THE BIOLOGY OF ANTIBIOTIC RESISTANCE PLASMIDS

by

HOWARD THIEN HUI SAW

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

School of Immunity and Infection

College of Medical and Dental Sciences

University of Birmingham

April 2015

ABSTRACT

Plasmids confer genes encoding clinically relevant antibiotic resistance. It was hypothesised that the AcrAB-TolC multidrug resistance efflux pump was required for clinically relevant levels of carbapenem resistance. However, carbapenemase-producing *Salmonella* TolC mutants were less susceptible to carbapenems. In the presence of the efflux inhibitor phe-arg-β-naphthylamide (PAβN), wildtype bacteria and 36/86 non-replicate clinical isolates of carbapenem-producing Enterobacteriaceae were ≥4-fold less susceptible to ertapenem. Experimental data suggested that OmpF repression conferred the increased carbapenem MICs. Two bla_{KPC}-encoding plasmids have been isolated in the UK; pKpQIL-UK was found in *K. pneumoniae*, but its variant, pKpQIL-D2 was also found in other species. Therefore, it was hypothesised that a region of pKpQIL-D2 either conferred a broader plasmid host range and/or a fitness benefit to the host bacterium. Fitness studies measuring growth rates, ability to form biofilm, conjugation frequency and plasmid persistence showed that both plasmids affected the host bacterium but in different ways. Compared to pKpQIL-UK, pKpQIL-D2 did not confer a significant fitness advantage to its host under the conditions tested. RNA-sequencing showed both plasmids affected a different set of genes related to metabolism. The understanding of the factor(s) contributing to the persistence and dissemination of successful plasmids may help to control antibiotic resistance.

Acknowledgements

Firstly, I would like to thank both of my supervisors, Laura Piddock and Mark Webber for their great guidance and support throughout my PhD studies.

I am also grateful for the presence of many kind colleagues in the Antimicrobial Research Group. The help and advice provided by them during my studies are very much appreciated.

The constant motivation from my friends in England and also those from Malaysia, Melbourne and Singapore has kept me moving even during very harsh times.

I appreciate the help provided by many of our collaborators namely, Neil Woodford, Michel Doumith, Shazad Mushtaq, Mark Sutton and Matthew Wand in Public Health England.

I am thankful for the help that has been provided by the staffs and colleagues in the School of Immunity & Infection, and Institute of Microbiology & Infection.

Lastly, I would like to thank the University of Birmingham for awarding me the Elite Doctoral Researcher Scholarship which funds my PhD studies in England.

Table of Contents

CHAPTER ONE: INTRODUCTION 1

1.1 Bacteria 1

1.1.1 *Salmonella* Typhimurium as a Model Organism 3

1.2 β-lactam Antibiotics 4

1.2.1 Mechanism of Action of β-lactams 8

1.2.2 Carbapenems 10

1.3 Antibiotic Resistance 13

1.3.1 Burden of Antibiotic Resistance 14

1.3.2 Antibiotic Resistance Mechanisms 15

1.3.3 Carbapenemase-mediated Antibiotic Resistance 19

1.3.4 Antibiotic Resistance Which Requires AcrAB-TolC 23

1.3.5 Efflux Inhibitors 25

1.4 Spread and Acquisition of Antibiotic Resistance 27

1.4.1 Lateral Gene Transfer (LGT) in Dissemination of Antibiotic Resistance 27

1.5 Plasmid-mediated Antibiotic Resistance 29

1.5.1 The bla_{KPC}-encoding pKpQIL Plasmid 30

1.6 Factors Important in Plasmid Dissemination in Enterobacteriaceae 32

1.6.1 Plasmid Stability and Segregation 32

1.6.2 Inhibition of Plasmid Transfer 34

1.7 Plasmid-mediated Antibiotic Resistance and Fitness 35

1.8 Background to This Project 37

1.8.1 Plasmid pCT 37

1.8.2 Plasmid pKpQIL-UK & pKpQIL-D2 38

1.9 Hypotheses to be Investigated in This PhD Project 43

1.10 Aims & Objectives 43

CHAPTER TWO: METHODS 44

2.1 Bacterial Strains, Plasmids & Culture Conditions 44

2.1.1 Bacterial Identification 44

2.2 Plasmid Extraction 56

2.3 Polymerase Chain Reaction 56

2.4 DNA Gel Electrophoresis 57

2.5 DNA Sequencing 57

2.6 Insertional Inactivation of Plasmidic Genes 62

2.6.1 Primer design 62

2.6.2 Electrocompetent Cell Preparation and Electroporation 62

2.6.3 Gene Inactivation by Homologous Recombination 63

2.7 β-lactamase Assay 64

2.8 Hoechst 33342 Accumulation Assay 65

2.9 Outer Membrane Protein Extraction 66

2.10 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) 67

2.11 Selection of Rifampicin Resistant Bacteria 67

2.12 P22 Transduction 68

2.13 Conjugation 69

2.14 Growth Kinetics 70

2.15 Determination of Conjugation Frequency 71

2.16 Minimum Inhibitory Concentration (MIC) Determination 72

2.17 Biofilm Assay 72

2.17.1 Biofilm Formation on Plastic in Microtitre Tray 72

2.17.2 Biofilm Formation on Glass Under Flow 73

2.18 Plasmid Persistence 75

2.19 Pairwise Competition Assay 76

2.20 *Galleria mellonella* Infection Model 77

2.21 RNA Sequencing 77

2.21.1 RNA Extraction 77

2.21.2 Ribosomal RNA (rRNA) Reduction 79

2.21.3 RNA Library Preparation 79

2.21.4 Sequencing 80

2.21.5 Data Analyses 81

CHAPTER THREE: THE CELL ENVELOPE AND CARBAPENEM RESISTANCE 83

3.1 Background 83

3.2 Hypothesis and Aims 84

3.3 Carbapenem Resistance by bla_{KPC} is Seen Even in the Absence of the Tripartite AcrAB-TolC Multidrug Resistance Efflux Pump 84

3.3.1 Strain Constructions 84

3.3.2 The MICs of Antibiotics in Strains Lacking a Component of the AcrAB-TolC Efflux Pump 103

3.4 The *Salmonella* TolC Mutant is Less Susceptible to β-lactam Antibiotics and This is Not Specific to Carbapenemase 106

3.5 Increased Carbapenem Resistance is Not Associated with Passive Release of Carbapenemase from the Periplasmic Space of the *Salmonella* Efflux Mutant..... 108

3.6 Efflux Inhibitor Alters Susceptibility to Some β-lactam Antibiotics 113

3.6.1 PAβN Reduces β-lactam Antibiotic Susceptibility in *K. pneumoniae*, *E. coli* and *Salmonella* 113

3.6.2 PAβN Increases Carbapenem Resistance in Clinical Isolates of Enterobacteriaceae 118

3.7 PAβN and Lack of TolC Alter the Porin Profile and Confers Decreased Susceptibility to β-lactam Antibiotics 124

3.7.1 Outer Membrane Protein Mutants Showed Reduced Susceptibility to Ertapenem 124

3.7.2 PAβN Reduces Outer Membrane Protein Expression in *Salmonella* Strains 129

3.7.3 Outer Membrane Protein Profile of Clinical Isolates of Enterobacteriaceae. 131

3.8 Discussion 133

3.9 Future Work 142

3.10 Key Findings 144

CHAPTER FOUR: COMPARISON OF PLASMIDS pKpQIL-UK WITH pKpQIL-D2 .. 145

4.1 Background 145

4.2 Hypothesis and Aims..... 145

4.3 Comparison of the Genetic Sequence of pKpQIL-UK with that of pKpQIL-D2 145

4.4 Transfer of Plasmid into Different Species of Enterobacteriaceae 146

4.5 Growth Rates of Enterobacteriaceae pKpQIL-UK and -D2 149

4.6 Plasmid Persistence 156

4.7 Pairwise Competition of *K. pneumoniae* Ecl8 carrying pKpQIL-UK and -D2 160

4.8 Conjugation Frequencies of the Plasmids into Various Enterobacteriaceae 164

4.9 Biofilm Formation 169

4.9.1 Biofilm Formation on Plastic 169

4.9.2 Biofilm Formation under Constant Flow of Medium 169

4.10 Infection of *Galleria mellonella* by Plasmid Carrying *K. pneumoniae* ST258 174

4.11 Minimum Inhibitory Concentration of Antibiotics for Various Plasmid-bearing Hosts 176

4.12 Summary of Phenotypes Conferred by Carriage of pKpQIL-UK vs -D2 183

4.13 Gene Expression Profiling of Plasmid Carrying *K. pneumoniae* ST258 with RNA Sequencing 183

4.13.1 Determination of Growth Phase for RNA Extraction 183

4.13.2 Optimisation of Total RNA Extraction 187

4.13.3 Ribosomal RNA Depletion and Library Preparation 191

4.13.4 RNA Sequencing 191

4.14 Discussion 207

4.15 Future Work 216

4.16 Key Findings 218

CHAPTER FIVE: OVERALL DISCUSSION 219

REFERENCES 226

List of Figures

Chapter One

Figure 1.1	Differences between Gram-positive and Gram-negative bacteria cell wall	2
Figure 1.2	Distribution of antibiotic usage in Europe	6
Figure 1.3	Structures of β-lactam antibiotics	7
Figure 1.4	Simplified scheme of peptidoglycan macromolecule	9
Figure 1.5	Structures of carbapenem antibiotics	12
Figure 1.6	Alignment of plasmid pKpQIL-UK and variants	39
Figure 1.7	Comparison of the substitution fragment of pKpQIL-UK and -D1	41
Figure 1.8	Comparison of the substitution fragment of pKpQIL-UK and -D2	42

Chapter Two

Figure 2.1	BioFlux system	74
Figure 2.2	RNA sequencing data analyses workflow	82

Chapter Three

Figure 3.1	Growth kinetics of rifampicin-susceptible/resistant mutants of *K. pneumoniae* Ecl8 and its efflux mutants	86
Figure 3.2	PCR amplification of *rpoB* of rifampicin-susceptible/resistant *E. coli* BW25113 for DNA sequencing	88
Figure 3.3	PCR verification of inactivated *acrB* and *tolC* genes in rifampicin-resistant mutants of Keio Collection strains	89
Figure 3.4	Growth kinetics of rifampicin-susceptible/resistant mutants of *E. coli* BW25113 and its efflux mutants	90
Figure 3.5	PCR amplification of *rpoB* of rifampicin-resistant *S.* Typhimurium ATCC14028s for sequencing and mutant verification	93
Figure 3.6	Growth kinetics of rifampicin-susceptible/resistant mutants of *S.* Typhimurium 14028s and its efflux mutants	94
Figure 3.7	PCR verification of *K. pneumoniae* Ecl8, *acrAB*, Ecl8^{Rif}/pKpQIL-UK and Ecl8^{Rif} *acrAB*/pKpQIL-UK	97
Figure 3.8	PCR verification for the presence of pKpQIL-UK in the *E. coli* BW25113^{Rif} and isogenic efflux mutants	98
Figure 3.9	PCR verification of 14028s^{Rif}/pKpQIL-UK transconjugants	99
Figure 3.10	PCR verification of 14028s^{Rif} *acrAB*/pKpQIL-UK, 14028s^{Rif} *tolC*/pKpQIL-UK and 14028s^{Rif} *acrAB*/pKpQIL-D2	100

List of Figures (Continued)

Chapter Three

Figure 3.11	PCR verification of 14028s^{Rif}/pKpQIL-D2 and 14028s^{Rif} *tolC*/pKpQIL-D2	101
Figure 3.12	PCR verification of 14028s^{Rif} *acrAB*/pKpQIL-D2 transconjugants	102
Figure 3.13	PCR verification of transconjugants of 14028s^{Rif} and its mutants carrying the plasmid pNDM-HK	109
Figure 3.14	β-lactamase activity assay for pKpQIL-UK carrying *Salmonella* and isogenic efflux pump component mutants	112
Figure 3.15	Hoechst 33342 accumulation assay for PAβN and varying concentrations of Compound A and NMP	117
Figure 3.16	PCR verification of SL1344 porin mutants carrying pKpQIL-UK	125
Figure 3.17	PCR verification of SL1344 porin mutants carrying pKpQIL-UK	126
Figure 3.18	Outer membrane protein profile of *Salmonella* Typhimurium SL1344 and its isogenic efflux pump mutants	127
Figure 3.19	Outer membrane protein of *E. coli* clinical isolates in the presence and absence of 25 μg/ml PAβN	132
Figure 3.20	Outer membrane protein of *Enterobacter* spp. clinical isolates in the presence and absence of 25 μg/ml PAβN	134
Figure 3.21	Outer membrane protein of *Klebsiella* spp. clinical isolates in the presence and absence of 25 μg/ml PAβN	135

Chapter Four

Figure 4.1	Alignment of pKpQIL-UK and -D2 with pKPN5	147
Figure 4.2	PCR verification of the *E. coli* DH10B transformants for the presence of pKpQIL-UK and -D2	150
Figure 4.3	PCR verification of the *Salmonella* SL1344^{Rif} transconjugants carrying pKpQIL-UK and -D2	151
Figure 4.4	PCR verification of the *E. coli* 10418^{Rif} transconjugants carrying pKpQIL-UK and -D2	152
Figure 4.5	PCR verification of the rifampicin-resistant *E. cloacae* and *S. marcescens* transconjugants carrying pKpQIL-UK and -D2	153
Figure 4.6	Repeat PCR verification of the rifampicin-resistant *S. marcescens* transconjugants carrying pKpQIL-UK	154

List of Figures (Continued)

Chapter Four

Figure 4.7	PCR verification of the *K. pneumoniae* ST258 transconjugants carrying pKpQIL-UK and -D2	155
Figure 4.8	Growth kinetics of various Enterobacteriaceae carrying pKpQIL-UK vs -D2	157
Figure 4.9	The percentage of pKpQIL-UK vs -D2 carrying cells in growth over a period of 20 days without antibiotic selection	159
Figure 4.10	PCR verification of the *E. coli* DH10B transformants for the presence of bla_{KPC} inactivated pKpQIL-UK and -D2	161
Figure 4.11	PCR verification of the *K. pneumoniae* Ecl8 transconjugants for the presence of bla_{KPC} inactivated pKpQIL-UK and -D2	162
Figure 4.12	Pairwise competition of pKpQIL-UK vs -D2	163
Figure 4.13	Growth rates of rifampicin-resistant *K. pneumoniae* Ecl8 carrying pKpQIL-UK & -D2 in the presence & absence of bla_{KPC}	165
Figure 4.14	Conjugation frequencies of pKpQIL-UK and -D2 from their respective clinical isolates into Enterobacteriaceae species	167
Figure 4.15	Biofilm formation on plastic for the various plasmid carrying Enterobacteriaceae strain	170
Figure 4.16	Images of biofilm formation in microfluidic channel at various time points	171
Figure 4.17	Percentage area of coverage by biofilm in microfluidic channel after 48 hours under constant flow of LB broth (without salt)	175
Figure 4.18	Virulence assay of *Galleria mellonella* infected by pKpQIL-UK and -D2 carrying *K. pneumoniae* ST258	177
Figure 4.19	Outer membrane profile of *S. marcescens* and *K. pneumoniae* Ecl8 carrying pKpQIL-UK and -D2	180
Figure 4.20	Growth kinetics of *K. pneumoniae* ST258/pKpQIL-UK vs -D2 in the presence of doripenem	182
Figure 4.21	Growth kinetics of *K. pneumoniae* ST258 and plasmid carrying isogenic strains in MOPS minimal medium, and bla_{KPC} expression	185
Figure 4.22	Purification of RNA from *K. pneumoniae* ST258 in the presence and absence of pKpQIL-UK and -D2	188
Figure 4.23	Purification of RNA from increasing volume of *K. pneumoniae* ST258 culture	190
Figure 4.24	Purification of RNA using RiboPure^{TM} RNA Purification Kit	192
Figure 4.25	Validation of DNA contamination in DNAse-treated RNA samples	193

List of Figures (Continued)

