB& colistin
Sample B												LB& colistin
Sample C												-
Sample C												-
Sample D												-
Sample D												-

2.6 Polymerase chain reaction (PCR)

PCR was carried out using illustra puReTaq Ready–To–Go PCR Beads (GE Healthcare Life Sciences, UK) in a final volume of 20 , also containing 1 µL DNA template, 1 µL loading dye, 0.5 µL forward primer(10–20 µmol), and 0.5 µL reverse primer(10–20 µmol) (**Table 2.4**). Primers were purchased from Urofins Genomics, UK. Molecular water (ThermoFisher Scientific, UK) was used to make up the final volume of 20 µL.

mcr–1 PCR was performed for 1 cycle at 94 for 5 minutes, then 30 cycles of sequential incubation for 30 seconds at 95 , 1 minute at 52 and 1 minute at 72 . To finish the reaction, an extension cycle at 72 for 10 minutes was taken.

Table 2.4 Sequences of primers and probes used in this study

Primer/Probe	Sequencing(5'-3')	Size (bp)	Reference
mcr-1 F	GCTACTGATCACCACGCTGT	953	(Yang *et al.,* 2017)
mcr-1 R	TGGCAGCGACAAAGTCATCT		
mcr-1-qF	TGGCGTTCAGCAGTCATTAT		
mcr-1-qR	AGCTTACCCACCGAGTAGAT		
mcr-1 probe	ROX-AGTTTCTTTCGCGTGCATAAGCCG- BHQ1		
rpoB-qF	TCCTTTCTATCCAGCTTGACTCGT	200	
rpoB-qR	CGCAGTTTAACGCGCAGCGG		
rpoB probe	HEX-ACGTCAGCTACCGCCTTGGCGAACCGGTGT BHQ1		
16S-qF	CATTGA CGTTACCCGCAGAA	100	
16S-qR	CGCTTTACGCCCAGTAATTCC		
16S probe	FAM-CGTGCCAGCAGCCGCGGTA-TAMRA		

2.7 Agarose gel electrophoresis

After the PCR reaction, the reaction mixture was loaded onto an agarose gel to size and fractionate the DNA fragment. Hi-Res Standard Agarose (Cambridge Reagents Ltd, UK) was used at 1.5% in 1 X TBE buffer. Ethidium bromide was added to a final concentration of 0.1 μg/mL in the gel. A 1kB plus DNA ladder (Lambda PFG Ladder, New England Biolabs, UK) was used to ascertain the size of DNA fragments. Electrophoesis was done

at 300V for 30 minutes. DNA patterns were visualised and recorded by G:BOX Chemi XX6 gel imaging system (Synoptics Ltd, UK).

2.8 Stability of HLCRMs

Stability of HLCRMs was detected by serial passaging in colistin-free medium. Overnight culture of HLCRMs was diluted by 1:500 into LB broth without colistin and was incubated for 18h at 37 at 220rpm. Same process was repeated for 14 days. To further measure the proportion of colistin resistant population, Overnight culture was diluted serially and was inoculated on antibiotic-free agar plates as well as on agar plates with 0.5 X MIC to colistin of respective HLCRMs. After 18–22h incubation at 37 colony-form units (CFU)/ml of colistin-resistant cells were counted.

2.9 Preparation for genomic DNA

Genomic DNA was extracted using QIAcube system (Qiagen, Germany), using QIAGEN spin-column kits (Qiagen, Germany)

2.10 Real time quantitative polymerase chain reaction (RT-qPCR)

2.10.1 Expression of *mcr*-1

Expression of *mcr*-1 for both wild-type isolates and HLCRMs were detected by a two-step qRT-PCR using primers *mcr-1*-qF, *mcr-1*-qR (Table 2.4) and *mcr-1* probe with Precision 2x qPCR Mastermix (PrimerDesign, UK).

1) Reverse Transcription: Total RNA was isolated using RNeasy Plus kit (Qiagen, Germany) with on column DNase digestion, followed by cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen, Germany).

2) Real–Time PCR: PCR amplification was done in a 20 µL reaction volume for the 96–well plates in the absence of reverse transcriptase, using a StepOnePlus System (Thermofisher Scientific, UK). *rpoB* expression level was used as positive control using primers *rpoB*–qF, *rpoB*–qR and *rpoB* probe (Table 2.4). Relative expression results were calculated by the comparative C_T analysis method using average C_T value.

2.10.2 *mcr-1* copy number

mcr–1 copy numbers per cell was determined by qPCR amplification. PCR amplification was done in a 20 µl reaction volume for the 96–well plates, using a StepOnePlus System (Thermofisher Scientific, UK). 0.1 ng of total genomic DNA was used as template with primers (*mcr–1* qF/qR, or 16S qF/qR) and probe (*mcr–1* or 16S probe) (Table 2.4). Standard curve for *mcr–1* was obtained using as template serial dilutions of *mcr–1*–carrying plasmid DNA extracted from pSU18–*mcr–1* strain (single copy of *mcr–1*per cell) (4.3 ng of DNA corresponding to 10^6 copies, calculated through the website: http://cels.uri.edu/gsc/cndna.html)(Yang *et al.,* 2017). And that for 16S was obtained using as template serial dilutions of *mcr–1*–carring plasmid DNA extracted from *E. coli* TOP10 (Invitrogen, UK) total genomic DNA (5 ng corresponding to 10^6 cells). The absolute copy number of *mcr–1* in the *E. coli* total DNA samples was determined from the

corresponding standard curves, using the C_T values.

2.11 S1 pulsed-field gel electrophoresis (S1-PFGE)

S1–PFGE was performed according to the standard operating procedure and DNA restriction was under (Centers for Disease Control and Prevention, 2013b; Toleman, 2018).

2.11.1 Buffers and reagents

1) TE buffer (10 mM Tris:1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0

2 ml of 0.5 M EDTA, pH 8.0

To a total volume of 1000 ml with sterile ultrapure clinical laboratory reagent water (CLRW)

2) Cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0)

100 ml of 1 M Tris, pH 8.0

200 ml of 0.5 M EDTA, pH 8.0

To a total volume of 1000 ml with sterile ultrapure water (CLRW)

3) Cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl)

50 ml of 1 M Tris, pH 8.0

100 ml of 0.5 M EDTA, pH 8.0

100 ml of 10% Sarcosyl (N–Lauroylsarcosine, Sodium salt)

To a total volume of 1000 ml with sterile ultrapure water (CLRW)

4) 10 X Tris–boric acid/EDTA (TBE) buffer

Tris base 108.0 g

Boric acid 55.0 g

0.5 M EDTA (pH 8.0) 20.0 mL

To a total volume of 1000 ml with sterile ultrapure water (CLRW)

5) 1/10 TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0).

5 mL of 1 X TE is diluted to 50 mL with sterile ultrapure water (CLRW) to produce 1/10 X TE buffer.

6) 1 X S1 buffer (30 mM sodium acetate pH 4.6, 1 mM $ZnSo_4$, 5% glycerol).

10 X S1 buffer is prepared as:

12.3 g of Sodium acetate

0.92 g Zinc acetate

200 mL sterile ultrapure water (CLRW)

250 mL of glycerol

Final pH 4.6

To a final volume of 500 mL with sterile ultrapure water (CLRW)

5 mL of 10 X S1 buffer is diluted to 50 mL with sterile ultrapure water (CLRW) to produce 1 X S1 buffer.

*10 X S1 buffer needs to be stored at –20 .

7) 0.5 X TBE (45 mM Tris–HCl, 45 mM boric acid, 1 mM EDTA).

100 mL of 10 X TBE is diluted to 2000 mL with sterile ultrapure water (CLRW) to produce 0.5 X TBE.

2.11.2 Making plugs with SeaKem Gold (SKG) agarose (Lonza, Switzerland)

Bacterial colonies were inoculated into 2 ml cell suspension buffer (with a final concentration of OD 0.8–1.0 at 600nm). 400 µl cell suspensions were mixed with 400 µl melted 1% SKG agarose and 20 µl of Proteinase K (20 mg/ml stock), before dispensed into clean plug mold.

2.11.3 Lysis of cells in agarose plugs

Plugs were transferred into 5 ml cell lysis buffer with 25 µl Proteinase K (20 mg/ml stock) and were incubated at 55 ℃ at 150–175 rpm for 1.5–2 hours.

2.11.4 Washing of agarose plugs after cell lysis

After Incubation with cell lysis buffer, plugs were washed with 10–15 ml pre-heated sterile ultrapure water (CLRW) for two times, then with 10–15 ml pre-heated sterile TE buffer for four times, shaking at 55℃ for 10–15 minutes each wash. Plugs could be stored in TE buffer at 4 ℃.

2.11.5 Restriction digestion of DNA in agarose plugs

After washing, plugs were incubated in 300 µL 1/10 TE for 5 minutes at room temperature and then pre-restricted by 200 µL S1 buffer for 5 minutes at room temperature. Restriction was done by 200 µl S1 enzyme master mix (1 unit of S1 nuclease

[Invitrogen, UK]) overnight at 4˚C. After restriction, plugs were treated with 200 µL 0.5 X TBE for 5 minutes at room temperature before loading into1% SKG agarose (melted with 0.5 X TBE). Lambda PFG Ladder (NEB, UK) was used as marker.

2.11.7 Electrophoresis Condition

Gel was run on a CHEF–DR III apparatus (Bio–Rad, Hercules, CA, USA) under following conditions.

Initial switch time: 4s

Final switch time: 45s

Voltage: 6 V

Included Angle: 120°

Run time: 18 hours

2.12 In gel hybridisation

After the electrophoresis, the gel was dried and then dealt with direct agarose gel probing (Toleman, 2018).

2.12.1 In gel hybridisation components

1) Denaturing solution (0.5 M NaOH, 1.5 M NaCl)

20 g NaOH

87.66 g NaCl

To a total volume of 1000 ml with sterile ultrapure water (CLRW)

2) Neutralising solution (0.5 M Tris–HCl, PH 7.5, 1.5 M NaCl)

60.5 g Tris base

87.6 g NaCl

Final pH7.5

To a total volume of 800 ml with sterile ultrapure water (CLRW)

3) Pre–hybridisation solution:

(6 X SSC, 0.1%[W/V]) polyvinylpyrrolidone

0.1% Ficoll,

0.5% SDS

150 mg/L Herring testes DNA

1 mL full cream milk

To a total volume of 20 mL with sterile water (CLRW)

2.12.1 Pre- hybridisation

Once dried, the gel was re–hydrated in the following steps: 200 mL deionized DNase–free water for 5 minutes, 200 mL denaturing solution at room temperature for 45 minutes and 200 mL neutralising solution for 45 minutes at room temperature. Then re–hydrated gel was moved to a hybridisation tube and incubated with 20 mL pre–hybridisation solution at 65 ℃ for 24 hours.

2.12.2 Probe preparation and hybridisation

mcr–1 probe was prepared by random priming labelling method using purified *mcr–1* positive PCR product (using QIAquick Gel Extraction Kit (Qiagen, Germany)) with radio-active ^{32}P dCTP (Stratgene, Amsterdam, Netherlands) as a label, according to . Once labelled, unincorporated ^{32}P dCTP and unlabelled nucleotides were removed by a sephadex G50 gravity flow gel filtration column (illustraTM NickTM Columns Sephadex G–50 DNA grade, GE Healthcare Life Sciences, UK). Labelled probe was boiled for 6 minutes before added into pre-hybridised gel overnight at 65 ℃.

2.12.3 Film development

After hybridisation, gel was washed with 100 mL 2 X SSC, 0.1% SDS for one hour at 65 ℃ before put into film cassette. Detection film used was Lumi-Film Chemiluminescent Detection film (Roche, Germany). Films were developed using standard film development and fixer solutions.

2.13 Growth rate

To test the growth curve, 10 µL overnight culture was added into 10 mL LB broth and incubate at 37ºC at 220 rpm for 8 hours. OD value at 492 nm was measured every one hour.

2.14 Competition assay by flow cytometry

Competition assay was tested for seven wild-type strains and seven HLCRMs using flow cytometry. Green fluorescent protein (GFP)-labelled DH5- was used as control. All competitions were carried out in M9 medium (Sigma-Aldrich, UK) with six replicates per strain/condition. Flow cytometry was performed on an Accuri C6 (BD Biosciences, UK). Briefly, overnight culture was diluted by 1:400 for each wild-type strain, HLCRM and GFP-labelled DH5- around 1000 events per 1 in M9 broth. Each diluted sample strain was mixed with GFP-labelled DH5- , and incubated at 37 ℃ at 220 rpm for 6 hours. The starting ratio between wild-type strains/HLCRMs and GFP-labelled DH5- was measured by flow cytometry. After 6 the bacterial mixture was diluted by 1: 400, and the final ratio between sample strains and GFP-DH5- was measured by flow cytometry. Relative fitness was calculated between each wild-type strain/HLCRM and GFP-DH5- by formula below.

$p0$ stood for the initial proportion of an unlabelled stain, and $p1$ stood for the final proportion of an unlabelled stain after competition. $n_{dilution}$ was the factor that reflected the fold difference in cell density at the beginning/end of the competition.

The relative fitness of HLCRMs to their parental strains was further calculated by $_{HLCRM}/_{parental}$ for each repeat, and error propagation was used to account for the uncertainty.

2.15 *Galleria mellonella* pathogenicity model

Pathogenicity *in vivo* was examined in a Galleria model. *G. mellonella* caterpillars (Live Foods UK Ltd., http://www.livefood.co.uk) were stored in the dark and were used within 7 days from shipment.

1) 10 mL overnight culture was centrifuged at 4000–6000 rpm for 10 minutes and bacterial pellet were washed with 5mL sterile normal saline twice.

2) Washed bacterial pellet were standardised in suspensions with sterile normal saline equating to $1x10^9$, 10^8, 10^7, 10^6 and 10^5 CFU/ml.

3) 10 healthy *G. mellonella* larvae with similar size (15–20 mm) and weight (approximately 0.2 g) were manually selected for each level of inoculation in triplicate. Each group of larvae were placed into a sterile 10 x 10 mm Petri dish.