Chapter Four

Figure 4.26	Depletion of ribosomal RNA	194
Figure 4.27	Validation of cDNA libraries	195
Figure 4.28	Quality check of RNA sequencing reads	196
Figure 4.29	Chromosome alignment of *K. pneumoniae* ST258 chromosome sequences with MGH78578	198
Figure 4.30	Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-UK on to substituted region on pKpQIL-UK DNA sequence	203
Figure 4.31	Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-UK on to substituted region on pKpQIL-D2 DNA sequence	204
Figure 4.32	Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-D2 on to substituted region on pKpQIL-D2 DNA sequence	205
Figure 4.33	Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-D2 on to substituted region on pKpQIL-UK DNA sequence	206
Figure 4.34	Amino acid alignment of ParA pKpQIL-UK vs -D2	208
Figure 4.35	Amino acid alignment of ParM pKpQIL-UK vs -D2	209

List of Tables

Chapter One

Table 1.1	Function of genes involved in conjugation	33
Table 1.2	Distribution of pKpQIL-UK and variants from medical centres in North Western England Region	40

Chapter Two

Table 2.1	List of antibiotics used in this study	45
Table 2.2	Plasmids	46
Table 2.3	Bacterial strains	47
Table 2.4	Morphological appearance on differential and selective agar	55
Table 2.5	Primers used in this study	58

Chapter Three

Table 3.1	Generation times of rifampicin-susceptible/resistant *K. pneumoniae* Ecl8 and its isogenic *acrAB* mutant	87
Table 3.2	Generation times of rifampicin-susceptible/resistant *E. coli* BW25113 and its isogenic efflux pump mutants	91
Table 3.3	Generation times of rifampicin-susceptible/resistant *S.* Typhimurium 14028s and its isogenic efflux pump mutants	95
Table 3.4	MIC values of various antibiotics for efflux pump component mutants carrying the pKpQIL-UK plasmid	104
Table 3.5	Protein sequence homology of AcrAB-TolC efflux system	107
Table 3.6	MIC values of various antibiotics for efflux pump component mutants carrying various plasmids	110
Table 3.7	Effects of PAβN and CCCP on MIC of β-lactam antibiotics	114
Table 3.8	Effects of PAβN and CCCP on MIC of β-lactam antibiotics	116
Table 3.9	Ertapenem MIC in the presence of various efflux inhibitors	119
Table 3.10	Changes in ertapenem resistance of clinical isolates in the presence of PAβN	122
Table 3.11	The changes in MIC_{50} and MIC_{90} of the clinical isolates in the absence and presence of PAβN	123
Table 3.12	Effects of PAβN and CCCP on MIC of β-lactam antibiotics	128
Table 3.13	Ertapenem MIC in the presence of various efflux inhibitors	130

Chapter Four

Table 4.1	Rifampicin-resistant mutants of five species of Enterobacteriaceae	148
Table 4.2	Generation time of various Enterobacteriaceae carrying pKpQIL-UK vs -D2	158
Table 4.3	Generation times of rifampicin-resistant *K. pneumoniae* Ecl8 carrying pKpQIL-UK & -D2 in the presence & absence of bla_{KPC}	166
Table 4.4	Conjugation frequencies of pKpQIL-UK and -D2 into various Enterobacteriaceae	168
Table 4.5	Minimum inhibitory concentration of pKpQIL-UK and -D2 carrying hosts	178
Table 4.6	Relative abundance of the major outer membrane proteins in the plasmid-carrying strains of *S. marcescens* and *K. pneumoniae*	181
Table 4.7	Summary of the fitness experiments for the various plasmid carrying Enterobacteriaceae	184
Table 4.8	Generation times of plasmid carrying *K. pneumoniae* ST258 in MOPS minimal medium	186
Table 4.9	Differentially expressed chromosomal genes in the presence of pKpQIL-UK vs -D2	199
Table 4.10	Differentially expressed plasmidic genes	202

CHAPTER ONE: INTRODUCTION

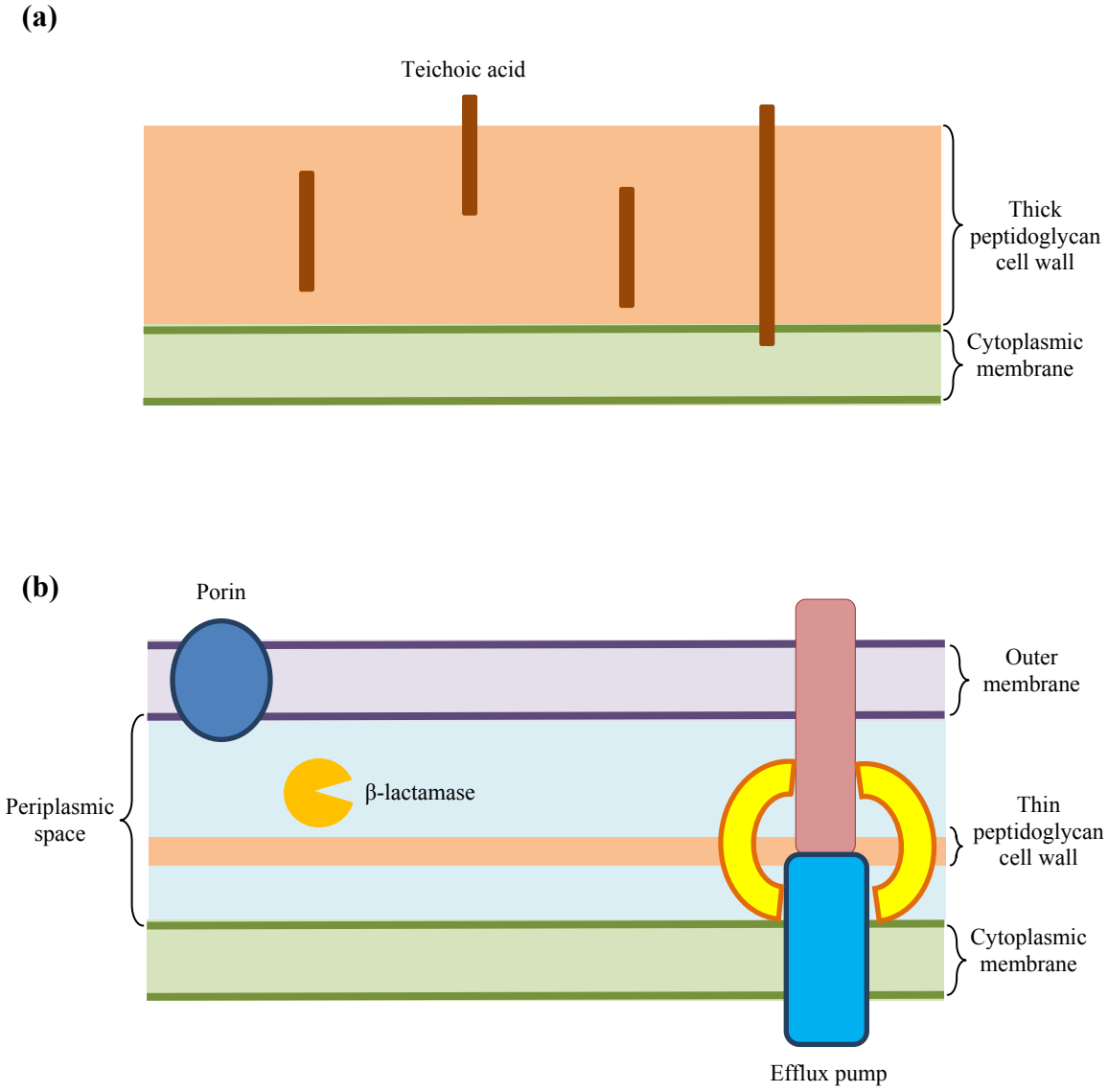
1.1 Bacteria

The earliest evidence for the existence of bacteria dates back to about 3.5 billion years (Schopf, 1993). Besides being ubiquitous, these microscopic organisms share an intimate relationship with humans. Newly born infants are colonised with a normal flora within days of birth (Hall et al., 1990). The presence of a normal flora has been suggested to benefit the host in many ways, such as modulation of host gene expression important in intestinal functions (Hooper et al., 2002), provision of vitamins (Hill, 1997), absorption of minerals (Younes et al., 2001), intestinal epithelial cell differentiation (Gordon et al., 1997), development of the immune system (Umesaki et al., 1993) and preventing colonisation of the host by harmful bacteria (Bernet et al., 1994). Although many bacteria benefit their hosts, there are also others which cause diseases.

Bacteria can be broadly differentiated into two groups based on the way they are coloured by Gram's stain i.e. Gram-positive and -negative bacteria (Moyes et al., 2009). As a result of differences in the cell wall of bacteria, Gram-positive bacteria are stained purple while Gram-negative bacteria will be stained pink (Moyes et al., 2009). The Gram-positive bacterium has a thicker, multi-layered peptidoglycan cell wall (Figure 1.1a). In comparison, a Gram-negative bacterium has a thinner, mostly single-layered peptidoglycan cell wall (Figure 1.1b) (Cabeen and Jacobs-Wagner, 2005). The Gram-negative bacterium also carries an outer membrane which is lacking among Gram-positive bacteria (Cabeen and Jacobs-Wagner, 2005).

The Enterobacteriaceae family consists of Gram-negative rod shaped bacteria and includes *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella enterica*. Many of these bacteria are commonly found as normal flora in human intestine and are capable of being

Figure 1.1 Differences between Gram-positive and Gram-negative bacteria cell wall



(a) The cell wall of a Gram-positive bacterium is made up of multiple layer of peptidoglycan polymer. Teichoic acids are found embedded in the cell wall to increase its rigidity. (b) Gram-negative bacterium possesses a thinner peptidoglycan in the space between the outer membrane and cytoplasmic membrane called the periplasmic space. The porin in the outer membrane acts as a non-specific channel for passive exchange of solutes between the extracellular environments with the bacterial cell. The efflux pump functions to extrude compounds in the cell to the external environment. β-lactamase enzymes inactivate β-lactam antibiotics allowing the bacterium to be resistant to the antibiotic.

opportunistic pathogen which cause nosocomial- and community-acquired infections (Nordmann et al., 2012a, Hurrell et al., 2009). Opportunistic pathogen refers to organisms which are living within a host (e.g. human) or originate from the environment and which are able to cause diseases upon successfully infecting their host (Brown et al., 2012).

Among the more notable of the bacteria which cause mortality and morbidity in both the clinical and community settings include *E. coli*, *K. pneumoniae*, *Enterobacter* spp. and *Proteus* spp. (Boucher et al., 2009, ECDC, 2013a). Being ubiquitous among humans and vertebrates, *E. coli* and *K. pneumoniae* are also the leading causative agents of urinary, respiratory and bloodstream infections worldwide (WHO, 2014). *Salmonella* is the leading cause of food borne outbreaks and the most frequent cause of enteric infection after *Campylobacter* in the EU (ECDC, 2015). In Europe, it was reported that about 3.2 million patients have at least one healthcare-associated infection annually (ECDC, 2013a). These include respiratory tract infections, surgical site infections, urinary tract infections and bacteraemia (ECDC, 2013a). The need to treat infection caused by pathogenic bacteria has driven medical research that has resulted in the discovery and development of various antibacterial compounds.

1.1.1 *Salmonella* Typhimurium as a Model Organism

Salmonella Typhimurium is a motile rod shaped Gram-negative bacterium and a member of the Enterobacteriaceae family. This facultative anaerobic bacterium is an important cause of food-borne diarrhoeal diseases (Fàbrega and Vila, 2013). Phylogenetic analyses using various genetic markers such as 16S RNA, *rpoB* and initiation factor 2, have shown that members of the Enterobacteriaceae family share high genetic similarities (Drancourt et al., 2001, Hedegaard et al., 1999, Moran et al., 2005, Roggenkamp, 2007). These analyses have also shown close phylogenetic relationship between *E. coli* and *S.* Typhimurium (Hedegaard et al.,

1999, Roggenkamp, 2007). About 72% of the genes in *S.* Typhimurium are homologous to that found in *E. coli* and *K. pneumoniae*, and these protein share on average, about 89% sequence identity (McClelland et al., 2001). Being the most studied laboratory organism for decades, *E. coli* has been the reference and model organism for studying various biochemical pathways and biological processes in other organisms (Keseler et al., 2005, Holden, 2002, Salgado et al., 2004). Although widely studied, there is a lack of suitable infection model available for this bacterium (Allen et al., 2006, Savkovic et al., 2005, Zhang et al., 2008). Similar to *E. coli*, *S.* Typhimurium is a highly tractable organism which has simple nutritional requirements, rapid growth rates and it is easy to genetically manipulate this bacterium (Garai et al., 2012). Due to the availability of various *in vitro* and *in vivo* infection models, *S.* Typhimurium is also an ideal model organism especially in studying host-pathogen interactions and bacterial pathogenesis (Garai et al., 2012).

1.2 β-lactam Antibiotics

One of the first antimicrobials to be discovered was penicillin in the late 1920s, which was found to exhibit a potent antibacterial effect (Fleming, 1929). For the next few decades, a range of novel antibacterial compounds of different classes were discovered, such as streptomycin, tetracycline, chloramphenicol, neomycin, gentamicin, erythromycin, vancomycin and rifampicin (Hopwood et al., 2007). The term 'antibiotic', which means 'against life' was coined by Salman Waksman no less than a decade after the discovery of penicillin to describe substances produced by microorganisms which possess growth inhibitory effect on other microorganisms (Waksman, 1973). At present, the definition of 'antibiotic' has been expanded to include semi- and fully-synthetic antibacterial agents, and is generally used to refer to compounds used clinically to treat human infections. This

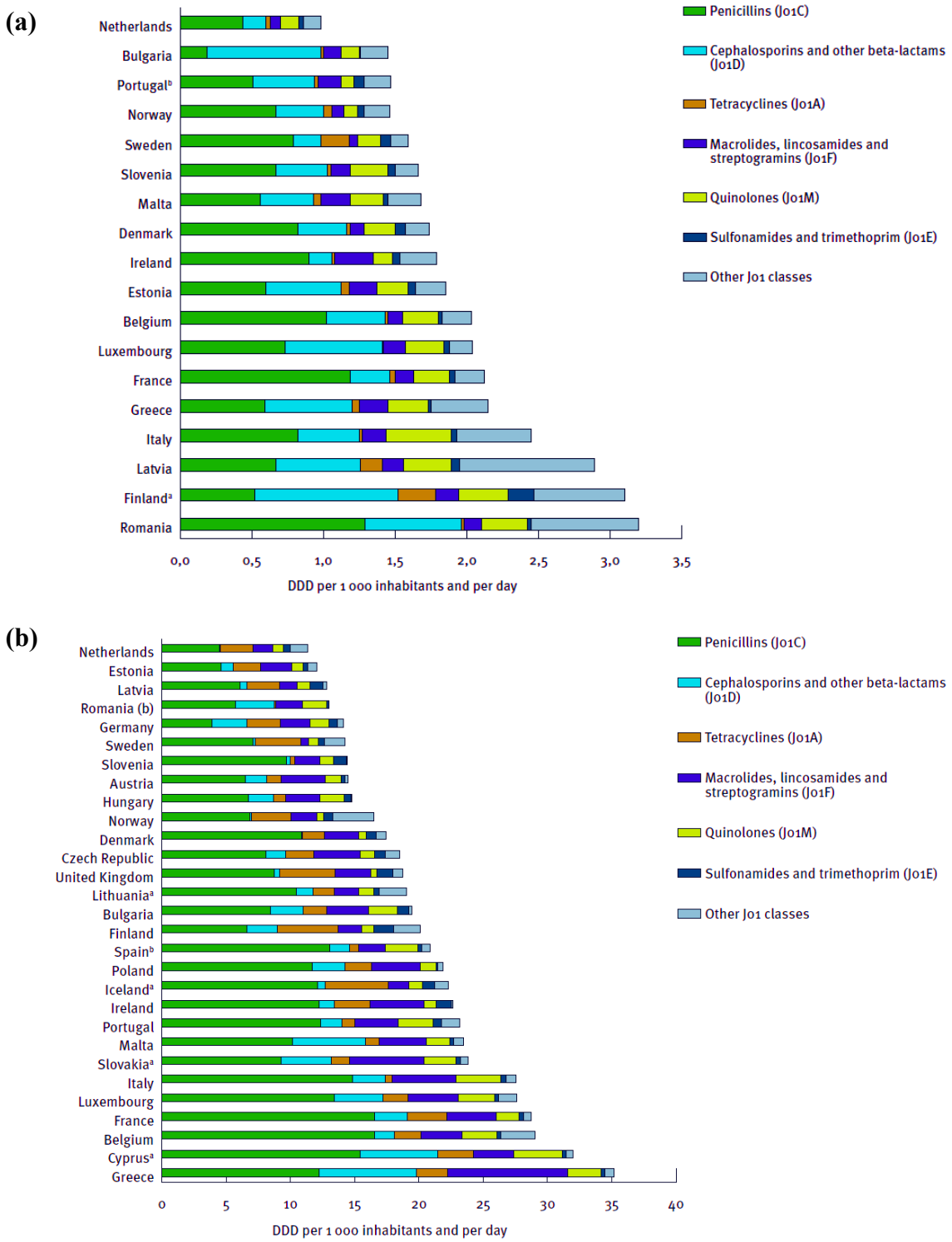
distinguishes antibiotics from biocides which are antimicrobial but not suitable for systemic use in humans (Yoneyama and Katsumata, 2006).

In 2009, antibiotics sales generated an estimated revenue of USD 42 billion globally (Hamad, 2010). Although there are a plethora of antibiotic classes being marketed, the β-lactam antibiotics are the largest class of antibiotic being manufactured and has the highest share in terms of sales, as they are widely used to treat Gram-positive and -negative bacterial infections (ECDC, 2013a, Hamad, 2010, Kresse et al., 2007, Woo et al., 2003). In most European countries, β-lactam antibiotics comprised of more than 50% of the antibiotics prescribed in the community and hospital settings (Figure 1.2) (ECDC, 2013a).

The β-lactam class of antibiotics encompasses several sub-classes such as penicillins (Fleming, 1929), cephalosporins (Burton and Abraham, 1951), clavams (Brown et al., 1976), carbapenems (Kahan et al., 1979) and monocyclic β-lactams (also known as monobactams (Aoki et al., 1976). These antibiotics are produced by fungi and prokaryotes, e.g. *Penicillium* spp., *Aspergillus nidulans*, *Cephalosporium* spp. and *Streptomyces* spp. (Demain and Elander, 1999). A common chemical structure i.e. the four-membered lactam ring, also termed β-lactam ring is shared among the sub-classes and plays an essential role in this antibacterial activity (Figure 1.3) (Demain and Elander, 1999, Donetz et al., 1984). Modification of chemical groups attached to the lactam ring results in changes in the antibacterial activity and stability of the antibiotic against inactivating enzymes as well as pharmacological properties (Yoshida, 1980, Chantot et al., 1992, Nayler, 1971).

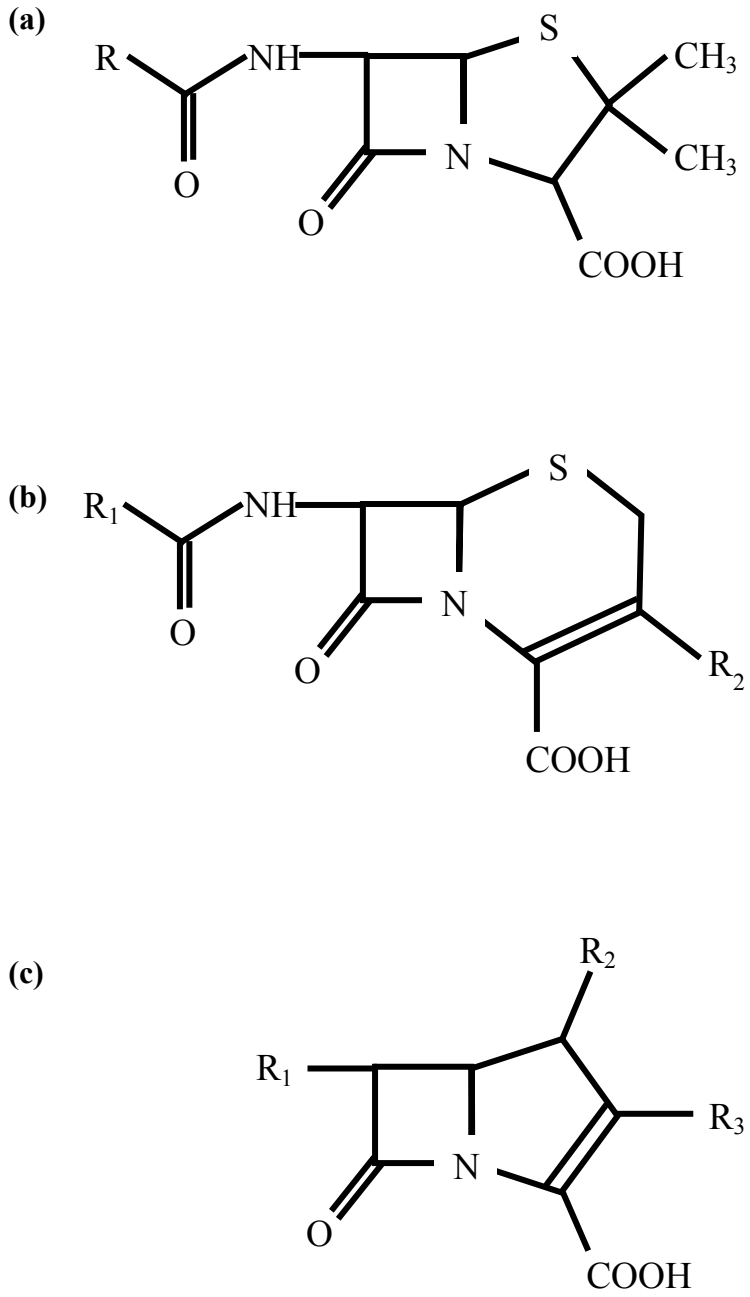
Due to their high clinical efficacy and low toxicity, β-lactam antibiotics play a major role in both clinical and veterinary medicine (Hornish and Kotarski, 2002, Timmerman et al., 2006). Therefore, β-lactam antibiotics are often the first choice for treating bacterial infections

Figure 1.2 Distribution of antibiotic usage in Europe



Antibiotic usage in the (a) hospital and (b) community setting, according to individual European countries [Source: ECDC (2013)]. 'DDD' refers to the 'defined daily dose' of an antibiotic.

Figure 1.3 Structures of β-lactam antibiotics



Core structure of (a) penicillin, (b) cephalosporin and (c) carbapenem. R is the side chain which differs among the individual members of the sub-class of the β-lactam antibiotics [Source: Demain & Elander (1999) and Hamilton-Miller & Brumfitt (1974)].

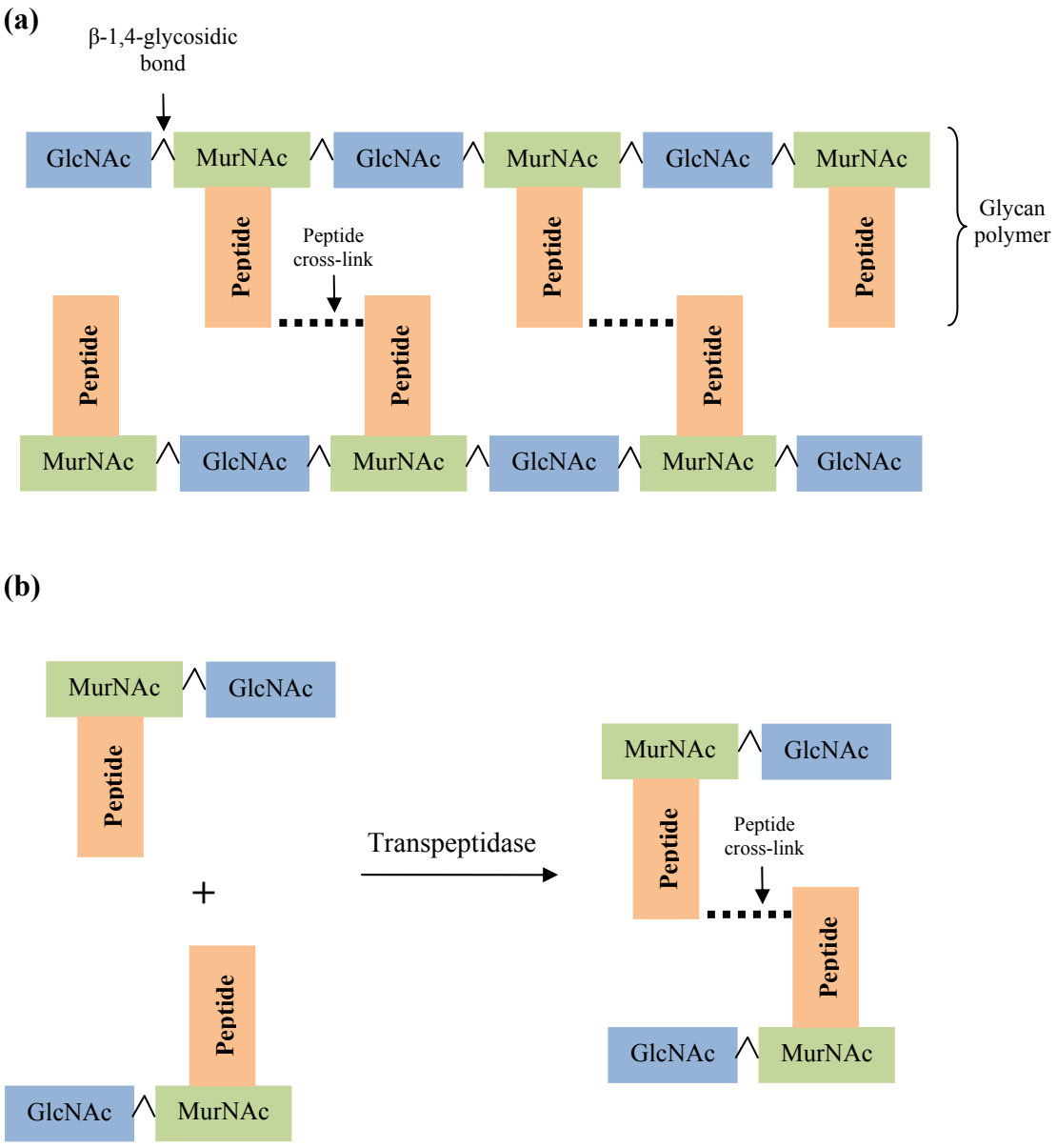
and are the most widely prescribed antibiotics in hospitals (Jovetic et al., 2010, Nicolau, 2008, Plüss-Suard et al., 2011, Liew et al., 2011). Broad-spectrum penicillins and cephalosporins are also used for growth promotion, disease and prophylactic treatments for animals, although this is country dependent (Timmerman et al., 2006, Phillips et al., 2004, Graham et al., 2007, Paphitou, 2013).

1.2.1 Mechanism of Action of β-lactams

All Gram-positive and -negative bacterial cells are separated from their surroundings by a cell wall made up of peptidoglycan which is important for preventing the cell from lysis due to the higher internal osmotic pressure of the cell relative to the external environment (Scheffers and Pinho, 2005). Peptidoglycans are made up of glycan chains which consist of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) subunits. Through transglycosylation reaction, these subunits are joined together by β-1,4-glycosidic bonds to form long polymer chains (Figure 1.4a) (Holtje, 1998). Peptide cross-links are formed between the glycan polymers through a reaction called transpeptidation, this allows the formation of a three dimensional structure that gives rise to the bacterial cell wall (Figure 1.4b) (Holtje, 1998, Scheffers and Pinho, 2005).

Both the transglycosylation and transpeptidation reactions are required for the development of the peptidoglycan cell wall. The reactions are catalysed by transglycosylase and transpeptidase enzymes (Scheffers and Pinho, 2005). Such enzymatic activities can be found on a group of proteins called penicillin-binding proteins (PBPs), identified for their binding affinity towards penicillin, from which they have been given the name (Blumberg and Strominger, 1974). The types of PBPs found in a particular bacterial cell vary across species (Georgopapadakou, 1993). Some PBPs, such as PBP2 and PBP3 are monofunctional, hence

Figure 1.4 Simplified scheme of peptidoglycan macromolecule



(a) The peptidoglycan cell wall of bacteria is made up of glycan polymers which consist of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) monomers linked together by β-1,4-glycosidic bonds through a process called transglycosylation. The glycan polymers are arranged in a three-dimensional structure via the peptide cross-links. (b) The cross-linking of the glycan chains occurs via transpeptidation catalysed by penicillin-binding proteins which forms the peptide cross-link between the peptide side chains of the GlcNAc and MurNAc monomers.

they are only capable of catalysing the transpeptidation reaction (Ghuysen, 1997, Goffin et al., 1996), whereas PBP1A and PBP1B are bifunctional, acting both as a transpeptidase and transglycosylase (Ishino et al., 1980, Nakagawa et al., 1979).

The PBPs' important role in maintaining cell integrity has been shown in many studies (Nelson and Young, 2000, Spratt, 1975, Wei et al., 2003). As the name of these enzymes suggest, they are the targets of β-lactam antibiotics. The antibiotic inhibits the transpeptidation step of bacterial cell wall synthesis (Tipper and Stroming, 1965). Naturally, the peptide stem of two peptidoglycan monomer would undergo transpeptidation to form the peptide cross-link. But in the presence of β-lactam antibiotics, the compound act as competitive substrates for the PBPs. During transpeptidation, the antibiotics are covalently linked to the PBPs and render the transpeptidase enzyme inactive (Tipper and Stroming, 1965, Holtje, 1998). These antibiotics have been shown to affect cell growth via binding to PBPs which may lead to cell death (Lleo et al., 1987, Tuomanen et al., 1986).

1.2.2 Carbapenems

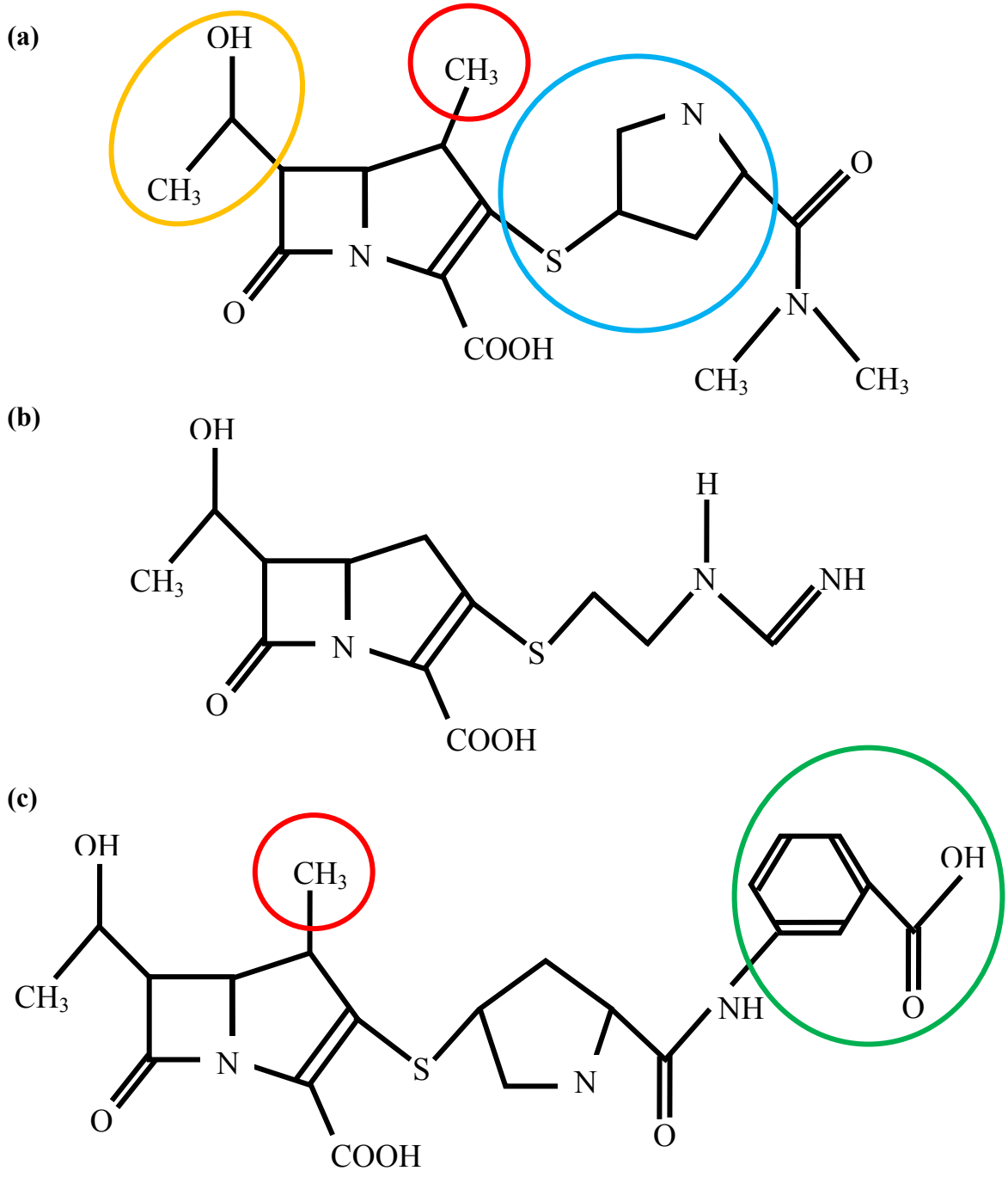
All β-lactam antibiotics share a similar mechanism of action by inhibiting bacterial cell wall biosynthesis. However, among all the β-lactam antibiotics, carbapenems are known to have a better efficacy against a broader spectrum of bacteria. The United States Food and Drug Administration (FDA) approved carbapenems are imipenem, meropenem, ertapenem and doripenem, with the latter being the newest member to this sub-class of β-lactam antibiotics (Hilas et al., 2008).