4) Selected larvae were injected with 10 of suspension into the hemocoel using a Hamilton Syringe, through the rear left pro-leg, and were incubated at 37 ℃ for 72 hours.

5) The amount of died and alive worms was checked every 24 hours. Death was denoted when larvae no longer respond to touch and exhibit grey/dark pigmentation.

6) Data were analysed by Kaplan-Meier survival curves using GraphPad Prism 7.0.

2.16 DNA sequencing

2.16.1 Illumina WGS and bioinformatics analysis

Whole genome sequencing (WGS) was carried out on the HLCRM isolates using the Illumina MiSeq platform (Illumina Inc., CA). Briefly, gDNA was extracted from an overnight culture (2 ml) using the QIAcube automated system (Qiagen, Germany), and resulting gDNA were quantified using the Qubit 3.0, using the dsDNA high sensitivity assay (Thermos Fisher Scientific), with quality ratios of gDNA (A260/280 and 260/230) determined via Nanodrop (ThermoFisher Scientific). DNA libraries were prepared for paired-end sequencing (2 x 301 cycles) using Nextera XT (Illumina). Quality control (QC) of raw sequence reads included fastqc (0.11.2), and quality and adaptor trimming were performed using Trim galore (0.4.3). For each *E. coli* isolate, at least 80 X coverage was generated. Reads were assembled in contigs using the de novo assembler SPAdes (3.9.0) (.fasta) and were aligned to the original fastq reads using BWA aligner (0.7.15). Any assembly mapping errors in the contigs was corrected, using Pilon (1.22). Assembly metrics were evaluated using Quast (2.1). MLST loci (by MLST 2.0), acquired resistance genes (by ResFinder 3.1, with minimum 90% identity and 80% coverage), and plasmid replicons (by PlasmidFinder-2.0, using Enterobacteriaceae database with minimum 95% identity and 80% query coverage) were retrieved from the online databases (CGE platform: https://cge.cbs.dtu.dk/services/).

2.16.2 Nanopore sequencing and bioinformatics analysis

High-molecular weight gDNA was extracted from the HLCRM following a chloroform-precipitation method. The total gDNA was quantified following a serial dilution using the Qubit 3.0 and the quality was assessed following the metrics as described above. Sequencing libraries were prepared using the Rapid Barcoding Kit (SQK-RBK004) following manufacturers' instructions. Following a QC check of the Nanopore flowcell (R9.4) just before use, over 1, 200 active pores were detected. The flowcell was primed according to the manufactures guidelines, and the library was gently mixed using a pipette tip, and loaded onto the Nanopore flowcell (R9.4). WGS was performed for 48 hours using a MinION and associated MinKNOW software. Raw fastq sequences were concatenated and the HLCRM were de-barcoded using Porechop. Unicycler (Wick, Judd, Gorrie, & Holt, 2017) was used to create a hybrid assembly with a combination of MiSeq short reads and MinION (Oxford Nanopore) long reads. The resulting hybrid assembly was visualised using Bandage to confirm complete genome assembly of the chromosome and plasmids.

Single nucleotide polymorphisms (SNPs) between wild-type strains and corresponding HLCRMs were analysed using Geneious 10.2.6. The circular comparisons among *mcr-1*-related IncX4 and IncI2 plasmid backgrounds were performed using BLAST Ring Image Generator (BRIG v0.95).

2.17 Bacterial morphology by transmission electron microscopy (TEM)

Briefly, overnight culture was diluted into 50 ml fresh LB media with 2 mg/L colistin. After an 8-hour incubation, glutaraldehyde was added to the sample with a final

concenreation of 1%. Sample was fixed in 4% low melting point agarose, dehydrated through graded propan–2–ol, and infiltrated and embedded through LR white acrylic resin (London Resin Company, UK). After embedding, sections of 80 nm thickness were cut on an Ultracut E. ultramicrotome with a glass knife and collected on mesh copper grids and strained by lead citrate before observation with a Philip CM12 TEM (FEI UK Ltd, UK) at 80 kv. Images were captured by a Megaview III digital camera and AnalySIS (Soft Imaging System GmbH, Germany).

Chapter 3: Results

3.1 Identification of *mcr-1* positive strains and acquisition of HLCRMs

3.1.1 Bacteria strains and susceptibility testing.

The aim of this study is to acquire high colistin resistant mutants (HLCRMs) and to examine the fitness cost of *mcr-1* to the host bacterium. For this purpose, seven *mcr-1* positive wild-type *E. coli* strains from different origins (PN16 from chicken meat, PN21 from chicken faeces, PN23, PN24, PN25 from duck faeces and PN42, PN43 from human faeces) from Phitsanulok, Thailand were selected for this study.

3.1.2 Generation of high-level colistin resistant mutants (HLCRMs)

MICs were performed by broth micro-dilution for each strain and colistin MICs ranged from 4 to 8 mg/L – all demonstrating low-level resistance to colistin (Liu *et al.,* 2016). This data is consistent with previous studies that report colistin MICs with *mcr-1* positive *E. coli* strains range from 4-16 mg/L (Liu *et al.,* 2016; Rhouma, Beaudry, Theriault, & Letellier, 2016). To produce HLCRMs, wild-type isolates were treated with increasing concentrations of colistin in continuous culture for 14 days. A portion of the population was removed each day and colistin MICs were determined by broth micro-dilution. *E. coli* ATCC 25922 was used as a QC strain.

After 14-days of passaging, all isolates demonstrated an increase in colistin MIC (**Table 3.1** and ***Figure 3.1***). The highest increase was seen in PN43 with a 64-fold increase in colistin MIC to 256 mg/L. The colistin MIC against PN23 HLCRM also reached 256 mg/L, followed by PN16,

PN21 and PN25 at 128 mg/L. Whilst PN24 and PN42 showed only a 4- and 8-fold increase to 16 mg/L and 32 mg/L, respectively. Throughout the passaging, the *E. coli* were stored daily at -80 ºC until further required. However, the Day14 mutant for PN16 was too weak to grow from -80 ºC, and therefore the Day13 mutant was used as the end-point HLCRM for PN16.

Table 3.1 MICs of colistin against wild-type strains for 14-day passaging

	MIC of colistin (mg/L)						
Day	**PN16**	**PN21**	**PN23**	**PN24**	**PN25**	**PN42**	**PN43**
1	8	4	8	4	8	4	4
2	8	8	8	4	8	8	4
3	8	8	8	4	8	8	4
4	8	8	16	4	8	8	8
5	16	8	16	4	8	8	8
6	32	8	32	8	16	16	16
7	16	16	32	8	16	16	16
8	16	16	32	8	32	32	16
9	16	32	128	16	32	32	32
10	16	32	128	16	32	32	64
11	16	64	128	16	64	32	128
12	16	64	128	16	64	32	128
13	32	64	128	16	64	32	256
14	128	128	256	16	128	32	256

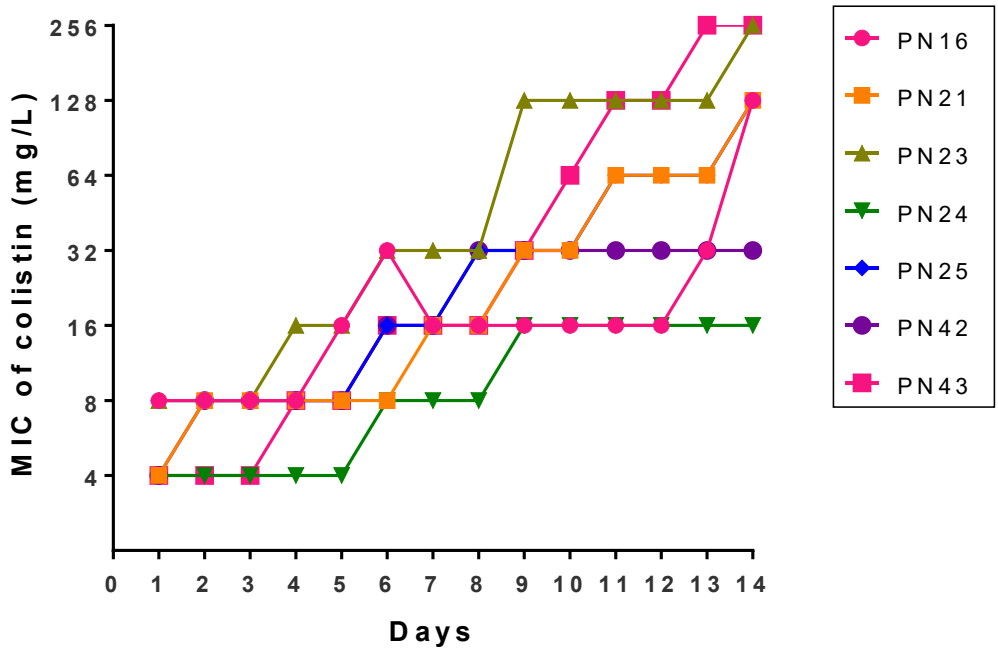


Figure 3.1 **Generation of HLCRMs over a passaging period of 14 days.** Seven wild-type MCRPEC were treated with increasing concentration of colistin for 14 days.

qRT-PCR was performed for *mcr-1* to examine whether the increase in colistin resistance is associated with *mcr-1* expression. Results indicated that the expression of *mcr-1* in 6 mutants increased compared to their respective parental strains. HLCRMs of PN21 and PN25 showed the highest increase in *mcr-1* expression with 11- and 3- fold, respectively. HLCRMs of PN16, PN23, PN24 and PN43 displayed a 1.2- to 1.9-fold change compared to their respective parent strains. However, the MCR-1 expression level of HLCRM PN42 showed no discernible increase in expression but a slight decrease (***Figure 3.2*** A).

Furthermore, *mcr-1* copy number was also examined in the HLCRMs by qRT-PCR. The copy number for wild-type isolates ranged from 1 (chromosomal copy for PN43) to 5.6 (PN23). Increase in the *mcr-1* copy number was seen in PN16, PN21 and PN25 mutants (**Table 3.2 and**

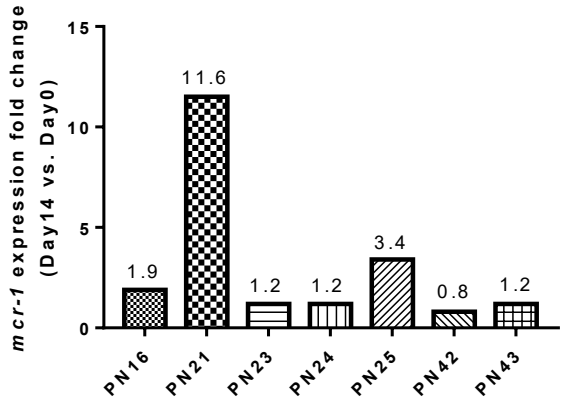
Figure 3.2 **B**).

Table 3.2 *mcr-1* copy number per cell comparing Day0 (parent) and Day14 (HLCRM)

(*mcr-1* copies/cell)

Strain	PN16	PN21	PN23	PN24	PN25	PN42	PN43
Day0	3.1	2.1	5.6	4.3	1.8	3.2	1
Day14	3.6	3.8	3.5	2.1	3.3	1.5	1

A



B

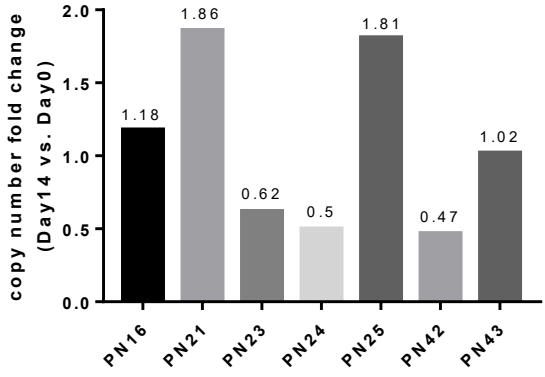


***Figure 3.2* A. mcr-1 expression represented as fold-change comparing Day14 to Day0.** Relative changes of *mcr-1* expression (Day14 compared to Day0) were calculated using ΔΔCT analysis method by mean CT value (n=2).

B. mcr-1 copy number expressed as fold-change comparing Day14 to Day0. Fold changes in *mcr-1* copy number were obtained by comparing each Day14 and Day0 strains.

3.1.3 Stability of HLCRMs

Stability experiments were undertaken for each HLCRM to examine their ability to maintain higher levels of resistance to colistin. This was achieved by passaging in LB broth without colistin over a period of 14 days and numerating the HLCRMs (CFU/ml) on Müller-Hinton agar plate with 16, 32, 64, 128 mg/L colistin and without colistin.

Difference in CFU/ml for each day was demonstrated in log phase (***Figure 3.3***). For three of the HLCRMs (PN24, PN25 and PN42) the CFU/ml (overall bacterial population) remained at the same level after 14 days passaging without colistin, indicating that their HLCRMs were highly stable. The other strains showed a varying decrease in CFU/ml after 14 days, especially for PN43 where the CFU/ml dropped gradually but continuously and after ten days, no growth on at 128 mg/L colistin could be detected. HLCRMs PN16, PN21 and PN23 also showed significant decrease in their HLCRM populations over 14 days, indicating that their respective HLCRMs are not particularly stable.

Colistin MICs were performed on strains isolated during the 14-day passaging without colistin The MICs were generally kept consistent with CFU/ml levels except for PN21 and PN43. Interestingly, even though the CFU/ml for strains PN21 and PN43 decreased to a very low-level after 14 days, their MICs were kept at 128 mg/L and 256 mg/L, respectively (**Table 3.3**).

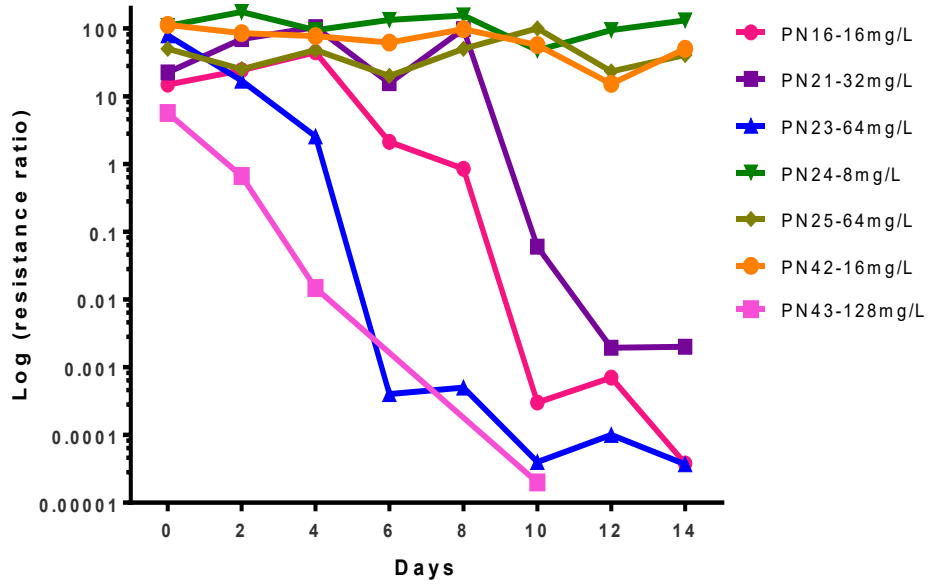


Figure 3.3 **Stability of HLCRMs after 14-day passaging without colistin.** CFU/ml was grown on media with/without colistin, ratio of CFU/ml with/without colistin was calculated.

Table 3.3 MICs of wild-type strains, high level mutants and stability strains

	MIC of colistin (mg/L)		
	Wild-type	**HLCRM(Day14)**	**Stability**
	8	32(Day13)	8
PN21	4	128	128
PN23	8	256	64
PN24	4	16	16
PN25	8	128	64
PN42	4	32	32
PN43	4	256	256

3.1.4 Location of *mcr-1* comparing Day 0 parent isolates and HLCRMS (Day 14)

To locate the *mcr-1* gene, S1-PFGE and southern blot were undertaken and analysed (***Figure 3.4***). Results showed that except for PN43, all *mcr-1* genes were located on different size of plasmids. No obvious plasmid size change was found between original wild-type isolates and respective mutants. The *mcr-1* gene of PN43 is located on chromosome, which is very unusual although has been previously reported (Falgenhauer, Waezsada, Gwozdzinski, *et al.,* 2016).

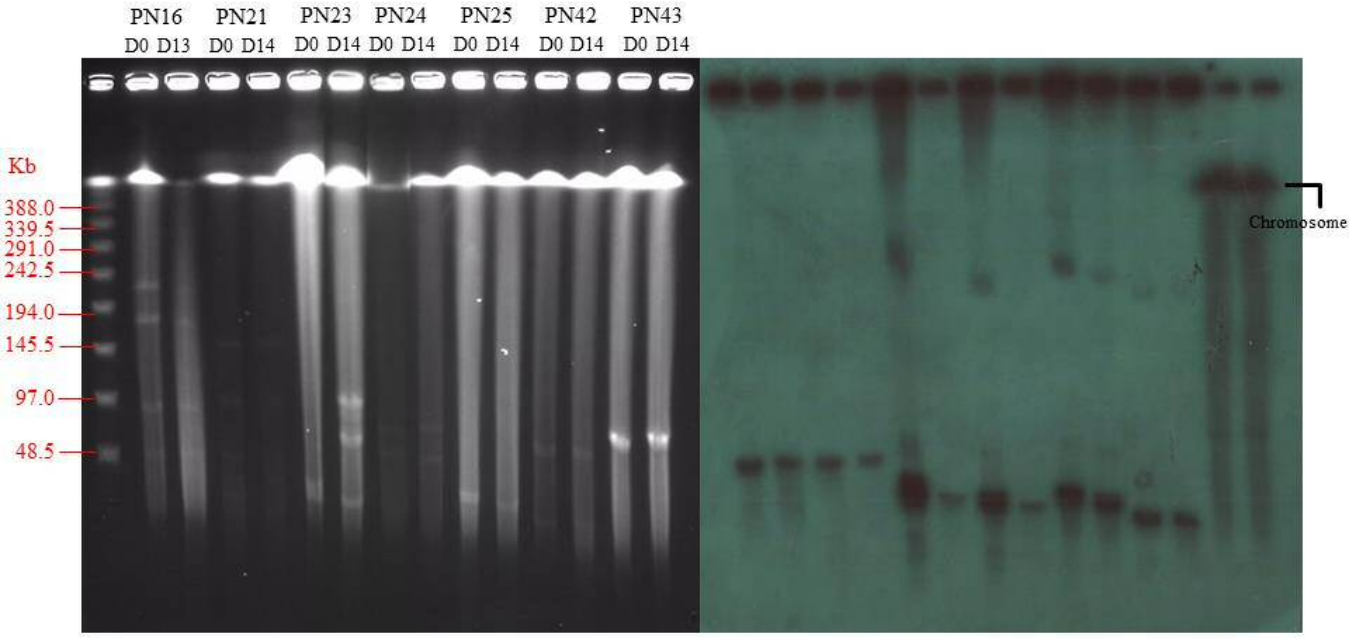


Figure 3.4 **S1-PFGE analysis of wild-type isolates and their corresponding Day14 HLCRMs.** In-gel hybridization with ^{32}P-labelled *mcr-1* gene probe after PFGE of nuclease S1 digested genomic DNA.

3.1.5 Whole genome sequencing (WGS) analysis

WGS was proposed on each wild-type isolate and HLCRM using Illumina. In addition, five HLCRMs (of PN16, PN24, PN25, PN42, PN43) were also sequenced by nanopore long reads and were corrected by Miseq short reads.

The *mcr-1* gene from these isolates were located on an Incl2 plasmid (PN16 and PN21) and IncX4 plasmid (PN23, PN24, PN25 and PN42), respectively. Unusually, *mcr-1* for strain PN43 was located on the chromosome. In addition to *mcr-1*, these isolates also harboured other resistance genes (see appendix section). The isolates were examined for different sequence types (MLSTs) and identified the following MLST groups: ST-2040, ST24*, ST-1121, ST-7986, ST-101, ST-744, and ST-410. I also determined the *mcr-1* copy number by qPCR and found the plasmid number to be ranging from 1 to 5.6 copies per cell.

In addition to PN43, the *mcr-1* harbouring plasmids of the six wild-type *E. coli* isolates belonged to IncI2 and IncX4 plasmid types, with sizes of ~60 kb and ~35-40 kb (**Table 3.4**), respectively. The GC content of all plasmids was approx. 42%, typical of plasmids associated with Enterobacteriaceae. Genetic structure of both plasmids were demonstrated in ***Figure 3.5*** **A, B**. Similar with previous studies, the insertion sequence, IS*Apl1*, was detected upstream of *mcr-1* in the IncI2 plasmid in PN16 and PN21 (***Figure 3.5*** **C**) (Liu *et al.,* 2016). However, no insertion sequence was found in front of the *mcr-1* gene in the IncX4-type plasmids (Gao *et al.,* 2016). *mcr-1* in PN43 is chromosomally located, and is flanked upstream by IS*Apl1* and adjacent to IS*1294* in the downstream (***Figure 3.5*** **C**). IS*Apl1* is always flanked at either or

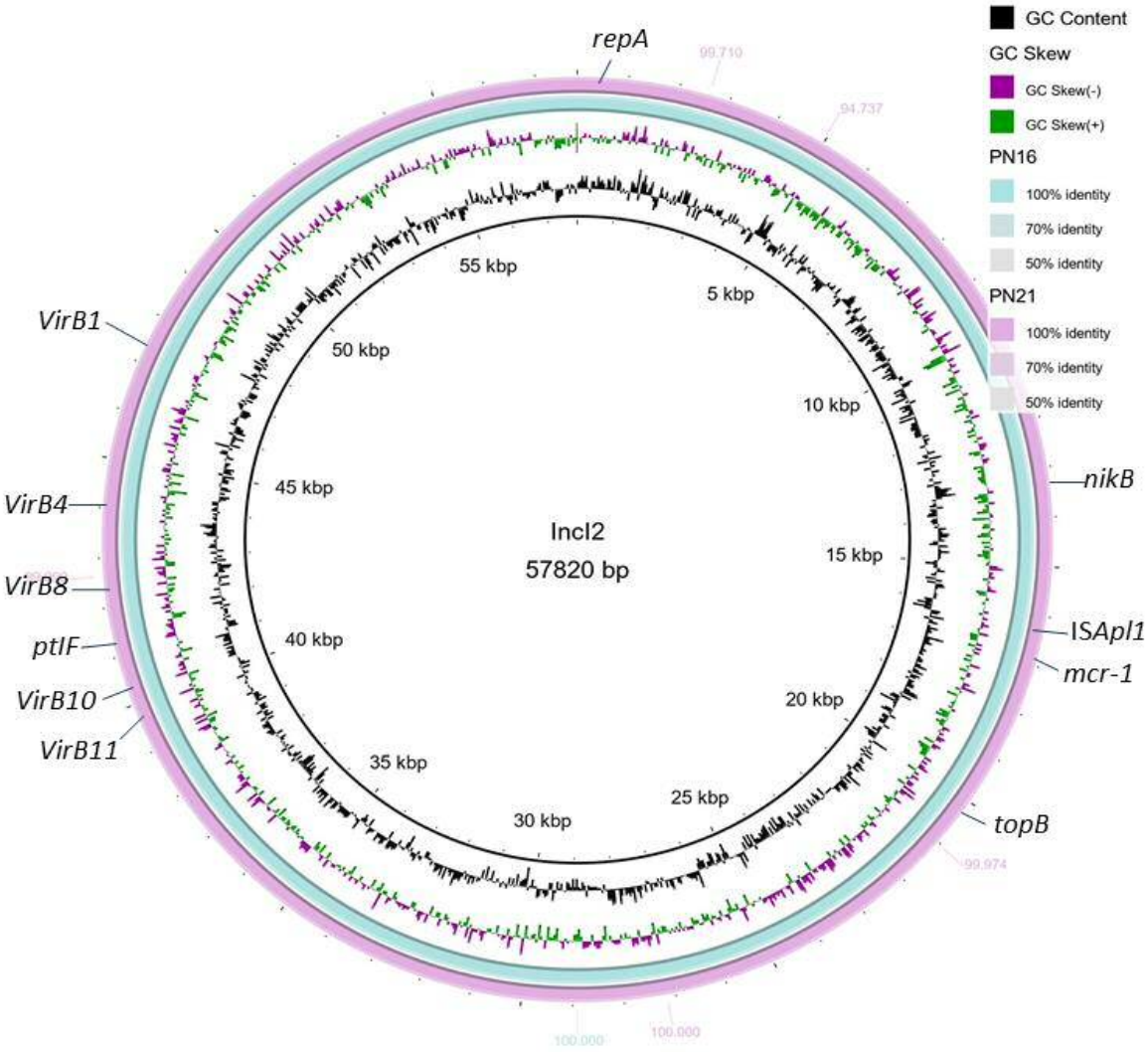
both side of *mcr-1*, and it has been reported that *mcr-1* is frequently transferred with IS*Apl1* composite transposon (Poirel, Kieffer, & Nordmann, 2017; Snesrud *et al.,* 2016). IS1294 is an IS91-like element, a rolling-cycle transposon (Tavakoli *et al.,* 2000), which suggests that IS1294 plays an important role in recombination of *mcr-1* segment.

Table 3.4 Characteristics of mcr-1 positive strains

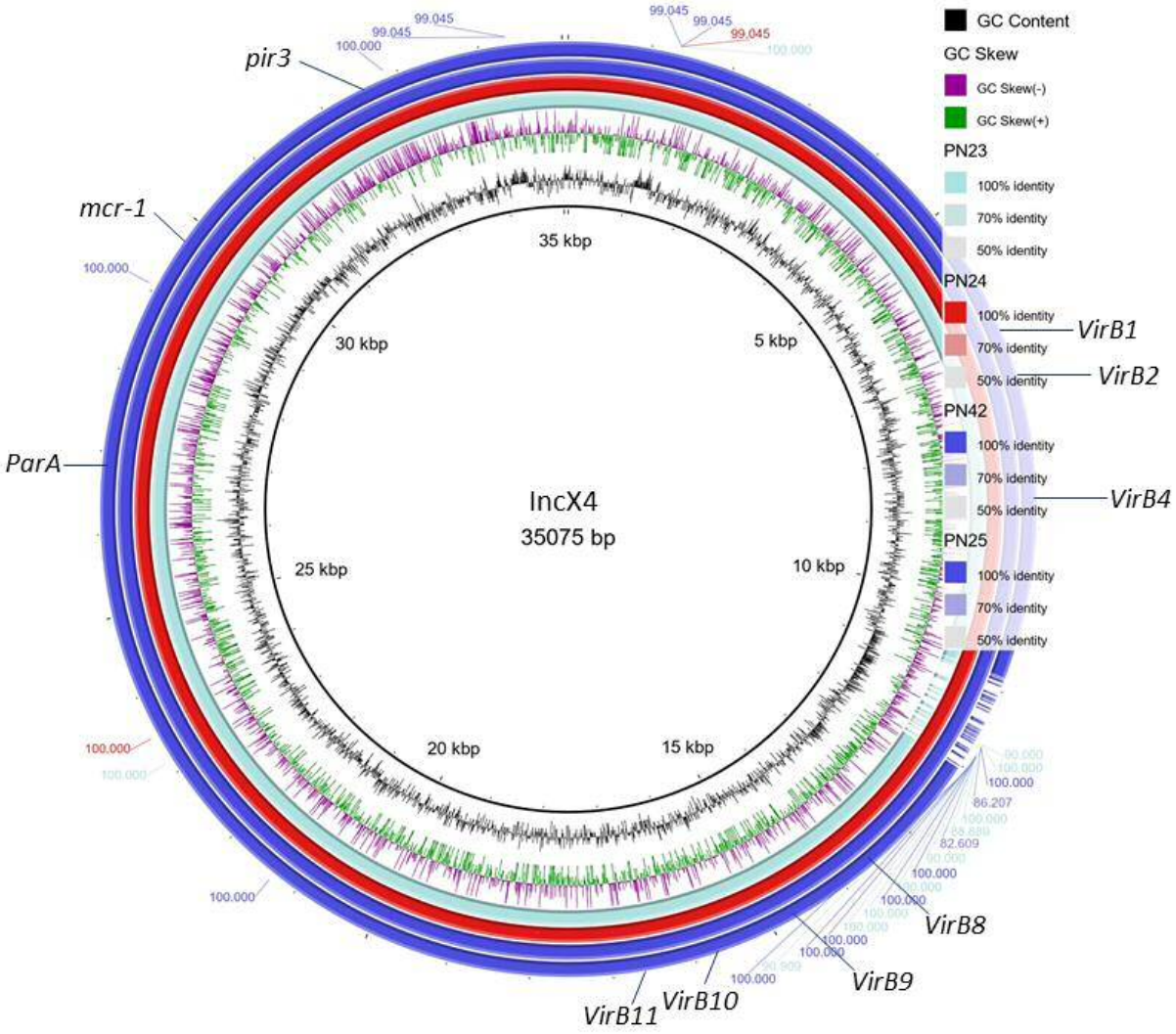
No.	MLST	*mcr-1* location	Plasmid** (type/size)	phylogenetic group
PN16	ST-2040	plasmid	IncI2 57,820 bp	A
PN21	Unknown (ST-24*)	plasmid	IncI2 ~ 60,989 bp	D
PN23	ST-1121	plasmid	IncX4 ~ 33,858bp	D
PN24	ST-7986	plasmid	IncX4 35,075 bp	D
PN25	ST-101	plasmid	IncX4 40,590 bp	D
PN42	ST-744	plasmid	IncX4 39,141 bp	A
PN43	ST-410	chromosome	NA	B2

*Sequence type for PN21 was determined using *E. coli*#2 configuration (Jaureguy *et al.,* 2008) .Other strains were identified following *E. coli*#1 configuration (Wirth *et al.,* 2006). **The plasmid size of PN21 and PN23 were estimated depend on illumina sequencing data. Others were on the basis of MinION long reads sequencing data.