Unlike most other β-lactam antibiotics for which their antibacterial activities are restricted by bacterial growth rate, carbapenem killing activity is growth-independent (Cozens et al., 1989, Eng et al., 1991). Carbapenems also outrank other β-lactam antibiotics in terms of bacterial cell penetration and stability against most β-lactamases (Franceschini et al., 2002,

Iaconis et al., 1997, Yang et al., 1995). Carbapenems also retain antibacterial activity against various genera of bacteria producing a range of extended-spectrum β-lactamases (Schwaber et al., 2005). Due to its low toxicity, high efficacy and ultra-broad spectrum activity against many Gram-positive, -negative and anaerobic bacteria, carbapenems are often regarded as the last line of defence we have for severe bacterial infections (Papp-Wallace et al., 2011). Hence, these antibiotics are not easily prescribed to patients to avoid the selective pressure on carbapenem resistant bacteria (Fong et al., 2012, Mohr, 2008). However, when an infection is caused by extended-spectrum β-lactamase (ESBL) producing bacteria, carbapenems are often the first line of treatment options employed (Colardyn, 2005, Moellering et al., 1989, Mohr, 2008, Hawkey and Livermore, 2012).

Carbapenems mainly differ from each other in the side chains they have. Compared to other β-lactam antibiotics (Figure 1.3), all carbapenems have a unique hydroxyethyl group (Figure 1.5; Orange circle) which provides better stability against β-lactamases (Hammond, 2004). Meropenem and ertapenem possess a C1 methyl group which confers stability to renal dehydropeptidase (DHP-I) (Figure 1.5a & c; Red circles) (Drusano, 1997, Blumer, 1997, Musson et al., 2003). The lack of this methyl group causes imipenem to be easily degraded by this enzyme (Figure 1.5b). Hence, imipenem is prescribed with cilastatin (DHP-I inhibitor) (Blumer, 1997). The presence of the C2 moiety confers meropenem with enhanced activity against Enterobacteriaceae, including *P. aeruginosa* (Figure 1.5a; Blue circle). The side chain in this position also results in the difference in activity between meropenem and imipenem where the latter is more active against *Staphylococcus* spp. (Drusano, 1997). The benzoate side chain in ertapenem (Figure 1.5c; Green circle) increases its protein binding ability which leads to a longer half life (4 hours) when compared to meropenem (1 hour) and imipenem (1 hour) (Hammond, 2004).

Figure 1.5 Structures of carbapenem antibiotics



Structure of (a) meropenem, (b) imipenem and (c) ertapenem. Unlike other β-lactam antibiotics, carbapenems have a unique hydroxyethyl group which allows better stability against β-lactamases (Orange circle). The methyl group (Red circle) in meropenem and ertapenem confers stability to renal dehydropeptidase-I. The C2 moiety (Blue circle) in meropenem confers enhanced activity against Gram-negative bacteria, including *P. aeruginosa*. The benzoate group in ertapenem (Green circle) confers ertapenem with a longer half life [Source: Demain & Elander (1999) and Hamilton-Miller & Brumfitt (1974)].

1.3 Antibiotic Resistance

The introduction of antibiotics into clinical therapy has successfully reduced the mortality rate associated to bacterial infections (IDSA, 2011). Antibiotic resistance is an evolutionary process of bacteria to overcome the inhibitory effects of antibiotics since their introduction into clinical therapy. This was supported by a study showing most clinical isolates collected from various continents before the antibiotic era were antibiotic susceptible (Hughes and Datta, 1983). However, a more recent study has provided evidence of antibiotic resistance existing long before the history of mankind when β-lactam, glycopeptides and tetracycline resistance genes were identified in 30,000-year old Beringian permafrost sediment (D'Costa et al., 2011). Since its introduction, the therapeutic effects of antibiotics have been countered by the emergence and expansion of antibiotic resistant bacteria (Hopwood et al., 2007).

Unlike the number of methicillin-resistant *Staphylococcus aureus* (MRSA) which is stabilising or showing downward trends in the EU, the reports of multidrug-resistant *K. pneumoniae* and *E. coli* have been increasing in one third of the EU countries (ECDC, 2013a). Non-susceptibility (i.e. intermediate and resistant) to third-generation cephalosporins and carbapenem in Enterobacteriaceae was reported to be 33.4% and 7.6%, respectively. In both cases, *K. pneumoniae* showed the highest percentage of non-susceptibility among the isolates tested (ECDC, 2013a). In 2014, the World Health Organisation (WHO) reported the detection of third generation cephalosporin resistant *E. coli* in 86 (44%) of its Member States. Similarly, resistance to third generation cephalosporin and carbapenem in *K. pneumoniae* was reported in 87 (45%) and 71 (37%) of WHO Member States, respectively (WHO, 2014). In these data, the countries which were included in the figures were only considered to have significant antibiotic resistant bacteria when studies were available showing more than 30% of the tested isolates were resistant. This depicts the dire situation where the standard intravenous

treatments with cephalosporins have been rendered ineffective and the last line therapy with carbapenem is also being threatened (WHO, 2014).

1.3.1 Burden of Antibiotic Resistance

With infectious disease being one of the leading causes of death around the world, the presence of antibiotic resistant bacteria further increases the burden of infectious diseases. In 2007, 25,000 deaths per year in the European Union (EU) were estimated to be due to infections caused by antibiotic resistant bacteria (ECDC and EMEA, 2009). Of note, about 67 % of this value is due to infection with Gram-negative bacteria. As only five antibiotic resistant bacteria, i.e. methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecium*, carbapenem-resistant *Pseudomonas aeruginosa*, third-generation cephalosporin resistant *E. coli* and *K. pneumoniae*, were taken into account in the survey, the total number of deaths by antibiotic resistant bacteria is underestimated. In the same report, the estimated financial cost related to infections caused by the antibiotic resistant bacteria amounted to EUR 1.5 billion annually. The cost included outpatient care cost (~EUR 10 million/annum), productivity losses due to absence of work (~EUR 150 million/annum), and productivity losses due to deaths (~EUR 450 million/annum). The infections were also associated with an extra 2.5 million days of hospitalisation.

In the US, the medical cost of a single patient infected with an antibiotic-resistant bacterial infection in a Chicago teaching hospital was estimated to be between USD 18,588 to USD 29,069 in 2000 (Roberts et al., 2009). The studies also estimated the mortality rate associated with antibiotic resistant infections to be 6.5% and the societal cost to be between USD 10.7 to 15.0 million per annum. Patients with antibiotic-resistant infections also had between 6.4 to 12.7 excess days of hospitalisation per annum. Extrapolation of these data indicates the excess healthcare and societal cost associated with antibiotic-resistant infections

in the US to be approximately USD 20 billion and USD 35 billion per annum, respectively, with an excess of 8 million hospitalisation days per annum (Bush et al., 2011). Every year, there are approximately 2 million healthcare-associated infections with a majority of the infections contributed by antibiotic-resistant bacteria and resulting in almost 99,000 deaths in the US (Klevens et al., 2007).

In 2006, a national survey showed 300,000 of the infections reported in the United Kingdom (UK) annually were healthcare-associated infections and the cost for the treatment of the patients amounted to over GBP 1 billion per annum (NAO, 2009). The estimated number of excess deaths attributed to MRSA and third-generation cephalosporin resistant *E. coli* in the UK was 1,600 cases in the 2007 (de Kraker et al., 2011). In the same study, the excess number of days of hospitalisation and excess cost due to these antibiotic-resistant bacteria were approximately 73,000 days and EUR 17 million (de Kraker et al., 2011).

With these data combined, it can be seen that antibiotic-resistant bacteria cause a significant burden to the society. Moreover, studies have shown association of higher death rates with infections caused by antibiotic-resistant bacteria in certain clinical situations such as bloodstream infection and infection during liver transplant (Esterly et al., 2012, Kalpoe et al., 2012). In particular, carbapenem-resistant Enterobacteriaceae associated infections have been shown to cause significantly higher mortality compared to infections caused by the corresponding susceptible species (Falagas et al., 2014). If the antibiotic resistance phenomenon is left unchecked, it is estimated that by 2050, there will be about 10 million deaths per annum associated to antibiotic resistant infections (O'Neill, 2014).

1.3.2 Antibiotic Resistance Mechanisms

Bacteria employ a myriad of mechanisms to survive the stresses imposed by the presence of antibiotics (Fisher et al., 2005). As the target of all β-lactam antibiotics is essentially the PBPs

involved in cell wall synthesis (Blumberg and Strominger, 1974), alteration of the target proteins resulting in lower affinity towards the antibiotics can confer the host cell with resistance to the antibiotics. This has been reported in various bacteria such as *Neisseria gonorrhoeae* (Ropp et al., 2002), *Neisseria meningitidis* (Antignac et al., 2001) and *Haemophilus influenzae* (Matic et al., 2003).

In addition to the cytoplasmic membrane and peptidoglycan cell wall that Gram-positive bacteria have, Gram-negative bacteria also possess an outer membrane which is impermeable to hydrophobic compounds (Nikaido, 2003). Hence, Gram-negative bacteria possess hydrophilic protein channels also called porins which allow non-specific exchange of small hydrophilic solutes such as glucose, sucrose, amino acids and some antimicrobial compounds across the outer membrane (Lugtenberg and Van Alphen, 1983, Yoshimura and Nikaido, 1985). The major porins found in Gram-negative bacteria are from the outer membrane protein F (OmpF) and OmpC families (Pages et al., 2008). These porins are non-specific to the solutes which pass through them. However, they are more efficient in allowing the diffusion of cation compounds (Nikaido and Rosenberg, 1983). Both porins can be transcriptionally and post-transcriptionally regulated in response to external signals which include medium osmolarity, pH, nutrient limitation and temperature (Nikaido, 2003, Pages et al., 2008). In general, OmpF has a larger pore size compared to OmpC (Nikaido and Rosenberg, 1983). In *E. coli*, the pore sizes of OmpF and OmpC are 1.2 nm and 1.1 nm, respectively (Pratt et al., 1996). Hence the repression of OmpF and the increase expression of OmpC will impair the influx of larger solutes across the bacterial outer membrane (Nikaido and Rosenberg, 1983).

Two major types of porins i.e. OmpK35 (OmpF homologue) and OmpK36 (OmpC homologue) are found in *K. pneumoniae* (Albertí et al., 1995, Hernandez-Alles et al., 1995).

The artificial expression of OmpK35 porin in an antibiotic-resistant *K. pneumoniae* strain lacking both of the major porins was shown to reduce the minimum inhibitory concentration (MIC) values of carbapenems, fluoroquinolones, second-, third- and fourth-generation cephalosporins (Domenech-Sanchez et al., 2003). The expression of OmpK36 in the same study also resulted in a decrease in the MIC values of a range of antibiotics tested but to a lesser extent when compared to the effects of OmpK35 expression. As most non-ESBL producing strains of *K. pneumoniae* express both of the major porins whereas the ESBL producing strains of this bacterium only expresses the OmpK36 porin, the possibility of an interplay between the expression of ESBL and the selective loss of porin expression to result in an increase in antibiotic resistance of the bacteria cannot be excluded (Hernandez-Alles et al., 1999, Martínez-Martínez et al., 2002).

The sub-inhibitory intracellular concentrations of antibiotics resulting from the restricted entry of antibiotics imposed by down-regulation of porin expression can also be achieved by the up-regulation of efflux pumps (Nikaido, 2001). Efflux pumps can be categorised into five families, i.e. major facilitator superfamily (MFS) (Marger and Saier Jr, 1993), adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Schneider and Hunke, 1998), small multidrug resistant (SMR) family (Paulsen et al., 1996), resistance-nodulation-cell division (RND) superfamily (Saier et al., 1994), and multidrug and toxic compound extrusion (MATE) family (Brown et al., 1999). Efflux is an active process which utilises energy in the form of ion or proton gradient or ATP for active transportation of compounds across the cell barrier (Kumar and Schweizer, 2005).

Amongst the best characterised efflux pumps are the AcrAB-TolC system of *E. coli* and the MexAB-OprM system of *P. aeruginosa* (Nikaido, 1998, Poole, 2000). Both are pumps of the RND superfamily and they are comprised of a tripartite system which includes

an inner membrane transporter protein (AcrB and MexB), efflux adaptor protein (AcrA and MexA) and the outer membrane protein (TolC and OprM) spanning both the inner and the outer membrane of Gram-negative bacteria (Symmons et al., 2009). Through kinetic studies, the *E. coli* AcrAB-TolC has been shown to have a high efflux capacity for penicillins (Lim and Nikaido, 2010). This tripartite pump system has been found to be a major efflux system among members of the Gram-negative Enterobacteriaceae (Poole, 2005). Other studies in *K. pneumoniae* and *S.* Typhimurium have also implicated this efflux system with resistance to β-lactam antibiotics (Nikaido et al., 1998, Pages et al., 2009).

Among the 12 predicted RND efflux systems on the genome of *P. aeruginosa*, a clinically important nosocomial bacterium, MexAB-OprM is the efflux system which has been studied most extensively (Stover et al., 2000, Gillis et al., 2005). The MexAB-OprM efflux pump has been associated with carbapenem resistance in one study where clinical isolates with the loss of OprD porin expression showed increased resistance to meropenem (Pai et al., 2001). The porin has been shown to act as a channel allowing diffusion of carbapenem across the cell barrier of *P. aeruginosa* (Wolter et al., 2004). However, another study found no correlation between OprD or MexAB-OprM expression and carbapenem resistance (El Amin et al., 2005).

Shortly after the introduction of penicillin into clinical use, the first β-lactamase enzyme, termed penicillinase was isolated (Abraham and Chain, 1940). The β-lactamase enzymes are one of the major mechanisms of resistance in Gram-negative bacteria and have been classified into four classes on the basis of protein sequence (Ambler Class A to D) and by their substrate and inhibitor profile (Group 1 to 3) (Bush et al., 1995, Ambler, 1980). The enzymes of clinical relevance can be generally differentiated into ESBLs, AmpC β-lactamases and carbapenemases. Although there is no specific definition of an ESBL, it is normally a β-

lactamase which can hydrolyse penicillins, cephalosporins (first-, second- and third-generations), and aztreonam; but is susceptible to β-lactamase inhibitors (Paterson and Bonomo, 2005). The ESBL-producing Gram-negative bacteria are becoming increasingly prevalent worldwide, albeit showing different antibiotic susceptibility depending on geographical locations (Reinert et al., 2007, Nijssen et al., 2004, Kallen et al., 2011). As ESBLs show no significant activity against carbapenems (Paterson and Bonomo, 2005), these antibiotics are often the preferred choice of therapy for ESBL-producing Gram-negative bacterial infections (Rupp and Fey, 2003). AmpC β-lactamases have similar activities to ESBLs but are resistant to inhibitors such as clavulanic acid (Bush et al., 1995). Lastly, the carbapenemases consist of various enzymes which are mostly resilient to β-lactamase inhibitors and possess varying hydrolytic ability against all β-lactam antibiotics including carbapenems (Queenan and Bush, 2007, Thomson, 2010).