A


repA
VirB1
VirB4
VirB8
ptlF
VirB10
VirB11
nikB
ISApl1
mcr-1
topB
IncI2
57820 bp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
50 kbp
55 kbp
GC Content
GC Skew
GC Skew(-)
GC Skew(+)
PN16
100% identity
70% identity
50% identity
PN21
100% identity
70% identity
50% identity

B


GC Content
GC Skew
GC Skew(-)
GC Skew(+)
PN23
100% identity
70% identity
50% identity
PN24
100% identity
70% identity
50% identity
PN42
100% identity
70% identity
50% identity
PN25
100% identity
70% identity
50% identity
pir3
mcr-1
ParA
VirB1
VirB2
VirB4
VirB8
VirB9
VirB10
VirB11
IncX4
35075 bp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp

C

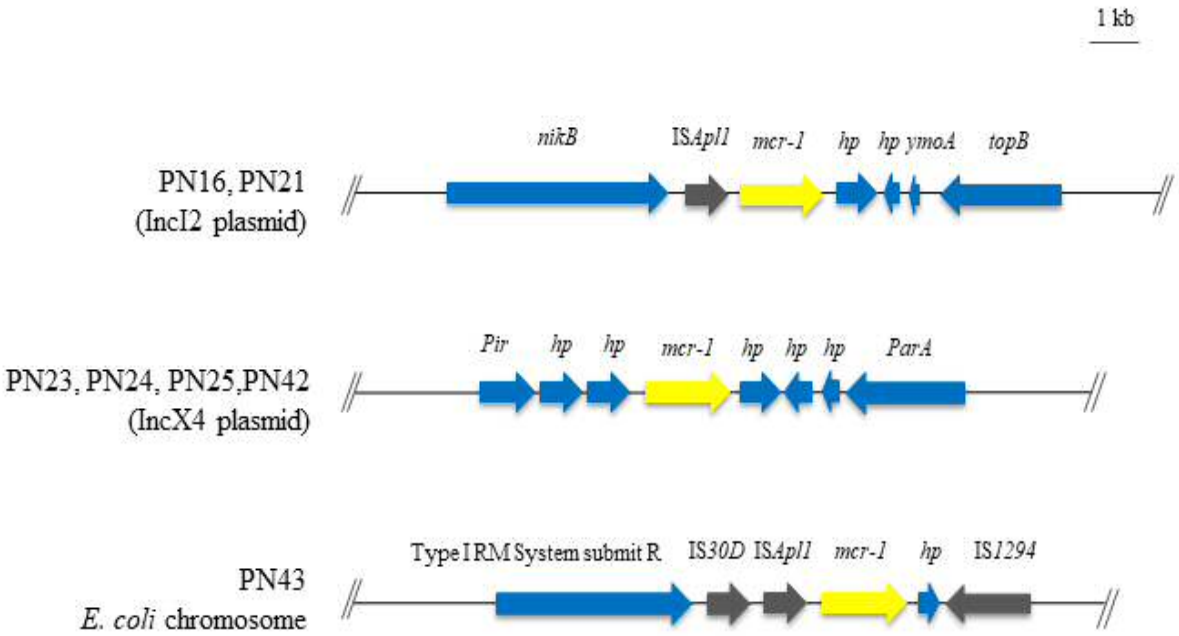


Figure 3.5 **Genetic contexts on plasmids and chromosome in *E. coli.* A.** Genetic structure of plasmid from PN16, PN21; **B.** Genetic structure of PN23, PN24, PN25 and PN42; **C.** Genetic environment of *mcr-1* on IncI2, IncX4 plasmids and chromosome. Arrows stand for open reading frame (ORFs) and the orientation of transcription. *hp*: hypothesis protein.

Sequencing analyses comparing the HLCRMs and their respective parental strains, showed no mutations in the *mcr-1* structural gene resulting in amino acid substitutions for MCR-1 and no other non-codon (silent) changes. The immediate genetic context of *mcr-1* in each of the isogenic sets was also analysed and no mutations in either the promoter or the broader adjacent genetic environment could be determined. Interestingly, point mutations were found in PN25 and PN42 in *pmrA* and *pmrB* genes, respectively. The genes *pmrB* and *pmrA* encode a two-component system (TCS) which is associated with chromosomally encoded colistin resistance although colistin resistance in *E. coli* through *pmrB* and *pmrA* changes are

considerably less frequent than in *Klebsiella* and *Pseudomonas* (S. Sun *et al.,* 2009). In PN25, the point mutation in *pmrA* results in the amino acid substitution R81S. This same substitution was previously reported in *E. coli* and has been inferred as a conserved position in the protein resulting in a raised colistin resistance (Alberto Quesada *et al.,* 2014). In PN42, a single nucleotide mutation in *pmrB* resulted in the substitution V161M. Similar substitution (V161G) was reported in *E. coli* previously and gave colistin MIC of 4 mg/L (Alberto Quesada *et al.,* 2014). Song Sun and colleagues found that the *pmrB* V161M substitution is associated with colistin resistance in *S. enterica Serovar Typhimurium* and resulted in 32-fold increase in colistin MICs (from 0.125 mg/L to 4 mg/L) (S. Sun *et al.,* 2009). However, these changes in *E. coli* are particularly rare (Alberto Quesada *et al.,* 2014).

Further sequencing analysis on single nucleotide polymorphisms (SNP) between HLCRMs and parental strains are ongoing. All SNPs recognised detected so far were listed in appendix. Interestingly, in PN25 HLCRM, except for the mutation in *pmrA*, several point mutations on *rfaY* gene (or *waaY*) resulted in amino acid substitutions T183Q, T184P, A185L, V186F, L187STOP were detected in HLCRM compared to its wild-type parental strain. *rfaY* gene is associated with the completion of the core region of bacterial lipopolysaccharide (LPS) and the attachment of *O*-antigen (Klena, Pradel, & Schnaitman, 1992; Z. Wang, Wang, Ren, Li, & Wang, 2015). However, no proof was found that mutation on *rfaY* gene was related to colistin resistance. Thus further experiments such as mutation detection in *rfaY* and *pmrA* genes, and *mcr-1* plasmid knockout are needed to understand the effect of *rfaY* gene on colistin resistance and the main cause of high MIC (128 mg/L) in PN25 HLCRM.

3.1.6 Discussion

In this study, samples from a variety of resources including human and animal and collected from Northern Thailand where colistin resistance in *E. coli* is becoming a national crisis (A. O. Olaitan *et al.,* 2016; Srijan *et al.,* 2018). Further analysis of additional isolates from Thailand (Uttapoln, personal communication) support my original findings and confirm that that *mcr-1* gene is widely disseminated in animals, humans and the environment in northern Thailand.

Similarly, to previous studies, many *mcr-1* positive strains also harbour other resistance genes (Schwarz & Johnson, 2016). For example, all of my isolates except for PN23, contained $bla_{\text{CTX-M}}$ or/and bla_{TEM}. CTX-M enzymes, which belong to extended-spectrum ß-lactamases (ESBLs), contribute to bacterial resistance to third generation cephalosporins, such as cefotaxime and ceftazidime (see appendix) – both commonly used to treat severe hospital infections throughout Southeast Asia (Lestari, Severin, & Verbrugh, 2012; Suwantarat & Carroll, 2016). As colistin is now recognised as a last resort antibiotic for infections caused by MDR Gram-negative bacteria, the co-existence of *mcr-1* and ESBL genes may reduce treatment options for MDR Gram-negative pathogens such as *E. coli* (Schwarz & Johnson, 2016). Moreover, we found that *mcr-1* positive isolates belonged to the global sequence types commonly associated with additional resistance mechanism such as KPC, OXA, NDM and *rmt*C/D **(Table 3.4)** (Deng *et al.,* 2015; Falgenhauer, Waezsada, Gwozdzinski, *et al.,* 2016; Hansen *et al.,* 2016; Kim *et al.,* 2017; Mavroidi *et al.,* 2012; Mushtaq *et al.,* 2011). Consequently, pan-drug resistance (PDR) in these MLST groups could potentially rapidly develop.

According to the S1-PFGE results, the size of *mcr-1* plasmids did not show noticeable changes (***Figure 3.4***). Combined with the analysis of WGS results, that no mutations were found in the contigs covering the *mcr-1* gene, their promoters or adjacent regions between Day0 and HLCRMs, these observations may indicate that the *mcr-1* gene and its immediate genetic context is very stable.

Although all of the seven isolates were *mcr-1* positive, as they were disparate wild-type strains with complicated genetic backgrounds, the differences in the generation of HLCRMs is, understandably, also likely to be different. In two of the HLCRMs, PN21 and PN25, mutants showed a 11- and 3-fold increase in *mcr*-1 expression. No considerable increase in *mcr*-1expression or *mcr-1* copy number was detected in the other HLCRMs. Interestingly, In PN25 and PN42, amino acid mutation in PmrA/B was detected in their respective HLCRMs. PN25 had R81-S substitution in PmrA, while PN42 had V161M in PmrB, and both mutations have been previously reported in *E.coli* and *S. enterica* (Alberto Quesada *et al.,* 2014; S. Sun *et al.,* 2009). R81 is in the phosphate acceptor domain of PmrA, the mutation leads to a totally conserved position of the protein, and V161 is in kinase domain of PmrB. Both substitutions may influence the phosphate transfer resulting in colistin resistance in *E. coli* (S. Sun *et al.,* 2009). However, for strains PN16, PN23, PN24 as well as PN43, the exact mechanism of increase of colistin resistance hasn't been elucidated. Combined with the results for the stability experiment, these data suggest the initiation of mechanisms for temporary acquisition of high colistin resistance (except for PN16 and PN24 HLCRMs with low MICs of 16-32 mg/L).

3.2 Reduction in bacterial fitness and virulence of HLCRMs

3.2.1 Growth rate

After the 14-day passaging, all mutants showed an increase in colistin resistance, some were particularly high with colistin MICs up to 256 mg/L. To examine whether this high resistance to colistin had an effect on bacterial physiology, a series of growth experiments were undertaken on both mutants and the original wild-type strains.

To examine the growth of the original strains and respective mutants, growth curves of Day0, Day3, Day7, Day11 and Day14 for each strain were evaluated in triplicate by testing the OD value at 492 nm for 8 hours. For PN16, the Day14 mutant was too weak to recover from -80ºC; and therefore, the Day13 HLCRM was chosen as its respective "end-point" mutant. As shown in ***Figure 3.6***, all the Day14 mutants (except for PN16) demonstrated a significant lower rate of growth compared with their respective wild-type isolates. After 8 hours, the OD value of HLCRMs was less than half of wild-type isolates ($p < 0.001$).

Although all of the isolates showed an overall gradual decrease in growth rate, the rate varied between each HLCRMs（shown in ***Figure 3.6***). For example, PN23 and PN43 HLCRMs showed a gradually lower rate of growth over time compared with their parental strains. For PN21 and PN25, the growth rate dropped on Day11, while no significant decrease was found in bacterial growth of PN16 or its corresponding HLCRMs. PN42 showed an interesting rate in growth - on Day3 it dropped; however, on Day7 it recovered and then dropped again on Day11.