Although each mechanism can individually confer resistance to β-lactam antibiotics, bacteria sometimes employ a combination of these mechanisms to achieve extremely high level of resistance to the antimicrobial compound, such as the mutations in PBP3 and elevated expression of AcrAB efflux pump in *H. influenzae* (Kaczmarek et al., 2004). Similarly, the production of β-lactamases (e.g. CTX-M-15) and deficiencies in porins (OmpK35 and OmpK36) confer high carbapenem resistance in *K. pneumoniae* (Jacoby et al., 2004, Poulou et al., 2013, Ruiz et al., 2012), and over-expression of MexAB-OprM efflux pump in the absence of OprD porin confers *P. aeruginosa* with carbapenem resistance (Pai et al., 2001).

1.3.3 Carbapenemase-mediated Antibiotic Resistance

Infectious disease is one of the leading causes of death worldwide, with pneumonia and diarrhoeal disease as the main global causes of deaths (WHO, 2011). The efforts in place to overcome infectious disease have been hampered by the worldwide emergence of antibiotic

resistant bacteria (Arias and Murray, 2009). Few antibiotics remain active for the treatment of severe and life-threatening infections by Gram-negative bacteria (Spellberg et al., 2004, Piddock, Gould, 2008). The increase in numbers of antibiotic resistant Gram-negative bacteria has been further exacerbated with the lack of new efficacious agents in the pipeline (Piddock, 2012).

Gram-negative bacteria are associated with the major types of infections, i.e. pneumonia and urinary tract infection (Gaynes et al., 2005, Hidron et al., 2008). The number of antibiotic resistant Gram-negative bacteria such as *E. coli* has increased in European countries (Gagliotti et al., 2011) and elsewhere e.g. Taiwan (Chuang et al., 2010). Among the commonest Gram-negative bacteria causing infections are *E. coli*, *K. pneumoniae*, *Enterobacter* spp., *Serratia marcescens*, *P. aeruginosa* and *Acinetobacter* spp. (Gaynes et al., 2005, Chuang et al., 2010). Although *Enterobacter cloacae* and *S. marcescens* associated infections occur less frequently as compared to *E. coli* and *K. pneumoniae*, but *E. cloacae* is able to cause a wide range of infections (including bacteraemia, endocarditis, septic arthritis, etc.) while *S. marcescens* is associated with sepsis, meningitis and lung inflammation in neonates that can be fatal (ECDC, 2013b, Pages and Davin, 2015, Polilli et al., 2011). The spread and persistence of ESBL-producing bacteria has driven more usage of carbapenems in treatment, but the emergence of carbapenemase-producing bacteria has threatened these 'last-line' defence agents in clinical therapy against bacterial infections.

Carbapenemases are categorised into Class A, B and D following the Ambler class system (Jean et al., 2015). Class A and D carbapenemases possess a serine residue in their active site while Class B requires a divalent ion for it to hydrolyse β-lactam antibiotics (Jean et al., 2015). The members of the Class A carbapenemase include *K. pneumoniae* carbapenemase (KPC), Guiana extended spectrum enzyme (GES), not metalloenzyme

carbapenemase (NMC), imipenem-hydrolysing β-lactamase (IMI) and *S. marcescens* enzyme (SME). Class B metallo-β-lactamase is consists of imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), German imipenemase (GIM), Sao Paulo metallo-β-lactamase (SPM), Seoul imipenemase (SIM) and New Delhi metallo-β-lactamase (NDM). The Class D carbapenemases are mainly oxacillinases (OXA-23-like, OXA-24-like, OXA-48-like and OXA-58-like) (Jean et al., 2015). The classes of carbapenemases vary in their spectrum of activity on β-lactam antibiotics. Class A carbapenemases can hydrolyse all sub-classes of β-lactam antibiotics but are susceptible to β-lactamase inhibitors. The Class B metallo-β-lactamases are only susceptible to aztreonam while Class D carbapenemases can hydrolyse all β-lactam antibiotics but they have weak hydrolytic activity on cephalosporins and carbapenems (Nordmann, 2014). Carbapenemases which are closely associated with mobile elements (such as plasmids) are able to spread widely across various countries and cause serious hospital outbreaks (Jean et al., 2015, Nordmann, 2014). Two carbapenemase enzymes of current clinical concern are the KPC and NDM enzymes (Yigit et al., 2001, Yong et al., 2009).

The KPC enzyme was first discovered in 1996 in a *K. pneumoniae* isolated from a hospital in North Carolina (Yigit et al., 2001). The ca. 32 kDa enzyme is encoded by an 879 bp coding region, $bla_{\text{KPC-2}}$ (originally called $bla_{\text{KPC-1}}$) (Yigit et al., 2008) and confers resistance to penicillin, cephalosporin, carbapenem and monobactam antibiotics. The hydrolytic activity of KPC-2 is not influenced by ethylenediaminetetraacetic acid (EDTA) or zinc (II) chloride ($ZnCl_2$), but is inhibited by clavulanic acid. Amino acid sequence homology and the presence of known or suggested catalytic residues places the KPC-2 enzyme as a member of the Class A family of β-lactamases. Among all the antibiotics tested, this enzyme had the highest activity against meropenem (Yigit et al., 2001).

Currently, there are 22 variants of KPC enzymes (www.lahey.org). In contrast to the first discovered KPC enzyme, most KPC enzymes identified recently are associated with conjugative plasmids (Moland et al., 2003, Naas et al., 2005, Woodford et al., 2004, Yigit et al., 2001). This carbapenemase has developed to become the most common carbapenemase in the US (Gupta et al., 2011). Although the geographical location of *K. pneumoniae*-producing KPC enzymes was initially in the US, these bacteria are now found around the world (Bradford et al., 2004, Naas et al., 2005, Leavitt et al., 2007). The enzyme is also found in other species of Gram-negative bacteria, such as *E. coli* (Morris et al., 2011), *Salmonella* spp. (Miriagou et al., 2003, Rodríguez et al., 2014), *S. marcescens* (Cai et al., 2008), *Pseudomonas putida* (Bennett et al., 2009), *Citrobacter freundii* (Rosa Gomez-Gil et al., 2010) and *E. cloacae* (Zavascki et al., 2009). The bla_{KPC} gene is located within a ca. 10 kb Tn*4401* transposon (Gootz et al., 2009). At least seven isoforms (a – f; 2 variants of isoform 'd') have been reported (Bryant et al., 2013). These isoforms contain varying length of deletions upstream of bla_{KPC} which affect the expression of the carbapenemase gene (Bryant et al., 2013, Seecoomar et al., 2013). This bla_{KPC} carrying transposon is known to be mobile and have been found in various plasmid backbones contributing to the dissemination of this carbapenemase gene among Gram-negative bacteria (Partridge, 2014).

A more recently identified carbapenemase is the NDM enzyme which was first identified in *K. pneumoniae* isolated from the urine of an Indian patient in 2008 (Yong et al., 2009). The enzyme, designated as NDM-1 is encoded by a gene of 807 bp, producing a protein of 269 amino acids with a molecular mass of ca. 27.5 kDa. The 180 kb plasmid carrying the bla_{NDM-1} gene in this patient, named pNDM-1 was shown to be conjugative. Interestingly, a carbapenem resistant *E. coli* was also isolated from the faecal sample of the patient, carrying a smaller, 140 kb plasmid which also harbours the bla_{NDM-1} gene. This led

the authors to suggest the possibility of *in vivo* conjugation and arrangement of the plasmid between the species resulting in two different plasmid sizes. The transfer of the pNDM-1 plasmid to *E. coli* J53 via *in vitro* conjugation conferred the recipient cell with resistance to penicillins, monobactam, carbapenems, first-, second-, third- and fourth-generation cephalosporins (Yong et al., 2009). Since the discovery of the NDM-1 enzyme, 15 other variants have been identified (www.lahey.org). Over a short period of time since its discovery, this enzyme and its variants have been isolated in various species of bacteria in countries such as Kenya (Poirel et al., 2011), Australia (Poirel et al., 2010), China (Chen et al., 2011), Japan (Yamamoto et al., 2011), Norway (Samuelsen et al., 2011), Belgium (Bogaerts et al., 2011), UK (Jain et al., 2014) and the USA (CDC, 2010).

The emergence of antibiotic resistant bacteria has been further compounded by the isolation and possible zoonotic transmission of these bacteria from companion animals (CDC, 2001, Shaheen et al., 2011). Advances in aviation industry contributing to increase international travel and the distribution of food have also played against the human race by facilitating rapid dissemination of multidrug-resistant bacteria (Kenyon et al., 1996, Fey et al., 2000, Hawkey, 2015). Poor hygiene practices observed in healthcare services may further encourage the spread of such bacteria (Pittet et al., 1999).

1.3.4 Antibiotic Resistance Which Requires AcrAB-TolC

Although target alterations have been known to generate antibiotic resistant strains of bacteria (Lambert, 2005); at times, this mechanism itself does not confer clinically relevant resistance level. Through multiple steps of selection in increasing concentration of enrofloxacin, *S.* Typhimurium mutants with increase resistance to ciprofloxacin were obtained (Giraud et al., 2000). Fluoroquinolone resistance has been shown to arise from mutation in the DNA gyrase gene which affects the binding of the antibiotic to the gyrase-DNA complex (Willmott and

Maxwell, 1993). However, Giraud et al. (2000) showed a mutant (BN18/21) had increased ciprofloxacin resistance compared to the parent *Salmonella* (BN18) strain without having any mutations in the DNA gyrase or topoisomerase gene. Moreover, two other mutants (BN18/41 and BN18/71) which had an identical substitution (Gly81Cys) in DNA gyrase (*gyrA*) showed four-fold difference in ciprofloxacin susceptibility. Immunoblotting showed that the increase in ciprofloxacin resistance correlated to the increase expression of efflux pump protein (AcrA), suggesting the role of active efflux in the reduce susceptibility towards ciprofloxacin (Giraud et al., 2000).

In another study, multidrug resistant *S.* Typhimurium strain which exhibited high level resistance towards various fluoroquinolones was also found to have no mutations in any of the DNA gyrase and topoisomerase subunit genes (Baucheron et al., 2004). In this study, it was shown that other strains carrying substitutions in *gyrA* were slightly more resistant to the antibiotics tested. Inactivation of the *acrB* and *tolC* genes in the multidrug resistant strains decreased the level of resistance towards the fluoroquinolones. Addition of efflux pump inhibitor, phenyl-arginine-β-naphthylamide (PAβN) also increased the susceptibility of these *Salmonella* strains (Baucheron et al., 2004). In addition to fluoroquinolone resistance, tetracycline resistance conferred by Tet(A) protein encoded on a pBR322 plasmid also showed large reduction in tetracycline MIC in the *E. coli* host with non-functional AcrAB-TolC efflux pump (180 μg/ml to 30 μg/ml) (de Cristóbal et al., 2006).

It has been well established that AcrAB-TolC efflux pump is associated with resistance to some β-lactam antibiotics, including penicillins and cephalosporins (Nikaido et al., 1998, Opperman et al., 2014, Piddock, 2006a, Poole, 2004). Data from a study have suggested a possible synergistic effect of AcrAB-TolC and β-lactamase enzymes in conferring resistance to β-lactam antibiotics in *K. pneumoniae* (Pages et al., 2009). In this

study, it was observed that various clinical isolates carrying the chromosomal SHV-1 β-lactamase enzyme showed increasing susceptibility to piperacillin in the presence of tazobactam (β-lactamase inhibitor) and PAβN (Pages et al., 2009). In the presence of tazobactam, the MIC values of piperacillin of the various isolates decreased 4- to 8-fold. However, in the presence of tazobactam and PAβN, the susceptibility of these isolates increased further by 4-fold. The authors suggested that tazobactam was possibly a substrate of the AcrAB-TolC efflux system. Hence, the inhibition of the efflux system by PAβN increased the intracellular concentration of tazobactam, allowing a stronger effect of the β-lactamase inhibitor on the SHV-1 enzyme (Pages et al., 2009). However, it has been previously shown that inactivation of AcrAB-TolC in *E. coli* resulted in 16-fold increase in susceptibility of the bacterium to piperacillin (Opperman et al., 2014). Therefore, it is also possible that the larger increase in susceptibility of the *K. pneumoniae* isolates to piperacillin in the presence of tazobactam and PAβN was a result of the combined loss of the functions of the SHV-1 β-lactamase and the efflux system.

These studies with others showed the synergistic role played by active efflux in the background for conferring clinically significant resistance in the presence of other resistance mechanisms (Chen et al., 2007).

1.3.5 Efflux Inhibitors

With the dwindling number of efficacious antibiotics against severe infections, search has been initiated for other treatment options. One of these is compounds which are able to potentiate the activity of currently available antibiotics (Fernebro, 2011). As efflux pump confers resistance to a broad spectrum of structurally distinct antibiotic classes and are in certain cases a pre-requisite for antibiotic resistance related mutations, studies are underway

to develop inhibitors for this particular resistance mechanism (Ricci et al., 2006, Van Bambeke et al., 2006).

An ideal efflux inhibitor (EI) will have to possess one or more characteristics which includes being inactive against eukaryotic cells, inexpensive to manufacture, stable against proteolysis, high specificity and efficacy towards targets, and low toxicity to human (Bhardwaj and Mohanty, 2012). Various strategies of efflux inhibition can or have been explored, such as newer antibiotic molecules which are less susceptible to efflux (e.g. tigecycline); blockage of substrate binding site (e.g. PAβN); allosteric site modulators of efflux pump which affects the conformation of the substrate binding sites; un-coupler of efflux pump's energy source; blockers for outer membrane protein found in Gram-negative bacteria which is an important channel for antibiotics to exit the bacterial cell membrane; inhibitors which affects the assembly of efflux pumps; and inhibitors which reduce or abolish expression of the efflux pumps (Poole and Lomovskaya, 2006).

Through a screening of synthetic compound and fermentation extract using *P. aeruginosa* overexpressing various efflux pumps, a peptide-like compound (peptidomimetic) known as PAβN (previously, MC-207,110) which acts as an EI against Mex family of efflux pumps was discovered (Renau et al., 1999). This inhibitor was further examined for its activity as an EI (Lomovskaya et al., 2001). Lomovskaya et al. (2001) showed that PAβN fulfilled many of the criteria set to be an ideal inhibitor. PAβN showed no significant antibacterial activity but significantly decreased the MIC values for levofloxacin of *P. aeruginosa* overexpressing various efflux pumps. Moreover, the EI successfully decreased spontaneous mutant selections in wildtype *P. aeruginosa* similar to the level observed when efflux pumps were deleted from the bacterium (10^{-7} to $<10^{-11}$ cfu/ml) (Lomovskaya et al.,

1999, Lomovskaya et al., 2001). Further studies of structurally similar peptidomimetic compounds also yielded similar inhibition activity (Renau et al., 2002).

1.4 Spread and Acquisition of Antibiotic Resistance

The development of antibiotic resistant bacteria can occur through two ways, i.e. spontaneous mutation or lateral gene transfer (LGT) (MacLean et al., 2010). For bacteria which are intrinsically resistant to many antibiotics, such as *P. aeruginosa*, these mechanisms can increase the resistant levels to the antibiotics, in addition to acquisition of new resistance phenotypes (Nicas and Hancock, 1983, Sacha et al., 2008). On the other hand, spontaneous chromosomal mutations have been associated with resistance to various β-lactam antibiotics due to increase of AmpC β-lactamase activity (Raimondi et al., 2001), inhibition of antibiotic target affinity via target alteration (Katayama et al., 2004, Sreevatsan et al., 1996) and decreased antibiotic accumulation due to regulatory mutations that alter porin production and increase efflux activity (Yoneyama and Nakae, 1993, Chuanchuen et al., 2001).