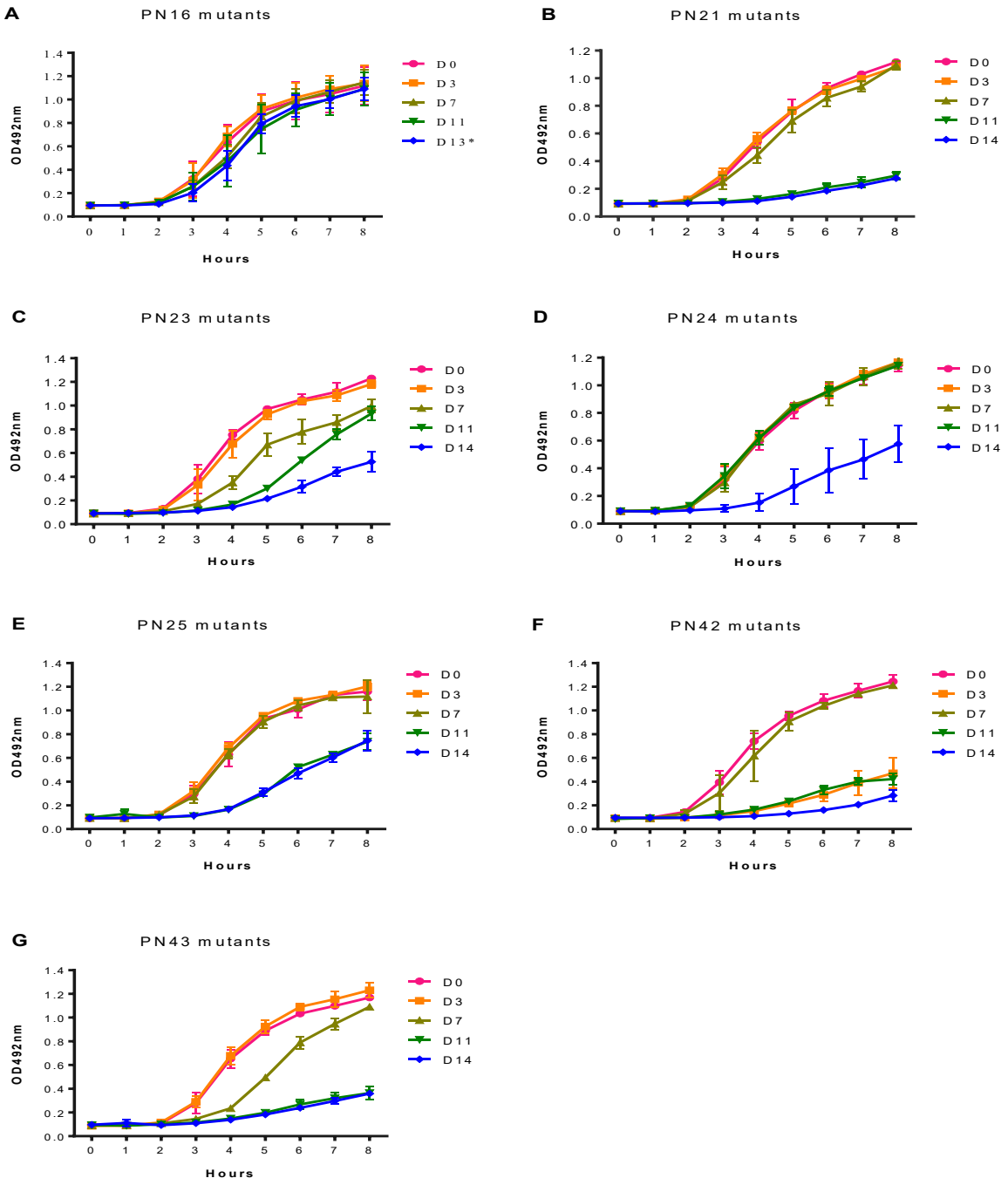


***Figure 3.6* Growth rate collected at OD492nm in 8 hours. A-G.** Growth rates for wild-type (Day0) isolates as well as Day3, 7, 11, 14 mutants for each strain were examined in colistin-free medium for 8 hours. The mean values in triplicate were shown. Error bars represented the SD. *PN16 Day14 mutant was unable to grow, so Day13 mutant was used for tests.

3.2.2 Competition assay

in vitro fitness cost was further evaluated by competition assay between wild-type strains and HLCRMs. *E. coli* DH5-α labelled by GFP was used as a control. Both wild-type isolates and mutants were mixed with GFP-labelled DH5α at approximately 50:50 and were incubated at 37ºC at 220rpm for 6 hours. The exact ratio at starting and ending points was measured by flow cytometry (***Figure 3.7***, statistical analysis is presented in **Table 3.5**). Relative fitness was calculated by relative ratio between each wild-type strain and HLCRM. Statistical analysis was performed using a paired t-test. *= ($p < 0.05$), **= ($p < 0.01$), ***= ($p < 0.001$).

Except for PN16, all HLCRMs were associated with a significant fitness cost compared with their respective wild-type strains. After 6 hours' incubation, the HLCRMs of PN21, PN23, PN24, PN25 and PN42, showed relative fitness of 0.4-0.6 (p =**) to their respective parental strain. HLCRM PN43 possessed a relative fitness of 0.78 (p =**) to its respective parent strain, yet HLCRM PN16 had a fitness rate similar to its original wild-type strain (with a relative fitness of 0.94, $p > 0.05$). Interestingly, PN21 and PN23 mutants, which showed 11- and 3-fold increase in *mcr-1* expression level, were associated with the highest fitness cost (relative fitness 0.41, p =**).

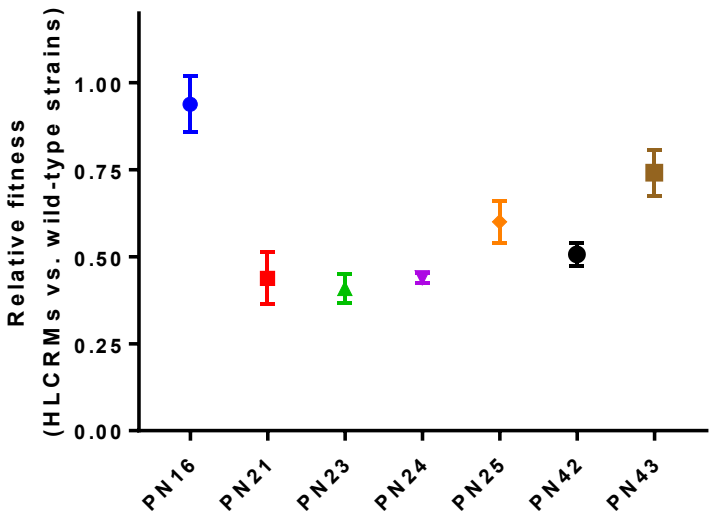


Figure 3.7 **Relative fitness of each HLCRM and wild-type strain.** Relative fitness was calculated by relative ratio between each wild-type strain and HLCRM. Means of 6 repeats were shown. Error bars represented SD.

Table 3.5 Statistic analysis of relative fitness

Relative fitness	**repeats**	**mean**	**SD**	**propagated errors**	***p*-value**
PN16-D13	6	0.9380	0.0809	0.0875	0.132
PN21-D14	6	0.4114	0.0293	0.0816	0.0022**
PN23-D14	6	0.4083	0.0385	0.1404	0.0022**
PN24-D14	6	0.4382	0.0133	0.0445	0.0022**
PN25-D14	6	0.6047	0.0557	0.1196	0.0022**
PN42-D14	6	0.5169	0.0309	0.0650	0.0022**
PN43-D14	6	0.7779	0.0534	0.1003	0.0022**

3.2.3 Galleria pathogenicity model

To test the potential decrease of pathogenic potential following generation of HLCRMs, *G. mellonella* were infected with both HLCRMs and their corresponding parental strains. Larvae of the wax moth (*G. mellonella*) are used as a reliable model to measure the pathogenesis of several pathogens including *E. coli* (Jønsson, Struve, Jenssen, & Krogfelt, 2016). Larvae were infected with 10^3 to 10^7 CFU/ml bacteria and were grown in 37°C for 72h. Death of Galleria was checked every 24 hours and survival rates for each strain are shown in ***Figure 3.9***.

In every case, the wild-type isolates had a higher killing rate than their respective HLCRMs (***Figure 3.8***). Comparing the Galleria killing rate for Day 14 with Day 0; HLCRMs of PN16, PN21 and PN23 showed a significantly reduced ability to kill larvae compared to their parent strains which is indicative of lower pathogenicity/virulence (pPN16=0.0056**, pPN21=0.0029**, pPN23=0.0003***). For strains HLCRM PN42 and PN43, although decrease of larvae killing was observed between Day 0 and Day 14, it was not statistically significant ($p > 0.05$).

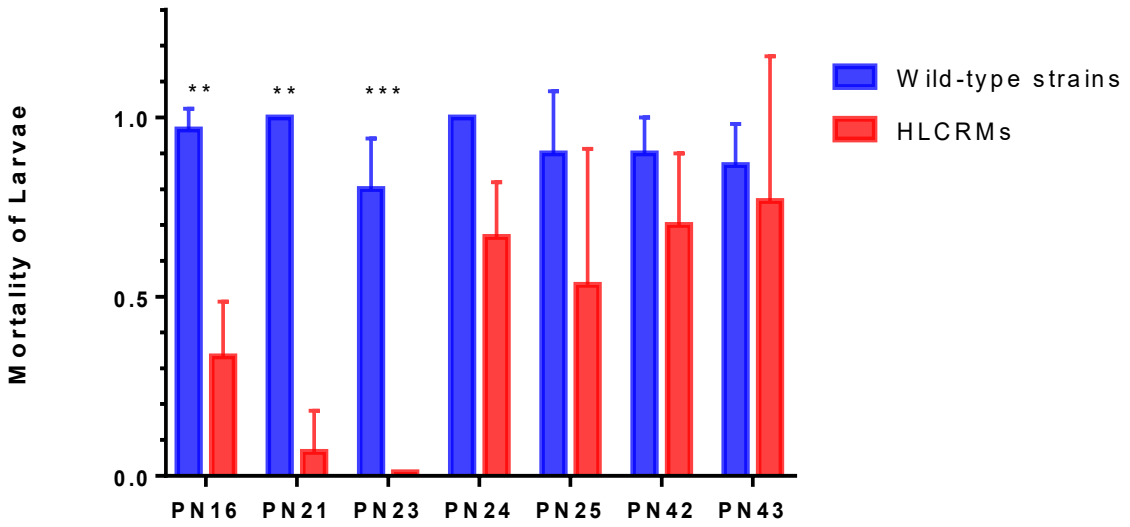


Figure 3.8 **Killing of Galleria by HLCRMs and their respective *E. coli* parental isolates expressed as mortality fractions.** Mortality of larvae after being injected bacteria for 72 hours. Ten larvae were tested in each treatment and means of 3 independent repeats were shown with SD. Statistical significance was calculated by t-test. *= ($p < 0.05$), **= ($p < 0.01$), ***= ($p < 0.001$). Concentration of bacteria injected: PN16, PN23, PN24, PN42 and PN43 and their corresponding mutants, 10^7 CFU/mL; PN21 and PN25 and their mutants, 10^6 CFU/mL.

Furthermore, to better understand the trend of decrease in virulence, mutants of Day3, Day7 and Day11 were also chosen to infect Galleria (***Figure 3.9***). Generally, the ability of killing larvae reduced by time of passage; for example, for HLCRM PN21 the survival rate of Galleria increased from 0 to 0.9 by Day0, Day3, Day7, Day11 and Day14. Similar trends were detected in strains PN16, PN23, PN24, and PN25. Thus, the HLCRMs show reduced ability to kill larvae which is indicative of lower virulence.

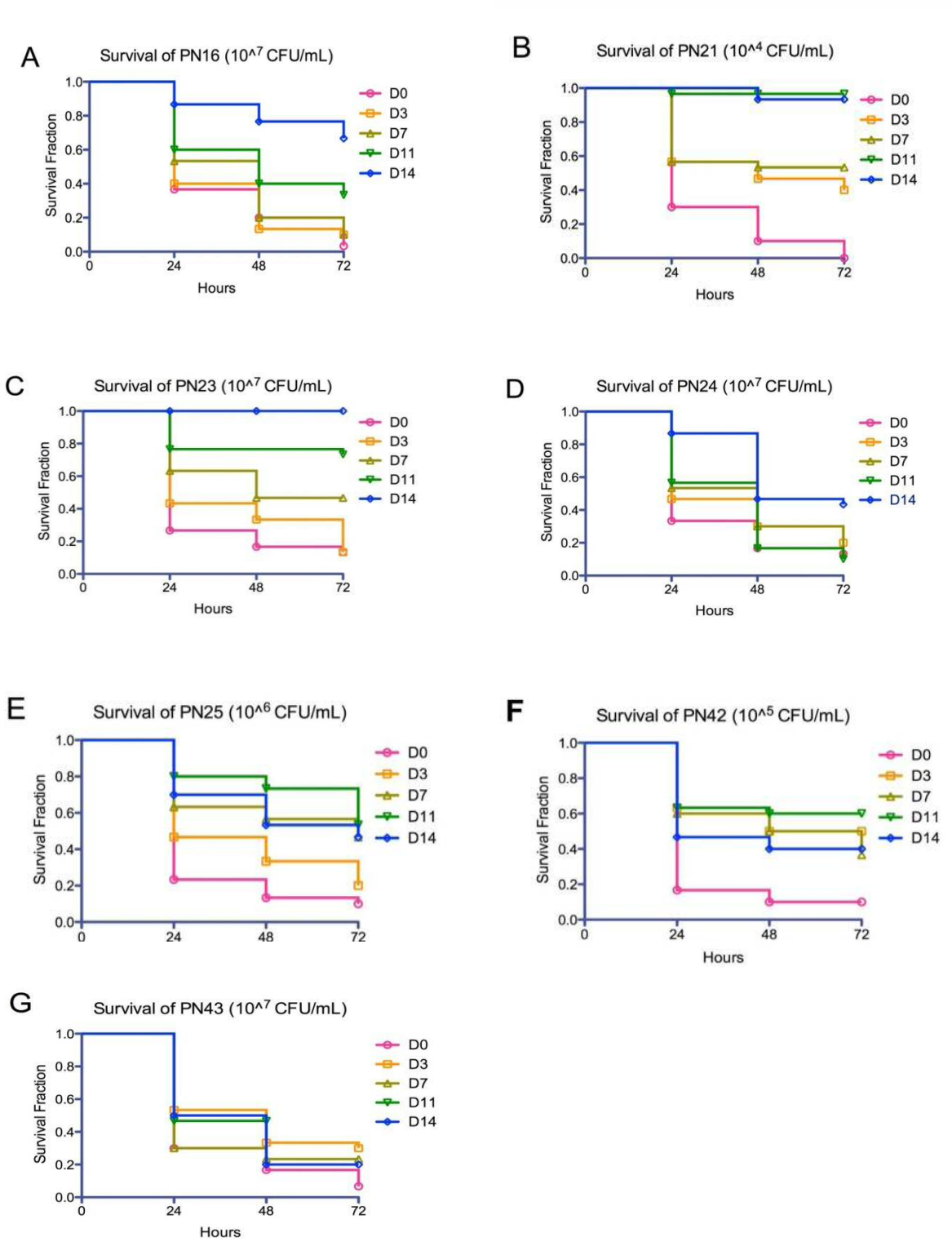
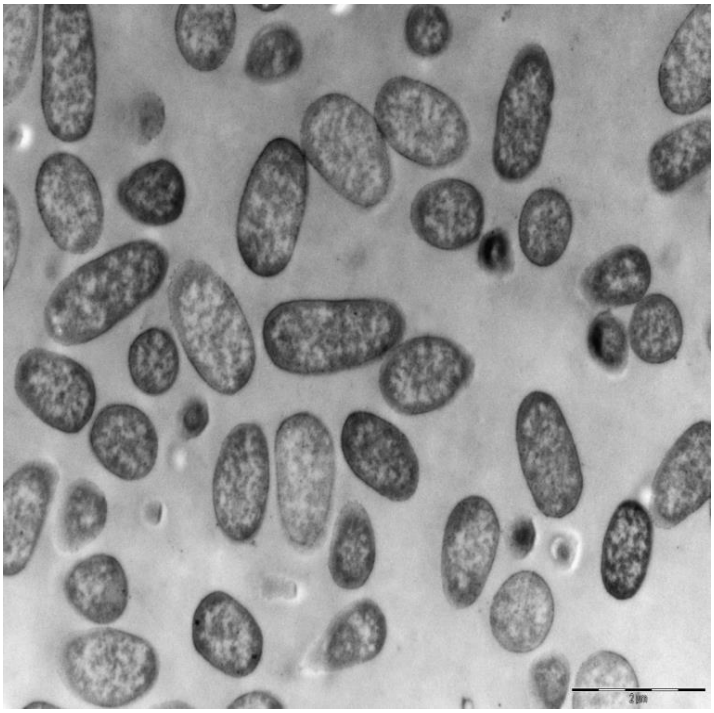


Figure 3.9 **Galleria survival rate for original strain and their respective mutants. A-G.** demonstrated the percentage of *G. mellonella* survival (n=30) for 72 hours after being treated with wild-type (Day0) strains, as well as Day3, 7, 11 and 14 mutants.

3.2.4 TEM on parent and HLCRM *E. coli*

Since HLCRM of PN21 showed the highest increase of *mcr-1* expression level (-11 fold increase), to further understand the effect of *mcr-1* expression on LPS and cell outer-membrane, transmission electron microscopy (TEM) was undertaken on PN21 and its respective mutant to examine the bacterial morphology. However, as shown in ***Figure 3.10***, no significant difference was observed at the cell outer-membrane between PN21 and its respective HLCRM. Moreover, no obvious cellular degradation of membrane was observed in HLCRMs. Both parental strains and HLCRMs possessed an integrated membrane with highly uniform electron density in the cytoplasmic region typical of normal cellular structure.

A. PN21



B. PN21-HLCRM

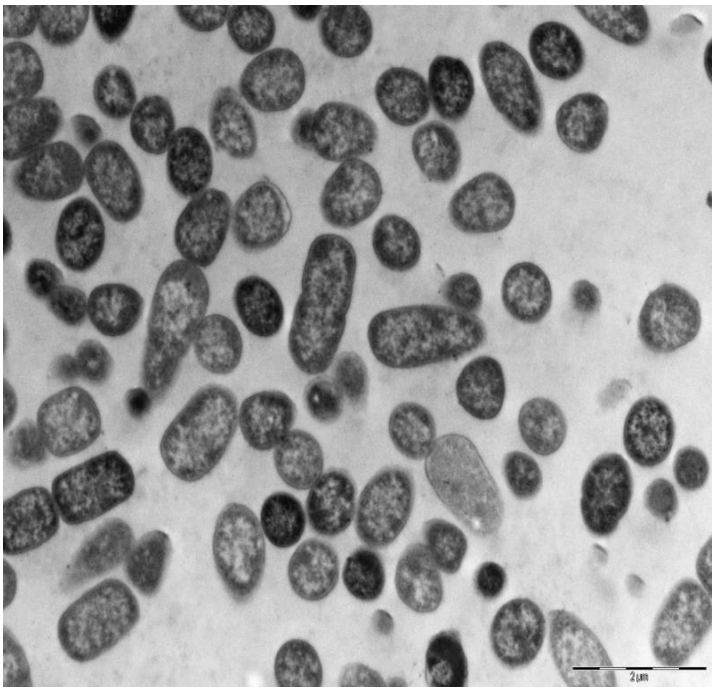


Figure 3.10 **Cell morphology of bacterial outer-membrane for PN21 and its HLCRM.**

3.2.5 Discussion

In section 3.1, HLCRMs were acquired after *in vitro* development at colistin resistance. All of the HLCRMs showed higher resistance to colistin than their respective wild-type strains, some of which possessed very high levels of resistance to colistin (MIC of 256 mg/L). In this section, a series of experiments were undertaken to measure the fitness and virulence of wild-type strains (Day 0) and their respective HLCRMs. For growth rates and Galleria models, Day3, Day7, Day11 mutants were chosen to examine their trend in fitness and virulence.

In *E. coli*, *mcr-1* is the main factor that leads to colistin resistance (Shen *et al.,* 2016). According to a previous report, the acquisition of *mcr-1* plasmids is not associated with a considerable fitness cost (Y. Zhang *et al.,* 2017). It can also be hypothesized that the global increase in colistin use, as well as the lack of fitness cost for acquisition of *mcr-1*, may lead to the outbreak of high-level colistin resistance in *E. coli* and the rapid spread throughout the world (Shen *et al.,* 2016; Zhou *et al.,* 2018). However, due to the fact that *mcr-1* always mediates low resistance to colistin in *E. coli* (Liu *et al.,* 2016), studying the effect of high colistin resistant *E. coli* in bacterial fitness is helpful to better understand the relationship of *mcr-1* and its host, and to predict future trends of *mcr-1* mediated resistance in clinical and agricultural sectors.

There results show that most HLCRMs possess a significant loss in fitness. Compared with parental strains, HLCRMs (except for PN16) showed slower growth in colistin free medium. Data from competition assays further support that HLCRMs are less competitive than their wild-type isolates (relative fitness 0.41-0.78, p=0.002**). The high fitness cost of HLCRMs

may limit the spread of *E. coli* with high colistin resistance in clinical and agricultural environments, and could explain the fact that *mcr-1* is often associated with a low level of resistance to colistin (Liu *et al.,* 2016). Loss of *in vitro* fitness cost was observed in the PN16 mutant – the Day13 mutant was used because of the lack of viability of the Day14 mutant from -80°C and had a relatively low resistance (MIC 32mg/L) compared with other HLCRMs.

The fact that HLCRMs are associated with reduction in fitness has been reported by our recent study in *Nature Communications*, where *E. coli* Top10, Top10 (pBAD) and Top10 (*mcr-1*/pBAD without L-arabinose induction) were passaged with colistin in the same way as the 7 wild-type strains in this study (Yang *et al.,* 2017). After 14 days, the colistin MIC of TOP10 (*mcr-1*/pBAD) HLCRMs increased from 0.5 mg/L to 32 mg/L, while TOP10 and TOP10 (pBAD) stayed susceptible（with MICs to colistin of 0.5 mg/L). The TOP10 (*mcr-1*/pBAD) mutant was correlated with a decrease in fitness compared with *E. coli* TOP10 and TOP10 (pBAD) (Yang *et al.,* 2017). These data indicate that HLCRM can be generated from laboratory-based isolates after acquisition of *mcr-1*, and in most cases, this comes with a price of lower bacterial fitness (Yang *et al.,* 2017).

Furthermore, a decrease in virulence by the HLCRMs determined *in vivo* Galleria models further supports this idea. In every case, survival rates of Galleria after being challenged with HLCRMs was higher than their respective wild-type parental strains, even for PN16. However, the decrease in PN24, PN25, PN42 and PN43 were insignificant ($p > 0.05$). Interestingly, PN24 and PN25 were assigned phylogenetic group D, PN43 was identified in group B2. *E. coli* strains can be mainly assigned into four phylogenetic groups: A, B1, B2, and D, and virulent

strains mainly belong to groups B2 and D, as these groups are normally associated with more virulence properties such as biofilm production and hemolysin secretion (Chakraborty *et al.,* 2015; Soto *et al.,* 2007). The virulent phylotypes may have an influence on the insignificant decrease of virulence in PN24, PN25, PN43 HLCRMs as these strains belong to more virulent groups. In PN42, the mutant in Day 14 showed higher mortality of larvae compared to Day 3, 7 and 11 mutants (***Figure 3.9*** **F**), which may indicate a "recovery" in virulence in Day14 HLCRM. However, the mechanism of this increase in virulence remains unclear.

Colistin resistance by chromosomal mutation is often associated with fitness cost and decrease in virulence in species, especially in *A. baumannii* (Beceiro *et al.,* 2014; Da Silva & Domingues, 2017). There are two mechanisms leading to colistin resistance in *A. baumannii*, one is by mutations in PmrA/B TCS that modifies LPS resulting in less affinity to colistin and hence resistance (Adams *et al.,* 2009), and the other is by mutations in *lpx* genes resulting in completely loss of LPS (Moffatt *et al.,* 2010). Both MCR-1 and mutations in PmrA/B (in *A. baumannii*) lead to resistance to colistin by the addition of phosphoethanolamine (PEA/ pEtN) to lipid A (Baron, Hadjadj, Rolain, & Olaitan, 2016; Gao *et al.,* 2016). Induced colistin resistant *A. baumannii* is often associated with a decrease in fitness and virulence (Da Silva & Domingues, 2017). López-Rojas *et al.* measured the fitness and virulence on ATCC19606 and colistin resistant mutant RC64. RC64 showed a 32-fold increase to 64 mg/L in MIC compared with ATCC19606 (López-Rojas *et al.,* 2011). The resistant mutant showed reduced fitness (with competition index of 0.016) and decreased virulence with a higher lethal dose (6.9 log colony-forming units vs. 4.9 log units of ATCC19606) (López-Rojas *et al.,* 2011). Beceiro *et al.*

compared the fitness and virulence ATCC19606 and its laboratory-selected mutant (*prmB* mutation). The colistin-resistant mutant showed lower *in vitro* growth (competition index of 0.35), and a slight decrease in virulence with a higher reduction in *C. elegans* brood size (Beceiro *et al.,* 2014). However, in the clinical sector, the situation is more complicated since resistant bacteria from clinical settings normally have an undefined genetic basis for resistance and fitness (Andersson, 2006; Holmes *et al.,* 2016). Loss of a fitness cost in colistin resistant bacteria has been reported (Da Silva & Domingues, 2017). For example, Durante-Mangoni *et al.,* collected both colistin susceptible and resistant *A. baumannii* during a long-term colistin therapy from an immunocompromised patient (Durante-Mangoni *et al.,* 2015). The lack of fitness cost and decrease in virulence were associated with colistin resistance due to P233S mutation in PmrB (Durante-Mangoni *et al.,* 2015). So far, it is unknown whether *mcr-1* is capable to induce very high colistin resistance in *E. coli* under clinical or agricultural pressures, or its effect on bacterial fitness. However, this study may indicate the possibility of emergence of *mcr-1*-mediated high colistin resistance, and the reversibility as a result of biological cost in HLCRMs.

Chapter 4: General Discussion

4.1 Aims of this study

Acquisition of antibiotic resistance has been recognised as a relevant problem to human health (Hernando-Amado *et al.,* 2017). Bacteria can develop resistance through two different ways: vertical evolution by mutations where antibiotic tolerant genotypes are selected and are passed to the next generation (***Figure 4.1*** **A**); or horizontal evolution where bacteria acquire resistance genes via horizontal transfer, including conjugation, transduction and transformation (***Figure 4.1*** **B**) (Sommer *et al.,* 2017).

Both of these mechanisms are important for the development of antibiotic resistance, especially horizontal transfer that contributes to the spread of resistant genes to sensitive organisms (Normark & Normark, 2002; Sommer *et al.,* 2017). For example, the "superbugs" mediated by bla_{NDM} have been spread to almost everywhere since its first detection in 2008 (C.-R. Lee *et al.,* 2016). The bla_{NDM} gene is mostly carried by plasmids and can be transmitted among strains, species and genus (Khong *et al.,* 2016). The dissemination of these superbugs has prompted widespread concern as some organisms are resistant to almost all antibiotics (World Health Organization, 2017b).

Colistin (Polymyxin E) is now listed as a last resort antibiotic to treat serious infections caused by MDR Gram-negative bacteria by WHO (World Health Organization, 2017a). Prior to *mcr-1*, resistance to colistin had been reported but was chromosomally mediated, and therefore, in terms of dissemination is limited (Liu *et al.,* 2016). The discovery of plasmid-mediated colistin resistance via *mcr-1*, caused worldwide concern as it potentially heralded the end of polymyxin

antibiotics (Liu *et al.,* 2016; Walsh & Wu, 2016). Moreover, *mcr-1* positive *E. coli* (MCRPEC) isolates were detected in a collection from 1987, which suggests that its appearance is earlier than anticipated (Shen *et al.,* 2016). This may explain the reason why MRCPEC had quickly been reported all around the world after the first publication of MCRPEC (Schwarz & Johnson, 2016).