1.4.1 Lateral Gene Transfer (LGT) in Dissemination of Antibiotic Resistance

While spontaneous mutation confers resistance to a particular bacterium and its progeny by cell division, LGT allows acquisition and dissemination of antibiotic resistance genes from and to other bacteria via transfer of genetic material (Mitsuhashi et al., 1967). The three mechanisms, whereby LGT occurs are: transformation which involves the uptake and subsequent incorporation of naked DNA from the environment into the genome of a competent host cell; transduction, in which the DNA from a bacterium is packaged into a bacteriophage and transferred into a recipient cell during infection by the bacteriophage; and conjugation which requires cell-to-cell contact for the transfer of genetic material from the donor to the recipient cell (Davison, 1999). An alternative pathway involving genetic material enclosed in membrane vesicles has also been described (Yaron et al., 2000). Such membrane

vesicles have been shown to be produced by *E. coli*, *N. gonorrhoeae*, *P. aeruginosa* and *Acinetobacter baumannii* (Pérez-Cruz et al., 2015, Yaron et al., 2000). These vesicles contain chromosomal DNA, plasmid and various proteins (Pérez-Cruz et al., 2015, Yaron et al., 2000). These membrane vesicles have been shown to be able to successfully transfer virulence and antibiotic resistance genes that are then expressed in the recipient cells (Yaron et al., 2000). However, the importance of such membrane vesicles and the mechanisms involving the packaging of the DNA in the vesicles remain unclear.

Although transformation is a very common methodology used in molecular biology research to confer a detectable trait in artificially competent host cells, this process also occur in the natural environment among naturally competent bacteria (Lorenz and Wackernagel, 1994). In the natural environment, DNA is released to the surrounding environment by excretion from viable cells and cellular lysis (Lorenz et al., 1991, Sinha and Iyer, 1971). Even though DNA is prone to degradation by nucleases, DNA adsorbed on to groundwater aquifer material is 1000 times more resistant to DNase I. This suggest that extracellular DNA found in the natural environment bound to mineral material containing inorganic precipitates and organic matter can be readily available for natural transformation (Romanowski et al., 1993). Acquired β-lactamase resistance via natural transformation has been demonstrated in Gram-negative bacteria such as *Helicobacter pylori* and *N. gonorrhoeae* (Kwon et al., 2003, Ohnishi et al., 2010).

Although evidence of transduction as a major method for antibiotic resistance dissemination between Gram-negative bacteria is lacking, a few studies have shown the occurrence of various β-lactamase genes in phage particles, suggesting the possibility of bacteriophage as a reservoir for these resistance determinants in the natural environment (Muniesa et al., 2004, Colomer-Lluch et al., 2011). As naked DNA is susceptible to

degradation in the natural environment, the organisation of DNA into a bacteriophage capsid may be protective and allow transduction to be an efficient process for the spread of antibiotic resistance genes (Colomer-Lluch et al., 2011). Some *in vitro* studies have also supported this notion where transduction carried out with bacteria such as *P. aeruginosa* and *S.* Typhimurium conferred antibiotic resistance to recipient cells (Schmieger and Schicklmaier, 1999, Blahová et al., 1999).

In conjugation, genetic material is transferred from the donor to the recipient cell, either as a conjugative plasmid, which is able to effect its own transmission between cells; or a mobilisable plasmid, which is non-self-transmissible but can be transferred to the recipient cell with the help of a conjugative plasmid; or via a conjugative transposon (Clewell et al., 1995, Davison, 1999). Plasmids are self-replicating covalently closed circular double-stranded extrachromosomal DNA molecules (Actis et al., 1999). Although not usually essential for the survival of host bacteria, plasmids can confer beneficial traits to its host, such as resistance to antimicrobial compounds, hence of benefit in terms of survival in a particular ecological niche (Foster, 1983). Similar to plasmids, conjugative transposons also play an important role in the carriage and dissemination of antibiotic resistance genes either by their own mobilisation or by effecting the transmission of non-conjugative plasmids (Clewell et al., 1995).

1.5 Plasmid-mediated Antibiotic Resistance

A study on Enterobacteriaceae found in the cultures collected by E.D.G Murray from 1917 to 1954 was carried out to compare the conjugative plasmids present during the 'pre-antibiotic' era and the period after introduction of antibiotics, which is believed to be the driving force for dissemination and proliferation of bacteria carrying plasmids with resistance determinants (Anderson, 1965, Hughes and Datta, 1983). Hughes and Datta (1983) showed at least 19% of the strains collected from various geographical locations, including Europe, Malta, India,

Northern Russia, the Middle East and North America, before the antibiotic era harboured conjugative plasmids. Of note, none of these conjugative plasmids carried any antibiotic resistance determinants (Hughes and Datta, 1983). By incompatibility (Inc) testing, most of these conjugative plasmids were in the same Inc groups as modern resistance plasmids (Datta and Hughes, 1983).

As conjugative plasmids were commonly found in Enterobacteriaceae during the 'pre-antibiotic' era and these plasmids belong to the same groups as the resistance plasmids observed after the usage of antibiotics, it is hypothesised that the modern resistance plasmids are of the same lineages as the former and were derived via the acquisition of resistance determinants by existing vectors and not simply by the selection of rare resistance plasmids due to antibiotic usage. This finding also suggests plasmids have a high rate for acquisition and dissemination of genes amongst bacteria (Datta and Hughes, 1983). The discovery of antibiotic resistance genes being at least 30,000 years old further supports the possibility of plasmids as a vehicle to acquire and spread antibiotic resistance determinant (D'Costa et al., 2011).

1.5.1 The *bla*$_{KPC}$-encoding pKpQIL Plasmid

The KPC enzyme was first detected in North Carolina in 1996 (Yigit et al., 2001). In the following decade, this carbapenemase has successfully spread across most of the states in the USA and across the globe (Arnold et al., 2011). In Israel, this enzyme was reported in 2005 and 2006, where 93% of the 46 carbapenem-resistant *K. pneumoniae* clinical isolates from various sites of infection carried this enzyme (Leavitt et al., 2007). Most isolates were from the same pulsotype determined by pulsed-field gel electrophoresis, designated as clone Q and carried the *bla*$_{KPC-3}$ gene. During the same period, a similar outbreak of carbapenem-resistant *K. pneumoniae* carrying *bla*$_{KPC-3}$ in patients from different clinical specialties was

documented in a nearby hospital (Samra et al., 2007). Clone Q was later found to be a hyper-epidemic clone carrying a common plasmid causing a nationwide outbreak in Israel. This clone was genetically related to the outbreak strains of *K. pneumoniae* isolated from the US (Navon-Venezia et al., 2009). This clone was later determined as multilocus sequence type (ST) 258 (Kitchel et al., 2009).

The common plasmid which was associated with carbapenem resistance of the *K. pneumoniae* ST258 isolates in Israel was named pKpQIL (Leavitt et al., 2010b). The plasmid was shown to be conjugative and harboured a $bla_{\text{TEM-1}}$ and $bla_{\text{KPC-3}}$ β-lactamase genes (Leavitt et al., 2010b). Sequencing of the plasmid determined it to be a 113,637 bp IncFII-like plasmid (Leavitt et al., 2010a). The majority of pKpQIL (86,610 bp) shares 99% identity with the pKPN4 plasmid from multidrug-resistant *K. pneumoniae* MGH 78578 (ATCC 700721). The $bla_{\text{KPC-3}}$ gene is organised within the Tn*4401* transposon as previously described (Naas et al., 2008). The plasmid was stable without apparent changes during the two years of the outbreak (Leavitt et al., 2010b). Recently, pKpQIL or its derivatives were detected in a majority of Polish bla_{KPC}-producing *Klebsiella* spp. clinical isolates (Baraniak et al., 2011). A pKpQIL-like plasmid has also been found to be involved in hospital outbreak in Italy, Korea and Czech (García-Fernández et al., 2012, Richter et al., 2012, Hrabák et al., 2013, Lee et al., 2014). A recent study suggested that the pKpQIL plasmid may have originated from the US as similar plasmids were identified from various Enterobacteriaceae isolated as early as 2003 (Chen et al., 2014a, Tang et al., 2014). These suggest the possibility of the success of pKpQIL plasmids across geographical borders since its isolation in the US and its stability as a platform for the dissemination of bla_{KPC} genes.

1.6 Factors Important in Plasmid Dissemination in Enterobacteriaceae

Although plasmid DNA can be transferred between bacterial cells via natural transformation and transduction (Garzon et al., 1995, Chamier et al., 1993), conjugation is the major mechanism of plasmid transfer (Barlow, 2009). Conjugation occurs in three steps: DNA substrate processing, substrate recruitment and translocation of substrate to recipient cell (Alvarez-Martinez and Christie, 2009). In conjugation, a nucleoprotein complex is formed between a relaxase and other auxiliary proteins with the origin of transfer (*oriT*) sequence on the plasmid DNA. This complex is termed the relaxosome and results in the nicking of the supercoiled plasmid to produce a relaxed form. The relaxosome then translocates across the conjugative pore into the recipient cell, where re-circularisation and establishment of the plasmid occurs (Frost and Koraimann, 2010).

Plasmids such as those from the IncF group contain a transfer region encoding more than 30 transfer (*tra*) genes, which play an important role in DNA metabolism, type IV secretion system (T4SS) and regulation of the plasmid transfer potential (Frost and Koraimann, 2010). There are eight core genes involved in regulation of conjugation (Table 1.1).

1.6.1 Plasmid Stability and Segregation

In the presence of a selective pressure such as antibiotics, plasmid-harbouring cells which possess the beneficial trait such as resistance to the antibiotic would survive in the population. However, in the absence of the selective pressure, the plasmid may incur a metabolic burden on its host cell (Jakobsen et al., 2006, Loftie-Eaton and Rawlings, 2010). In order to avoid itself from being lost when the host cells are actively dividing, plasmids have acquired properties such as toxin-anti-toxin systems and active segregation machineries to allow plasmid persistence in the host (Engelberg-Kulka and Glaser, 1999, Greenfield et al., 2000).

Table 1.1 Function of genes involved in conjugation

Gene	Function
traM	Processing of DNA for transfer Regulation of its own promoter, P_m
traJ	Activator of *tra* gene expression Activates P_y
traY	Processing of DNA prior to transfer Regulation of its own promoter, P_y and activates P_m
finP	Antisense RNA inhibitor of TraJ
finO	Stabilises FinP to avoid degradation by RNase E
hfq	Promotes TraJ degradation, hence reducing transfer potential
arcA	May be involved in activation of P_y ArcAB represses succinate dehydrogenase (*sdhABCD*)
sdhABCD	May be involved in reduction of transfer potential by regulation of TraJ

Adapted from Frost and Koraimann (2010). The *tra* locus of a conjugative plasmids may contain about 30 genes which are involved in the conjugation process. Eight genes which play the core regulative functions of conjugation are listed in the table.

Plasmid encoded toxin-anti-toxin systems play a role in postsegregational killing. In the presence of the plasmid, both the stable toxin and labile anti-toxin are produced. When the plasmid is lost, the host bacterium is unable to survive as there is no production of the labile anti-toxin to inhibit the effects of the more stable toxin still present in the cell. This ensures the permanent linkage between survival of the host cell with the presence of the plasmid (Yamaguchi and Inouye, 2011). Disruption of the interaction between the toxin and anti-toxin therefore eliminates the plasmid by killing the cell (Lioy et al., 2010).

Although high-copy number plasmids may stably propagate by random segregation during cell division, low-copy number plasmids require an active partitioning system to ensure each daughter cell carries at least one copy of the plasmid. Most of the active partitioning systems are comprised of three components, i.e. ParA (a nucleotide-binding motor element), ParB (a DNA-binding adaptor protein), and a centromere-like region. In general, the ParB protein binds to the centromere-like region to form a nucleoprotein complex, which then interacts with the ParA motor element to allow the active partitioning of the plasmid at the opposite pole of the cell to ensure the persistence of the plasmid in each daughter cell after cell division (Ghosh et al., 2006, Salje, 2010).

1.6.2 Inhibition of Plasmid Transfer

Due to their importance in dissemination of antibiotic resistance, plasmids have been targeted by strategies designed to prevent the spread of plasmid DNA (Williams and Hergenrother, 2008).

An *in vitro* study has shown that targeting F plasmid relaxase, the enzyme important for the formation of the relaxosome which allows the translocation of plasmid into the recipient cell, inhibits conjugation and selectively kills bacteria harbouring the conjugative plasmids through an unknown mechanism (Lujan et al., 2007). The authors showed that the

inhibition of relaxase was achieved using bisphosphonates, etidronate and clodronate, which are clinically approved for bone loss treatment. The results suggest an opportunity for the combination therapy between the bisphosphonates with antibiotics for the treatment of bacterial infections (Lujan et al., 2007). However, this hypothesis has yet to be investigated *in vitro*.

Plasmid incompatibility has also been exploited as a possible mechanism for the elimination of specific plasmids as those with the same incompatibility group cannot stably co-segregate into the daughter cells (Novick, 1987). The replication of IncB plasmids is regulated by the RepA protein which in turn is regulated by RNA I (Thomas et al., 2005). Screening of various aminoglycosides found apramycin to mimic the function of RNA I, by inhibiting the translation of RepA protein, which in turn inhibits plasmid replication and subsequently resulting in plasmid elimination and increased susceptibility of the host cell to antibiotics (Thomas et al., 2005, DeNap et al., 2004).

1.7 Plasmid-mediated Antibiotic Resistance and Fitness

A study competing plasmid RSF2124-carrying *E. coli* against plasmid-free *E. coli* without antibiotic showed an initial decrease in fitness of the plasmid-carrying strain (Helling et al., 1981). However, after 18 generations of growth, it was found that the plasmid-carrying strain possess a better fitness relative to the plasmid-free strain. When the evolved plasmid after 18 generation was introduced into the initial plasmid-free *E. coli*, no fitness advantage was observed. Hence, the authors concluded that adaptive changes in the host chromosome conferred the fitness advantage after a certain number of generations of growth (Helling et al., 1981).

A similar study also compared the fitness of plasmid-carrying and plasmid-free *E. coli* (Bouma and Lenski, 1988). The authors showed that *E. coli* carrying the pACYC184 plasmid

which encodes resistance determinants for chloramphenicol and tetracycline possessed a higher fitness cost compared to the plasmid-free strain. Interestingly, after the *E. coli* carrying the plasmid was grown for 500 generation (75 days) in the presence of chloramphenicol, the evolved host (H_{500}) carrying the ancestral plasmid ($H_{500}P_0$) showed significant increase in fitness compared to plasmid-free host (H_{500}) in the absence of antibiotic. This study also suggested the adaptive changes were found only on the host chromosome (Bouma and Lenski, 1988).

Further investigation was pursued to identify the determinants on the plasmid pACYC148 which resulted in the fitness cost of the naive host (H_0) and the resulting fitness benefit of the pACYC148 coevolved host (H_{500}) (Lenski et al., 1994). By constructing various deletions on the pACYC148 plasmid, the authors were able to show that the chloramphenicol resistance gene was responsible for the fitness cost incurred on the naive host. As the evolved host carrying the plasmid devoid of the chloramphenicol resistance still possesses a fitness advantage against plasmid-free evolved host, this suggested the chloramphenicol resistance gene was not involved in the fitness advantage observed. When the tetracycline resistance cassette was deleted, it was found that this function had a major role in the fitness advantage observed in the coevolved host as the coevolved host carrying this plasmid had a fitness cost. However, the importance of the tetracycline resistance gene in the evolution which ameliorates the fitness cost is unclear (Lenski et al., 1994).

In contrast to the above experiments which were using non-conjugative laboratory plasmids, similar study was conducted on natural conjugative plasmids (ca.100 kb plasmid R1 and ca. 60 kb plasmid RP4) in *E. coli* to assess the fitness impact of natural conjugative plasmids on their host (Dahlberg and Chao, 2003). Serial propagation of the plasmids with their host over a period of 166 days (ca. 1100 generation), showed the initial fitness cost

exhibited by the plasmid-carrying host was reduced significantly at the end of the study. Unlike the previous studies (Bouma and Lenski, 1988, Lenski et al., 1994), the compensatory adaptation was suggested to be in both the chromosome and plasmids. Although the chromosomal compensatory mutation was not determined, but the changes in the plasmids which led to a lower fitness cost were found to be deletions of loci associated with plasmid transfer function and antibiotic resistance (Dahlberg and Chao, 2003). Under the conditions tested, this study also found that the host which evolved in the absence of the plasmid was able to reduce the cost of the plasmids introduced into it. This suggests that the fitness cost alleviating adaptation which occurred in the host was also in part due to its own response to its growth conditions (Dahlberg and Chao, 2003).

1.8 Background to This Project

1.8.1 Plasmid pCT

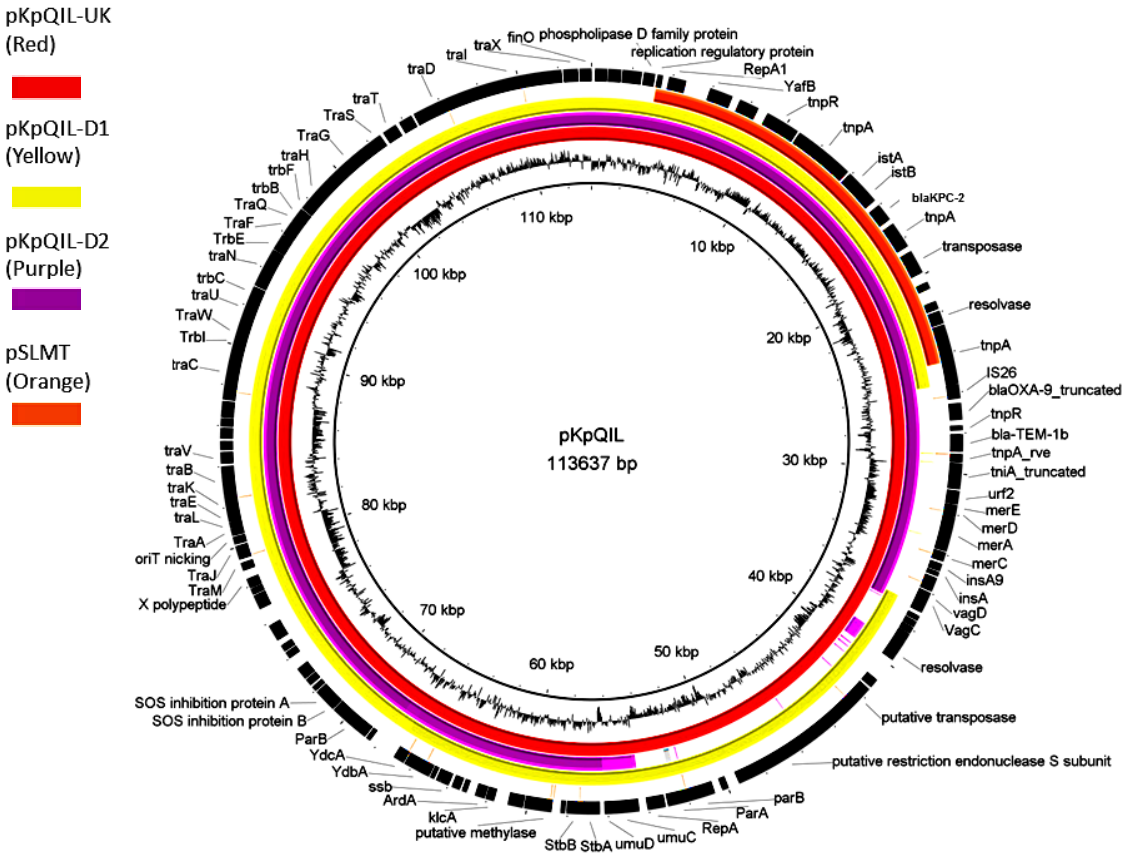
An IncK plasmid carrying a single $bla_{\text{CTX-M-14}}$ ESBL gene was isolated from scouring (diarrhoea) cattle in the UK (Liebana et al., 2006). The pCT plasmid and pCT-like plasmids have since been detected in human clinical isolates from the UK, Spain, Australia and China (Cottell et al., 2011, Dhanji et al., 2012). Previous study of the pCT plasmid by inactivation of the $bla_{\text{CTX-M-14}}$ ESBL gene found that it carries no fitness benefit or cost on its host in the presence or absence of antibiotic selection pressure (Cottell et al., 2012). Inactivation of various genes on the pCT plasmid also found no significant effect on the maintenance of the plasmid in the bacterial cell or in a bacterial population when compared to the wildtype plasmid (Cottell et al., 2014). This finding challenges the dogma which relates the persistence of antibiotic resistance gene carrying plasmids to antibiotic selective pressure (Anderson, 1965).

1.8.2 Plasmid pKpQIL-UK & pKpQIL-D2

Carbapenemase-producing Enterobacteriaceae was first isolated in the UK in 2003. However, it was not until 2008 that a drastic increase in the number of annually reported carbapenemase-producing isolates was observed. Until the second quarter of 2011, the dominant carbapenemase, i.e. bla_{KPC} accounted for 384 (58%) of 657 carbapenemase-producing Enterobacteriaceae isolates reported since 2003. Although the KPC enzymes have spread within some UK hospitals, this carbapenemase has been a problem particularly in the North Western (NW) region of England (HPA, 2011).

A study by Public Health England on 17 bla_{KPC}-harbouring clinical isolates referred by various referral centres in the NW England region showed that 14 of the isolates carried the plasmid pKpQIL and its variant (Figure 1.6), with the remaining three isolates undetermined (Table 1.2). Most of the isolates were *K. pneumoniae* with the rest being other species of the Enterobacteriaceae family. Sequence analyses showed that most of the plasmids were similar to the one reported in Israel (henceforth, termed pKpQIL-UK). Five from the 14 isolates harboured a derivative plasmid of pKpQIL-UK (Table 1.2). One derivative, named pKpQIL-D1 carries a new ca. 19 kb DNA segment which replaces the original ca. 11 kb segment beginning from the truncated bla_{OXA-9} to the *vagC* gene in the pKpQIL-UK (Figure 1.7). Another derivative, named pKpQIL-D2 had a ca. 20 kb DNA segment between *vagC* and *stbA* of the pKpQIL-UK substituted with a new ca. 16 kb DNA segment (Figure 1.8). While most pKpQIL-UK plasmids were found in *K. pneumoniae* ST258, its derivative, i.e. pKpQIL-D2 was found in other Enterobacteriaceae species and sequence types of *K. pneumoniae*. It is possible the substitution with the new ca. 16 kb DNA segment has provided the variant plasmid with the ability to better disseminate across the species barrier.

Figure 1.6 Alignment of plasmid pKpQIL-UK and variants



The diagram was generated using Blast Ring Image Generator. It shows the plasmid alignments of pKpQIL-UK with pKpQIL-D2, pKpQIL-D1 and pSLMT. From the inner most ring, (1) Size of the plasmid, (2) GC content of the sequence, (3) pKpQIL-UK (red), (4) pKpQIL-D2 (purple), (5) pKpQIL-D1 (yellow), (6) pSLMT (orange) and (7) plasmid genes of pKpQIL-UK (black) (Source: Woodford, N. Public Health England).

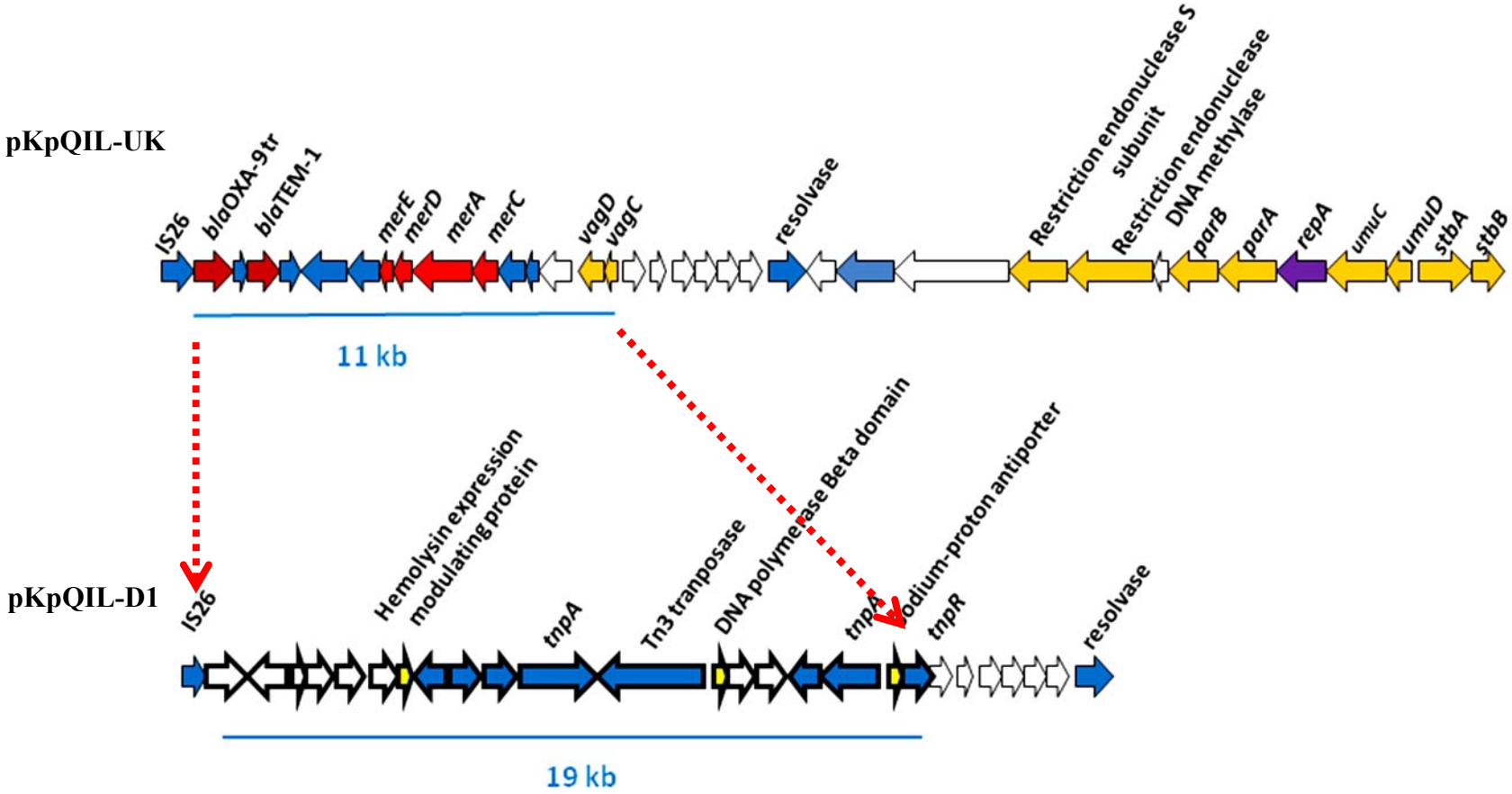
Table 1.2 Distribution of pKpQIL-UK and variants from medical centres in North Western England Region

Isolates	Species	ST-type	Centre	*bla*$_{KPC}$	Inc_type	Plasmid Name
T4	*K. pneumoniae*	258	Centre_6	2	FIIK2	pKpQIL-UK
T6	*K. pneumoniae*	258	Centre_2	3	FIIK2	pKpQIL-UK
T8	*K. pneumoniae*	258	Centre_7	2	FIIK2	pKpQIL-UK
T11	*K. pneumoniae*	258	Centre_8	2	FIIK5	ND
T13	*K. pneumoniae*	11	Centre_1	3	FIIK2	pKpQIL-UK
T19	*K. pneumoniae*	11	Centre_1	2	FIIK2	pKpQIL-D1
T27	*K. pneumoniae*	258	Centre_5	2	FIIK2	pKpQIL-UK
L16	*K. pneumoniae*	258	Centre_3	2	FIIK2	ND
L19	*K. pneumoniae*	258	Centre_1	2	FIIK2	pKpQIL-UK
L33	*K. pneumoniae*	468	Centre_3	2	FIIK2	pKpQIL-UK
L27	*K. pneumoniae*	321	Centre_1	2	FIIK2	pKpQIL-D2
L37	*K. pneumoniae*	491	Centre_1	2	FIIK2	ND
L38	*K. pneumoniae*	490	Centre_1	2	FIIK2	pKpQIL-D2
LENT	*Enterobacter spp.*	ND	Centre_1	2	FIIK2	pKpQIL-D2
LESC	*E. coli*	ND	Centre_1	2	FIIK2	pKpQIL-D2
I2	*Escherichia coli*	ND	Centre_4	2	FIIK2	pKpQIL-UK
I1	*K. pneumoniae*	ND	Centre_4	2	FIIK2	pKpQIL-UK

ND: Not determined

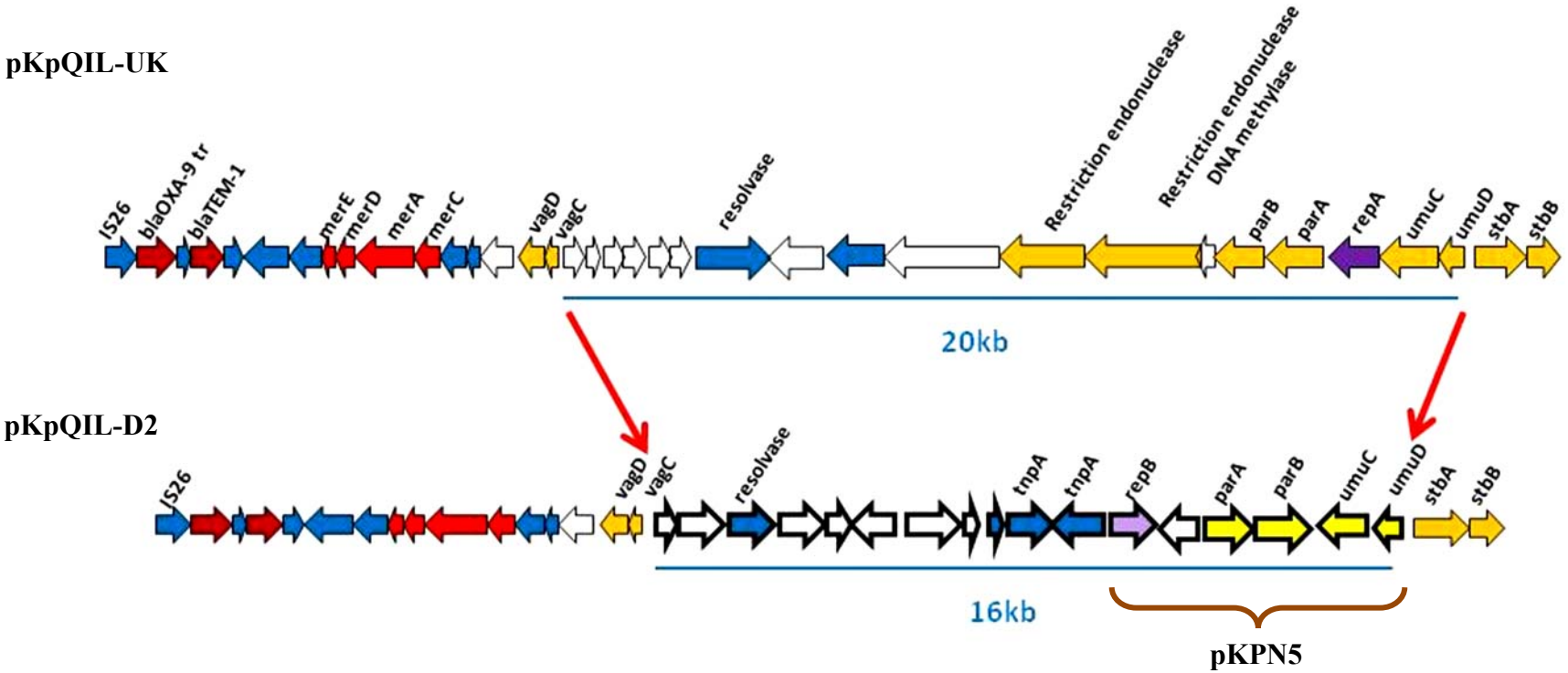
The pKpQIL-UK and -D2 plasmids used in this PhD study were from the clinical isolate L33 and L27, respectively.
Source: Woodford, N. Public Health England

Figure 1.7 Comparison of the substitution fragment of pKpQIL-UK and -D1



The diagram shows the 11 kb region on pKpQIL-UK which has been substituted with a new 19 kb fragment to form the pKpQIL-D1 plasmid. The open reading frames (ORF) include genes encoding resistance (Red), transposable elements (Blue), plasmid replicon (Purple), genes with no significant homologies to infer functions (White) and other plasmid genes (Yellow). Hypothetical protein (White) had no significant hit when analysed with BlastP (to search for protein homology), PROSITE (to detect conserved protein motif) and PSORT (protein localisation predictor).