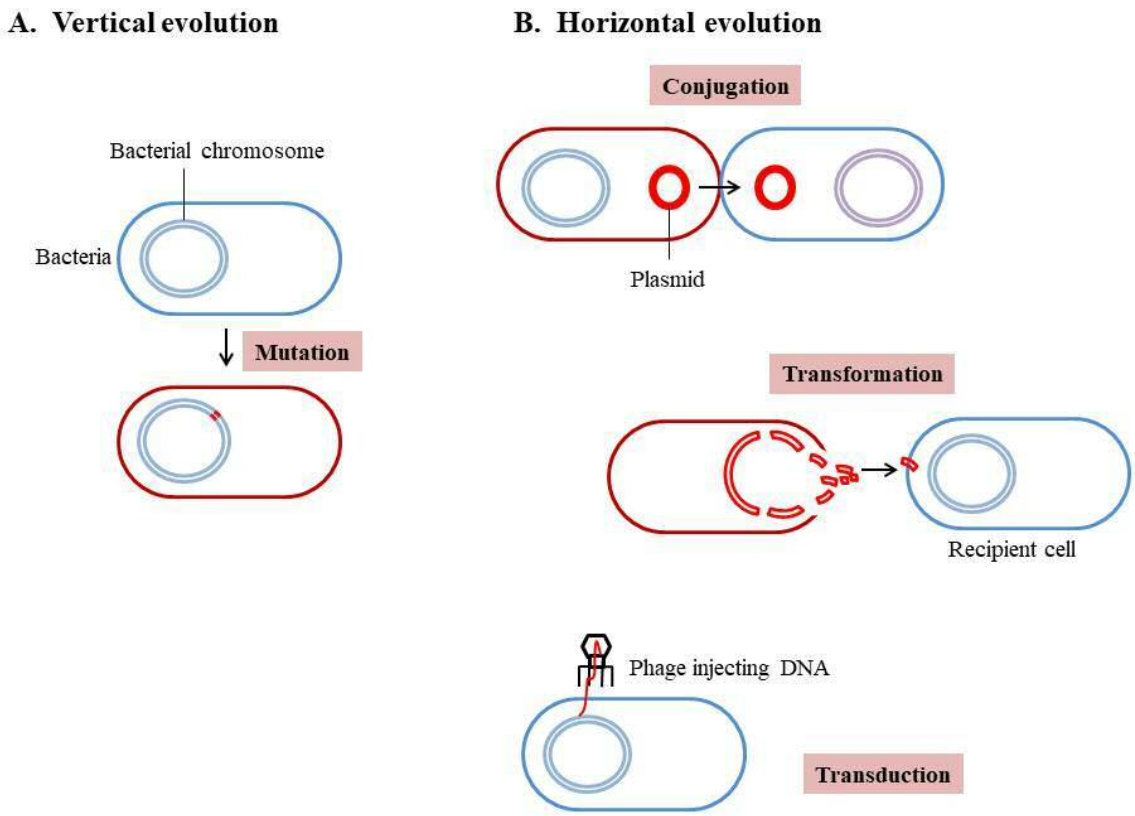


Figure 4.1 **Mechanisms of antibiotic resistance acquisition. A.** Bacteria gain resistance by vertical evolution: mutation(s) within the bacterial genome. Blue cells represent antibiotic-sensitive bacteria, and red cells represent resistant organisms; **B.** Acquisition of antibiotic resistance by horizontal evolution: horizontal gene transfer through conjugation, natural transformation and phage transduction (Sommer *et al.,* 2017).

It is generally accepted that antibiotic resistance is associated with a cost of fitness to the bacterial cell. This cost includes decreased bacterial growth rate, survival rate, transmission of resistance rate and disease-causing properties (Sommer *et al.,* 2017). Colistin resistance has been reported with a fitness cost in other species such as *A. baumannii* and *K. pneumoniae* due to chromosomal mutations in PmrA/B or PhoP/Q TCS (Choi & Ko, 2015; López-Rojas *et al.,* 2011). In *A. baumannii*, colistin resistance due to mutations in *lpxA*, *lpxC*, and *lpxD* genes leads to a complete loss of LPS and is associated with a high fitness cost (Moffatt *et al.,* 2010). However, few studies have studied the fitness cost of *mcr-1* mediated colistin resistance, especially when *mcr-1* can only cause comparative low-level colistin resistance (Liu *et al.,* 2016; Zhang *et al.,* 2017).

According to previous studies which are somewhat limited, fitness cost studies on acquisition of *mcr-1* positive plasmids in *E. coli*, suggest that acquisition of *mcr-1* is either associated with no reduction or a slight decrease in bacterial fitness (Kong *et al.,* 2017; Liu *et al.,* 2016; Yang *et al.,* 2017; Y. Zhang *et al.,* 2017; Zhou *et al.,* 2018). Fitness cost associated with the acquisition of antibiotic resistance might reduce the spread of resistance in an antibiotic-free environment as it helps sensitive bacteria outcompete resistant organisms (Hernando-Amado *et al.,* 2017). No cost resistance may make resistance less reversible (Sommer *et al.,* 2017). Thus the lack of fitness cost of acquisition of *mcr-1* may aid the maintenance of *mcr-1* plasmid under colistin-free conditions (Hernando-Amado *et al.,* 2017).

As *mcr-1* expression in *E. coli* is attenuated to mitigate cellular toxicity, the colistin MICs for MCRPEC are invariably 4-16mg/L (Liu *et al.,* 2016). Therefore, the aim of this study was to

acquire high-level colistin resistant MCRPEC mutants (termed HLCRMs), and to assess whether high-level colistin resistance is associated with a fitness burden and subsequent reduction in virulence. To acquire HLCRMs, seven non-clonal wild-type MCRPEC isolates were grown under increasing concentrations of colistin for 14 days (Methods and materials 2.4). I then examined the fitness and virulence for both wild-type and HLCRMs, including growth rate, competition assay and their pathogenic potential in *in vivo* Galleria models. Additionally, the stability of HLCRMs was examined by passaging in the absence of colistin for 14 days and reversion frequency calculated. S1-PFGE and WGS analysis were performed to identify DNA differences between parental strains and HLCRMs.

4.2 Creation and Assessment of HLCRMs

After being treated with gradually increasing concentrations of colistin, the level of colistin resistance for each wild-type MCRPEC strain showed varying degrees (4- to 64-fold increase) of increased resistance. *E. coli* Top10 was used as negative control being treated in an identical manner; however, after 14 days, no considerable increase in colistin resistance was observed (data not shown).

In MCRPEC, the level of colistin resistance can be readily increased under the pressure of colistin within a short-time frame (14 days). In contrast, when treated in an identical manner, *mcr-1* negative *E. coli* invariably maintained their susceptible phenotypes. Unlike *K. pneumoniae* or *P. aeruginosa*, *E. coli* rarely are able to mediate resistance to colistin via chromosomal mutations – this was the underpinning reason why *mcr-1* was first discovered (Liu *et al.,* 2016; Urban, Tiruvury, Mariano, Colon-Urban, & Rahal, 2011). Therefore, the fact that *mcr-1* can enhance *E. coli* in gaining higher levels of resistance to colistin is perhaps not expected. Normally, with MCRPEC isolates, the colistin MICs range from 4-16mg/ml and occasionally reaches 32mg/ml (Huang *et al.,* 2017; Liu *et al.,* 2016). However, it is clearly possible that with the presence of *mcr-1* high-levels of colistin resistance can be obtained.

In 2/7 HLCRMs, PN21 and PN25, the *mcr-1* expression level was increased 11- and 3- fold, respectively, compared with the wild-type parental strains, which may support, in part, that the expression of *mcr-1* attributes to high-level colistin resistance. However, in the other five mutants, the increase in colistin resistance cannot be attributed to *mcr-1* expression or the *mcr-1*

copy number (Results 3.1.2). This may indicate that although *mcr-1* expression is directly associated with *E. coli* colistin resistance also insusceptibility, other mechanisms must contribute to the development of HLCRMs. Interestingly, HLCRMs created from PN25 and PN42, showed chromosomal mutations in the PmrA/B TCS. In the case of HLCMR PN25, an R81S mutation in PmrA was demonstrated, whilst in the PN42 HLCRM, a V161M mutation in PmrB was evident. Both mutations have been previously associated with colistin resistance in *E. coli* (Alberto Quesada *et al.,* 2014); however, the V161M substitution in PmrB from PN42 HLCRM did not exactly match the variant (V161G) previously reported in *E. coli* (Alberto Quesada *et al.,* 2014). Both substitutions might mediate colistin resistance by affecting phosphate transfer between the PmrA/B TCS as R81 is located in the phosphate receiver domain of PmrA and V161 is located in the kinase domain of PmrB (S. Sun *et al.,* 2009). However, it remains unknown whether these colistin resistance strains in Quesada's study harboured *mcr-1*, as *mcr-1* was reported one year later in 2015 (Quesada *et al.,* 2014, Liu *et al.,* 2016). Also it is not clear whether the existence of *mcr-1* may have an effect on enhancing mutations in the PmrA/B TCS under the selective pressure of colistin. It may indicate that the presence of *mcr-1* affords a degree of protection and cell survival enabling these mutations to occur and be selected by the presence of colistin.

Furthermore, some of the HLCRMs showed the ability of keeping the high-level colistin resistance even without presence of the drug. PN25 and PN42 HLCRMs, in which mutations in the PmrA/B TCS were identified (HLCRM of PN25 also showed 3- fold increase in *mcr-1* expression), did not demonstrate decrease in bacterial growth or considerable reduction

(one-fold decrease) in colistin MICs for 14 days. Therefore, it can be hypothesized that these mutations are indeed stable. For HLCRM PN21, no significant decrease in MIC was detected even though the growth rate of PN21 reduced by 50% after passaging. In HLCRMs PN16, PN23, PN24 and PN43, the exact mechanisms of increasing resistance to colistin were not identified, but the result of stability experiments demonstrated that those mechanisms could only give a temporary increase in colistin resistance (except for PN24). Further studies are needed to understand the mechanisms of these HLCRMs.

4.3 How does HLCRMs affect the bacterial fitness and virulence?

Colistin is a critical important antibiotic, and plasmid-mediated colistin resistance (*mcr* genes) is a cause of great concern to public health (Schwarz & Johnson, 2016). It has been generally accepted that acquisition of antibiotic resistance by either target alteration or other mechanisms resulting in reduction in bacterial fitness (Melnyk *et al.,* 2015). MCR-1 mediates resistance to colistin by modification of the LPS on the bacterial outer-membrane, which is important for solute and protein translocation and signal transduction of the cell (Hinchliffe *et al.,* 2017; Koebnik, Locher, & Van Gelder, 2000). Some studies recently have shown that acquisition of a *mcr-1* plasmid has no considerable effect on bacterial fitness, (Kong *et al.,* 2017; Y. Zhang *et al.,* 2017) but it remains unknown whether high colistin resistance in *E.coli* is associated with a biological cost.

In general, the seven HLCRMs showed slower growth (except for PN16) and less competitive ability as compared to wild-type parental strains. It can be assumed that under clinical conditions or in farm productions, the high resistance will also impose a biological cost (dan i Andersson & hughes, 2010; Hernando-Amado *et al.,* 2017). This loss of fitness due to high resistance to colistin may explain why *mcr-1* is often associated with low MIC values (Liu *et al.,* 2016). What is more, MCR-1 induced colistin resistance is based on addition of PEA which decreases the net negative charge of lipid A and reduces the avidity of colistin binding (Hinchliffe *et al.,* 2017). The PEA modification reduces net negative charge of lipid A from -1.5 to -1, while another cationic substitution by 4-amino-4-deoxy-l-arabinose (L-ara4N) which is more efficient decreases negative charge to 0 (Nikaido, 2003). The lower ability of charge

alteration by PEA may also have an effect on resistance level to colistin.

So far, chromosomal mutation based resistance to colistin is mostly dependent on the addition of PEA or L-ara4N to the Lipid A moiety of LPS, or rarely by complete loss of LPS (Abiola O Olaitan *et al.,* 2014). In many studies, resistant mutants are induced by *in vitro* treatment with sub MIC colistin and meanwhile show decrease in fitness and virulence in *A. baumannii* and *K. pneumoniae* (Beceiro *et al.,* 2014; Choi & Ko, 2015; Fernández-Reyes *et al.,* 2009; Mu *et al.,* 2016). Clinical studies in *A. baumannii* demonstrate that resistant isolates are often detected after treatment with colistin (López-Rojas *et al.,* 2013; Rolain, Roch, Castanier, Papazian, & Raoult, 2011). Although reduction of fitness has been identified when compared with susceptible isolates and in some instances the lack of biological cost or retention of virulence has also been identified (Cannatelli *et al.,* 2014; Durante-Mangoni *et al.,* 2015). In this study, 6 out of 7 HLCRMs are linked to considerable fitness cost and all mutants displayed a decrease in virulence (3 were significant, $p < 0.01$), which may limit the resistant level in *mcr-1* harbouring *E.coli* (Liu *et al.,* 2016).

4.4 How might expression of *mcr-1*effect bacterial fitness and virulence?

In this study, two of the HLCRMs showed a significant increase in *mcr*-1 expression. PN21 and PN25 showed 11- and 3-fold increase in *mcr*-1 expression levels, respectively. At the same time, the HLCRMs demonstrated a significant biological cost (including decrease in growth rates and inferiority in competition assay with the exceptation of PN16 and a decrease in pathogenic potential). These data would suggest that high *mcr*-1 expression levels can lead to lower fitness and pathogenicity in *E. coli*.

MCR-1 is a member of phosphoethanolamine (PEA) transferase enzyme family and can mediate resistance to colistin by modification of LPS on bacterial cell outer-membrane (Liu *et al.,* 2016) The MCR-1 enzyme catalyses the addition of PEA to lipid A, which alters the surface charge and subsequently leads to a reduction in colistin binding, resulting in colistin insusceptibility (Hinchliffe *et al.,* 2017). In our recent report, the increased expression of *mcr-1* not only mediates a significant biological cost (even cell death), but a degradation of the *E. coli* outer-membrane (Yang *et al.,* 2017). LPS, a critical feature of the outer-membrane in Gram-negative bacteria, is also likely to be significantly altered (Moffatt *et al.,* 2010). The modification of LPS by over-expression of *mcr-1* can lead to leakage of the cellular cytoplasm resulting in cell death (as shown below in ***Figure 4.2*** [reproduced from Yang *et al.,* 2017]).

However, in HLCRM PN21, even though it possessed very high level of resistance to colistin (MIC of 128 mg/L), no significant modification in the cell wall (thickening of outer-membrane, loss of membrane definition and abnormal cytoplasmic morphology) was detected. Compared

with the inducible *mcr-1* expression model in pBAD vector reported by Yang *et al.,* where the over expression of *mcr-1* can contribute up to more than 1000- fold increase than un-induced pBAD/*mcr-1*, the *mcr-1* expression of PN21 HLCRM showed only an 11- fold increase (Yang *et al.,* 2017). As PN21 is a wild-type strain, understanding the mechanism of HLCRM might be more complicated. However, it could still suggest that high *mcr-1* expression can significantly contribute to a reduction in bacterial fitness and virulence.

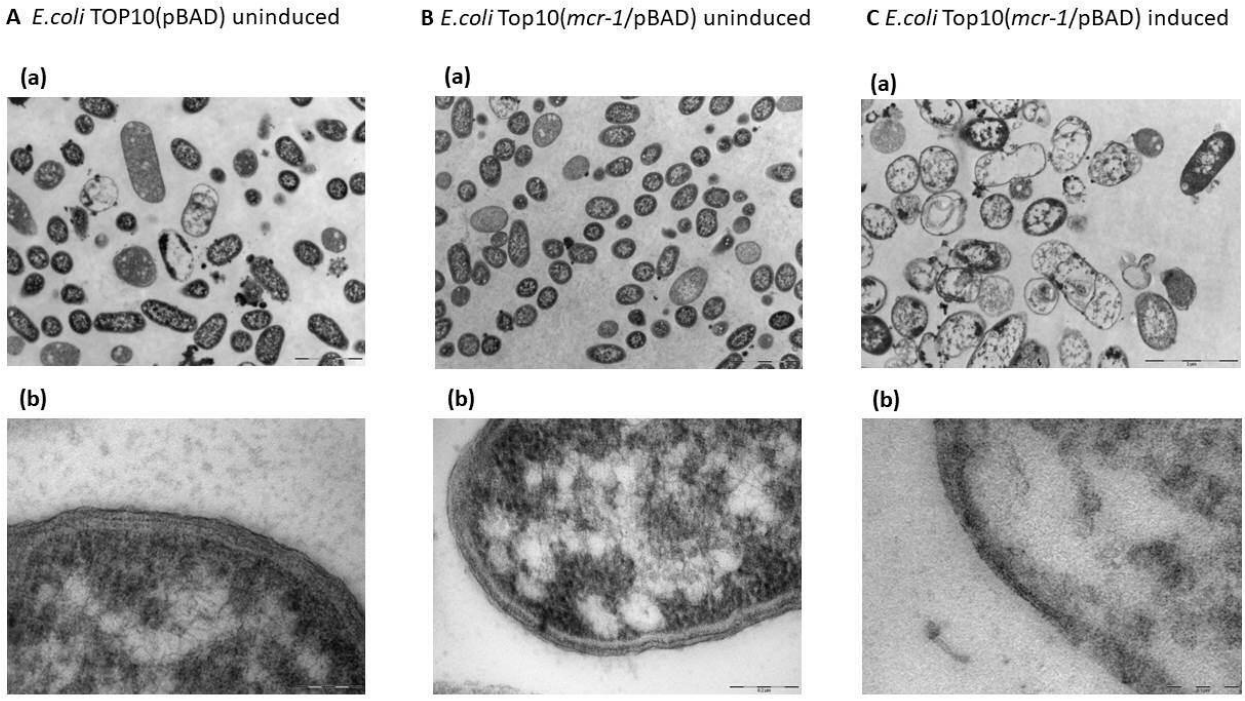


Figure 4.2 **Cellular morphology of inducible *mcr-1* in *E. coli* Top10 by transmission electron microscopy (TEM).** Over-expression of *mcr-1* is induced by 0.2% (w/v) L-arabinose. A. B. TEM micrographs of un-induced *E.coli*; C. TEM micrographs of induced MCRPEC (Yang *et al.,* 2017).

4.5 Does PmrA/B mutation have an effect on bacterial fitness and virulence in *mcr-1* harbouring *E.coli*?

As the isolates were wild-type strains with various genetic backgrounds, the exact mechanism of HLCRM will be challenging to identify. To further understand the mechanism of HLCRMs, we compared the genetic context of both HLCRMs and their parental strains. As described above, amino acid mutations on PmrA (R81S) and PmrB (V161M) are detected in PN25 and PN42 (***Figure 4.3***), respectively.

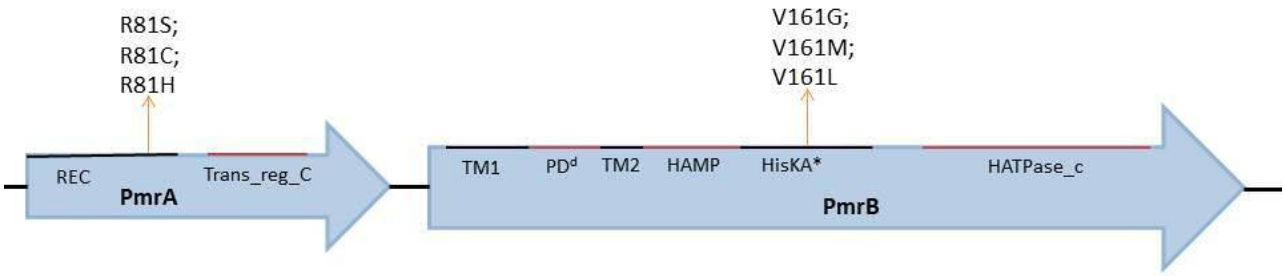


Figure 4.3 **Domains of the PmrA/B TCS and positions of mutations conferring colistin resistance in this study.** PmrA domains, amino acid(aa) positions 1-112: cheY-homologous receiver domain(REC); aa 145-216: Transcriptional regulatory protein, C-terminal domain (Trans_reg_C). PmrB domains, aa 13-35: First transmembrane domain (TM1); aa 35-66: dPeriplasmic domain (PD); aa 66–88: Second transmembrane domain (TM2); aa 89-141: Histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases (HAMP domain); aa 142–202: Histidine kinase A domain (HisKA); aa 249-356: Histidine kinase-like ATPases (HATPase_c). (Abiola O Olaitan *et al.,* 2014)

Antibiotic resistance caused by chromosomal mutation is often associated a fitness cost, as acquisition of resistance often depends on modification of essential targets such as ribosome, DNA gyrase, RNA polymerase or the cell wall (Melnyk *et al.,* 2015; Sommer *et al.,* 2017). The alteration of these targets normally has harmful effects on the host and results in reduced fitness (Hernando-Amado *et al.,* 2017). Both mutations identified in this study have been reported by Quesada *et al.,* whether these mutation mediating colistin resistance are linked to a decrease in fitness is not clear (Alberto Quesada *et al.,* 2014).

In the study by Sun *et al.,* mutations in Salmonella PmrA/B (including R81-H mutation) lacked of fitness cost and had a high mutation rate (S. Sun *et al.,* 2009). However, the level of colistin resistance was very low (with MICs to colistin < 5mg/L). In other studies, *A. baumannii* (mainly), *K. pneumoniae* and *P. aeruginosa*, the reduction in fitness or virulence caused by PmrA/B mutation was variable depending on the location, type, and the number of mutations (Beceiro *et al.,* 2014; Da Silva & Domingues, 2017). Thus, further studies are needed to understand the fitness cost of chromosome-mediated colistin resistance in *E. coli*.

4.6 Limitations and improvements

According to this study, colistin resistance in isolates harbouring *mcr-1* could be enhanced under the pressure of increasing colistin concentrations. However, this increase in resistance came with a price in fitness as well as virulence. Although it has been shown that high level colistin resistance in *mcr-1* isolates is associated with a reduction in fitness and virulence; how *mcr-1* mediates or modulates this process is not clear. Since they are wild-type isolates, the genetic basis for their resistance remains uncertain but is part of my future work. As no mutation was detected in the *mcr-1* structural gene or its surrounding context, and there was no obvious increase in *mcr-1* copy number, there might exist other mechanisms the bacteria as employed to adapt to the pressure of higher concentration of colistin. Further sequencing analysing on HLCRMs and parental strains are ongoing and following experiments will be undertaken to better understand the mechanism of HLCRMs in this study (eg. Genetic expression analysis on efflux pump systems linked to colistin resistance [Warner & Levy, 2010], detection of novel *mcr* genes, and mutation deletion analysis in LPS related genes identified in this study.)

As MCR-1 is a lipid A modifying enzyme, it is not clear that the rate or amount of modified LPS is proportional to the level of *mcr*. With regard to the cell morphology image on PN21 and its corresponding HLCRMs (***Figure 4.2***), the modification on the outer-membrane in the HLCRMs could be moderate or even small. Additionally, it is not clear whether the decrease in bacterial pathogenicity is associated with the modification of LPS, an important virulence factor in Gram-negative bacteria pathogenesis (Kömerik, Wilson, & Poole, 2000; Steimle,

Autenrieth, & Frick, 2016). Ergo, further studies are also needed to understand effects of modification on LPS or Lipid A and its subsequent effects on bacterial virulence/pathogenicity.

4.7 Future work

The phenomenon of fitness cost associated with AMR is a potential hope to reduce the frequency of resistant bacteria, and possibly to reverse antibiotic resistance (dan i Andersson & hughes, 2010; Holmes *et al.,* 2016). Experimental studies and theoretical modelling supports the reduction of antibiotic use and thereby benefitting fitter susceptible strains over less fit resistant strains such that susceptible organisms may outcompete resistant ones in the absence of selection (dan i Andersson & hughes, 2010; Hernando-Amado *et al.,* 2017). However, several studies have demonstrated that fitness cost can be reduced by compensatory mutations and hence help to support the maintenance of antibiotic resistance (Qi, Toll-Riera, Heilbron, Preston, & MacLean, 2016).

In this study, HLCRMs showed a significant biological cost and reduction in virulence compared to wild-type parental strains. The results support current surveillance and prevalence data that *mcr-1* normally only contributes to low-level colistin resistance (Liu *et al.,* 2016). My work shows that with a high fitness cost, *mcr-1* positive isolates are able to gain very high colistin resistance (up to 256 mg/L) within a short time. This significant reduction in fitness shows potential reversibility of colistin resistance; however, there is the possibility that after compensatory mutations high-level colistin resistant *E. coli* are stabilized in the population with less reversibility of resistance (Qi *et al.,* 2016).

For future work, it would be interesting to explore fitness adaptations of *mcr-1* HLCRMs. As shown in stability experiments in this study, some of the mutants showed no decrease in

CFU/ml on colistin after passaging for 14 days LB suggesting that HLCRMs can be stable. Thus further studies are needed to detect how *E. coli* would get used or can be attenuated to high-levels of colistin pressure, and to predict the reversibility of antibiotic resistance under the competition with susceptible phenotypes. Also, further analysing of whole genome sequencing in HLCRMs and mutants after stability is needed to better understand the cause of fitness cost and potential compensatory evolution. At last, further exploration in SNPs between PN21, PN23 and their HLCRMs are needed, especially for PN23, as it contains only one plasmid according to S1-PFGE image (***Figure 3.4***) and result of plasmidFinder (see Appendix).

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Appendix

Table 1 Acquired antimicrobial resistance genes

	PN16	PN21	PN23	PN24	PN25	PN42	PN43
Aminoglycoside	aadA1	aac(3)-IId	-	aadA1 aac(3)-IId aadA2	-	aph(3')-Ia aph(3")-Ib aph(6)-Id aadA1 aac(3)-IId aadA5 aadA2	aadA2 aadA1 aac(3)-IId
Beta-lactam	blaTEM-1B blaCMY-2	blaTEM-1A blaCTX-M-55	-	blaTEM-1B blaCTX-M-55	blaCTX-M-14	blaCTX-M-55 blaCTX-M-14 blaTEM-1B	blaTEM-1B blaCTX-M-55
Fluoroquinolone	qnrS1	qnrS1	-	qnrS1	-	qnrS1	qnrS1
Fosfomycin	-	fosA4	-	-	-	-	-
MLS - Macrolide, Lincosamide and Streptogramin B	mdf(A)	mdf(A)	mdf(A)	mdf(A) mef(B)	mdf(A)	mph(A) mdf(A) mef(B)	mdf(A) lnu(F)
Phenicol	cmlA1	-	-	catA2 cmlA1	-	catA1 cmlA1 -	cmlA1 catA2 floR
Sulphonamide	sul3	-	-	sul3	-	sul2 sul3 sul1	sul2 sul3
Tetracycline	tet(M)	tet(A)	tet(B)	tet(A)	tet(B)	tet(B)	tet(A) tet(M)
Trimethoprim	-	-	-	dfrA12		dfrA17 dfrA12	dfrA12

Table 2 Plasmid types in seven wild-type strains

Strain	Plasmid type
PN16	Col(BS512) IncFIA(HI1) IncFIB(AP001918) IncFIC(FII) IncHI1A IncHI1B(R27) IncI1 IncI2
PN21	Col(BS512) Col440II IncFIA IncFIB(AP001918) IncFII IncI1 IncI2
PN23	IncX4
PN24	IncFII IncN IncR IncX1 IncX4
PN25	Col(BS512) IncX4
PN42	IncFII IncI1 IncX1 IncX4
PN43	Col(BS512) IncFIB(AP001918) IncFII

Table 3 SNPs identified in HLCRMs assembled to wild-type strains

Strain	Gene	Substitution in HLCRMs
PN16	*gspA2*	F73Y
	hp	V5M, S38P, D42A
	panF	several mutations
	garK	*A9S*
	hp	C5S, S65R
	hp	nucl deletion after K195
	hp	D101N
PN24	*hp*	K229T, R327H
	hp	M10L, G14E, M17L, S18G
	uxuA	V160E
	hp	C187R
PN25	*rfaY*	L187STOP
	rpoB	H551P
	basR (*pmrA*)	R81S
	rhsC	V67D
	hp	R51K
PN42	*rpob*	Y1281D
	basS (*pmrB*)	V161M
	aceE1	nuc deletion after M775
	hp	G60S, N20D
	hp	T13A, L33F
	fadH	S97R
	dmlR	I174K
	ywnH	Y28C
	rshC 7	A984E
	glmS	C134W
	polA	K124E
PN43	*aceE*	E448STOP
	skp	Q103STOP
	rhsC	A807S, D814E, F815Y, R845S, Q852L, Y853S
	xylE	R540W
	malG	S24W