Figure 1.8 Comparison of the substitution fragment of pKpQIL-UK and -D2



The diagram shows the 20 kb region on pKpQIL-UK which has been substituted with a new 16 kb fragment to form the pKpQIL-D2 plasmid. The open reading frames (ORF) include genes encoding resistance (Red), transposable elements (Blue), plasmid replicon (Purple), genes with no significant homologies to infer functions (White) and other plasmid genes (Yellow). The fragment on pKpQIL-D2 (highlighted with brown bracket) shares 98% DNA sequence identity with pKPN5 plasmid from *K. pneumoniae* MGH78578. Hypothetical protein (White) had no significant hit when analysed with BlastP (to search for protein homology), PROSITE (to detect conserved protein motif) and PSORT (protein localisation predictor).

1.9 Hypotheses to be Investigated in This PhD Project

Although the use of antibiotics has a role in the dissemination of antibiotic resistance plasmids, this may not account for the spread of all antibiotic resistance currently observed, especially since conjugative plasmids without any resistance determinants were already common among bacterial strains prior to the antibiotic era (Datta and Hughes, 1983, Hughes and Datta, 1983). The bla_{KPC} gene has been observed to be transmitted by the pKpQIL plasmid in various countries around the world. The emergence of pKpQIL-D2 in the UK along with the original pKpQIL-UK plasmid warrants further investigation on both of these plasmids. The hypotheses to be investigated in this project are as follow:

i) The ca. 16 kb substitution region in pKpQIL-D2 confers a fitness benefit to the plasmid and/or plasmid-carrying host when compared to pKpQIL-UK.

ii) A functional AcrAB-TolC efflux pump is required to confer carbapenemase-producing bacteria with high carbapenem resistance.

1.10 Aims & Objectives

The objective of the research was to investigate plasmid mediated carbapenemase resistance and identify the differences in effects caused by the two plasmids (pKpQIL-UK and pKpQIL-D2) on the host. The specific aims were:

1. To evaluate the role of a functional AcrAB-TolC efflux pump in plasmid-mediated carbapenem resistance.

2. To assess the fitness cost or benefit of pKpQIL-UK and -D2 on their hosts.

3. To identify the potential gene(s) involved in conferring the fitness cost or benefit in the host.

CHAPTER TWO: METHODS

2.1 Bacterial Strains, Plasmids & Culture Conditions

Bacterial strains were routinely cultured in Luria Bertani (LB) agar (Sigma-Aldrich, Missouri, USA, Catalogue No.: L2897) and LB broth (Sigma-Aldrich, Missouri, USA, Catalogue No.: L3022) at 37°C. All bacterial cultures were stored on Protect Microorganisms Preservation System Beads (Thermo Fisher Scientific, Massachusetts, USA, Catalogue No.: TS80) at -80°C (*Salmonella* strains were kept at -20°C). When required, a Protect Bead was used to streak the bacteria of interest on to LB agar to obtain single colonies. The culture was then incubated overnight (12 – 16 hours) at 37°C. When liquid cultures were required, a single colony was used to inoculate 10 ml LB broth and incubated at 37°C with shaking (200 rpm) for 12 – 16 hours. Temperature for incubation was reduced to 30°C for temperature sensitive strains and plasmids. When necessary, growth medium was supplemented with the relevant antibiotics (Table 2.1). All experiments involving bacterial cultures were carried out aseptically. The plasmids and bacterial strains used in this study are listed in Table 2.2 and Table 2.3, respectively.

2.1.1 Bacterial Identification

In order to ensure the bacterial strains used in the study were correct, the strains were frequently grown on differential and selective media such as Xylose-Lysine-Deoxycholate (XLD) agar (Oxoid, Massachusetts, USA, Catalogue No.: CM0469) and MacConkey agar (Oxoid, Thermo Fisher Scientific, Massachusetts, USA, Catalogue No.: CM0007). The bacterial strains were differentiated by their morphological appearance on the differential agar according to the manufacturer's descriptions (Table 2.4). Gram staining (Sigma-Aldrich, Missouri, USA, Catalogue No.: 77730) was also used as a rapid test to confirm the type of bacteria as all the strains used in this study were Gram-negative bacteria. When a mutant

Table 2.1 List of antibiotics used in this study

Antibiotic	Supplier	Catalogue No.	Solvent
Ampicillin	Calbiochem, Darmstadt, Germany	171254	Sterile water
Biapenem	Sigma-Aldrich, Missouri, USA	SL0306	Sterile water
Cefotaxime	Sigma-Aldrich, Missouri, USA	C7912	Sterile water
Ceftazidime	Sigma-Aldrich, Missouri, USA	C3809	Sterile water
Cephalothin	Sigma-Aldrich, Missouri, USA	C4520	Sterile water
Chloramphenicol	Sigma-Aldrich, Missouri, USA	C0378	70% (v/v) ethanol in water
Doripenem	Sigma-Aldrich, Missouri, USA	32138	Sterile water
Ertapenem	MSD, New Jersey, USA	FR3843	Sterile water
Erythromycin	Sigma-Aldrich, Missouri, USA	E5389	70% (v/v) ethanol in water
Imipenem	Sigma-Aldrich, Missouri, USA	I0160	Sterile water
Kanamycin	Sigma-Aldrich, Missouri, USA	60615	Sterile water
Meropenem	Sigma-Aldrich, Missouri, USA	M2574	Sterile water
Piperacillin	Cambridge Bioscience, Cambridge, UK	P3462	100% DMSO
Rifampicin	Sigma-Aldrich, Missouri, USA	R3501	100% DMSO
Tetracycline	Sigma-Aldrich, Missouri, USA	T3383	70% (v/v) ethanol in water

DMSO: Dimethyl-sulfoxide (Sigma-Aldrich, Missouri, USA, Catalogue No.: D8418)

Table 2.2 Plasmids

Plasmid	Antibiotic Resistance Gene	Description	Source
pKpQIL-UK	*bla*$_{KPC-2}$	Plasmid pKpQIL [Leavitt et al. (2010)] which was isolated in the UK	Woodford, N.
pKpQIL-UK *bla*$_{KPC}$::*aph*		Plasmid pKpQIL-UK with the inactivated *bla*$_{KPC-2}$ carbapenemase	This study
pKpQIL-D2	*bla*$_{KPC-2}$	A variant of plasmid pKpQIL-UK	Woodford, N.
pKpQIL-D2 *bla*$_{KPC}$::*aph*		Plasmid pKpQIL-D2 with the inactivated *bla*$_{KPC-2}$ carbapenemase	This study
pNDM-HK	*bla*$_{NDM-1}$	IncL/M plasmid isolated in Hong Kong carrying a *bla*$_{NDM-1}$ carbapenemase gene	Ho, et al. (2011)
pUC18	*bla*$_{TEM-1}$	Cloning vector	Norrander, et al. (1983)
pCT	*bla*$_{CTX-M-14}$	IncK plasmid encoding a *bla*$_{CTX-M-14}$ isolated from scouring calves	Teale, et al. (2005)

Table 2.3 Bacterial strains

Strains	Code	Description	Source
K. pneumoniae			
K. pneumoniae ST468/pKpQIL-UK	ST468/pKpQIL-UK	Clinical isolate of *K. pneumoniae* ST468 carrying plasmid pKpQIL-UK, $Dor^{0.25}$	Woodford, N.
K. pneumoniae ST321/pKpQIL-D2	ST321/pKpQIL-D2	Clinical isolate of *K. pneumoniae* ST321 carrying plasmid pKpQIL-D2, $Dor^{0.25}$	Woodford, N.
K. pneumoniae ST258	ST258	Carbapenemase susceptible *K. pneumoniae*, Kan^{50}	Kreiswirth, B.
ST258/pKpQIL-UK	-	ST258 transconjugants carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study
ST258/pKpQIL-D2	-	ST258 transconjugants carrying pKpQIL-D2, Kan^{50} & $Dor^{0.25}$	This study
K. pneumoniae Ecl8	Ecl8	-	Forage & Lin (1982)
Ecl8 *acrAB*::*aph*	-	Ecl8 which the efflux pump *acrAB* has been inactivated	Veleba, et al. (2012)
$Ecl8^{Rif}$	-	Rifampicin-resistant mutant of *K. pneumoniae* Ecl8, His537Leu, Rif^{100}	This study
$Ecl8^{Rif}$ *acrAB*	-	Rifampicin-resistant mutant of *K. pneumoniae* Ecl8 *acrAB*::*aph*, His537Leu, Rif^{100}	This study
$Ecl8^{Rif}$/pKpQIL-UK	-	$Ecl8^{Rif}$ transconjugants carrying pKpQIL-UK, Rif^{100} & $Dor^{0.25}$	This study
$Ecl8^{Rif}$ *acrAB*/pKpQIL-UK	-	$Ecl8^{Rif}$ *acrAB* transconjugants carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study

Where necessary growth media were supplemented with Rif^{100} (100 µg/ml rifampicin), Kan^{50} (50 µg/ml kanamycin), $Dor^{0.25}$ (0.25 µg/ml doripenem)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
K. pneumoniae			
Ecl8^{Rif}/pKpQIL-UK *bla*$_{KPC}$::*aph*	-	Ecl8^{Rif} transconjugants carrying pKpQIL-UK *bla*$_{KPC}$::*aph*, Rif^{100} & Kan^{50}	This study
Ecl8^{Rif}/pKpQIL-D2	-	Ecl8^{Rif} transconjugants carrying pKpQIL-D2, Rif^{100} & Dor$^{0.25}$	This study
Ecl8^{Rif}/pKpQIL-D2 *bla*$_{KPC}$::*aph*	-	Ecl8^{Rif} transconjugants carrying pKpQIL-D2 *bla*$_{KPC}$::*aph*, Rif^{100} & Kan^{50}	This study
E. coli			
E. coli SW105	-	*mcrA* Δ[*mrr-hsdRMS-mcrBC*] Φ*80*d*lacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ[*ara*, *leu*]7697 *galU pgl*Δ*8 rpsL nupG* ʎ(*cI*857*ind1*) Δ{(*cro-bioA*))<>*araC*-P$_{BAD}$ *flpe*8	Warming, S. et al. (2005)
E. coli NCTC 10418	10418	Control strain for antibiotic susceptibility testing	National Collection of Type Cultures (NCTC)
10418^{Rif}	-	Rifampicin-resistant 10418, Ser531Phe, Rif^{100}	This study
10418^{Rif}/pKpQIL-UK	-	*E. coli* 10418^{Rif} transconjugant carrying pKpQIL-UK, Rif^{100} & Dor$^{0.25}$	This study
10418^{Rif}/pKpQIL-D2	-	*E. coli* 10418^{Rif} transconjugant carrying pKpQIL-D2, Rif^{100} & Dor$^{0.25}$	This study

Where necessary growth media were supplemented with Rif^{100} (100 μg/ml rifampicin), Kan^{50} (50 μg/ml kanamycin), Dor$^{0.25}$ (0.25 μg/ml doripenem)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
E. coli			
E. coli BW25113	BW25113	Parental strain of Keio Collection	Baba, T. et al. (2006)
BW25113 *acrB*::*aph*	-	BW25113 with inactivated *acrB* efflux pump gene, Kan^{50}	Baba, T. et al. (2006)
BW25113 *tolC*::*aph*	-	BW25113 with inactivated *tolC* efflux pump gene, Kan^{50}	Baba, T. et al. (2006)
$BW25113^{Rif}$	-	Rifampicin-resistant BW25113, Pro574Leu, Rif^{100} & Kan^{50}	This study
$BW25113^{Rif}$ *acrB*	-	Rifampicin-resistant BW25113 *acrB*::*aph*, Pro574Leu, Rif^{100} & Kan^{50}	This study
$BW25113^{Rif}$ *tolC*	-	Rifampicin-resistant BW25113 *tolC*::*aph*, His526Tyr, Rif^{100} & Kan^{50}	This study
$BW25113^{Rif}$/pKpQIL-UK	-	BW25113 ^{Rif} transconjugant carrying pKpQIL-UK, Rif^{100} & $Dor^{0.25}$	This study
$BW25113^{Rif}$ *acrB*/pKpQIL-UK	-	*E. coli* $BW25113^{Rif}$ *acrB* transconjugant carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study
$BW25113^{Rif}$ *tolC*/pKpQIL-UK	-	*E. coli* BW25113C *tolC* transconjugant carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study

Where necessary growth media were supplemented with Rif^{100} (100 μg/ml rifampicin), Kan^{50} (50 μg/ml kanamycin), $Dor^{0.25}$ (0.25 μg/ml doripenem)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
E. coli			
ElectroMAX^{TM} DH10B^{TM}	DH10B	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ* ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ - *rps*L *nup*G *ton*A	Invitrogen (California, USA)
DH10B/pKpQIL-UK	-	*E. coli* DH10B transformant harbouring the pKpQIL-UK	This study
DH10B/pKpQIL-D2	-	*E. coli* DH10B transformant harbouring the pKpQIL-D2	This study
DH10B/pNDM-HK	-	*E. coli* DH10B transformant harbouring the pNDM-HK	This study
***S.* Typhimurium**			
S. Typhimurium SL1344	SL1344	Wildtype *Salmonella enterica* serovar Typhimurium strain SL1344 isolated from cow	Wray & Sojka (1978)
SL1344^{Rif}	-	Rifampicin-resistant SL1344, Ser522Phe, Rif^{100}	This study
SL1344^{Rif}/pKpQIL-UK	-	SL1344^{Rif} transconjugant carrying pKpQIL-UK, Rif^{100} & Dor$^{0.25}$	This study
SL1344^{Rif} /pKpQIL-D2	-	SL1344^{Rif} transconjugant carrying pKpQIL-D2, Rif^{100} & Dor$^{0.25}$	This study
SL1344 *ompC*::*aph*	SL1344 *ompC*	SL1344 with inactivated *ompC* gene, Kan^{50}	Lawler, A.
SL1344 *ompF*::*aph*	SL1344 *ompF*	SL1344 with inactivated *ompF* gene, Kan^{50}	Lawler, A.
SL1344 Δ*ompC ompF*::*aph*	SL1344 *ompCF*	SL1344 with inactivated *ompC* & *ompF* genes, Kan^{50}	Lawler, A.

Where necessary growth media were supplemented with Rif^{100} (100 µg/ml rifampicin), Kan^{50} (50 µg/ml kanamycin), Dor$^{0.25}$ (0.25 µg/ml doripenem)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
***S.* Typhimurium**			
SL1344 *ompC*/pKpQIL-UK	-	SL1344 *ompC* transconjugant carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study
SL1344 *ompF*/pKpQIL-UK	-	SL1344 *ompF* transconjugant carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study
SL1344 *ompCF*/pKpQIL-UK	-	SL1344 *ompCF* transconjugant carrying pKpQIL-UK, Kan^{50} & $Dor^{0.25}$	This study
SL1344/pUC18	-	SL1344 carrying pUC18 plasmid, Amp^{100}	Blair, J.
SL1344 *acrA*::*aph*/pUC18	-	SL1344 with inactivated *acrA* gene carrying pUC18 plasmid, Amp^{100} & Kan^{50}	Blair, J.
SL1344 *acrB*::*aph*/pUC18	-	SL1344 with inactivated *acrB* gene carrying pUC18 plasmid, Amp^{100} & Kan^{50}	Blair, J.
SL1344 *tolC*::*aph*/pUC18	-	SL1344 with inactivated *tolC* gene carrying pUC18 plasmid, Amp^{100} & Kan^{50}	Blair, J.
S. Typhimurium ATCC 14028s	14028s	Wildtype *Salmonella enterica* serovar Typhimurium strain ATCC14028s isolated from chicken	American Type Culture Collection
$14028s^{Rif}$	-	Rifampicin-resistant 14028s, Ser522Tyr , Rif^{100}	This study

Where necessary growth media were supplemented with Rif^{100} (100 μg/ml rifampicin), Kan^{50} (50 μg/ml kanamycin), $Dor^{0.25}$ (0.25 μg/ml doripenem), Amp^{100} (100 μg/ml ampicillin)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
***S.* Typhimurium**			
14028s *acrAB*::*cat*	14028s *acrAB*	14028s with inactivated *acrAB* efflux pump gene, Cm^{15}	Nishino, et al. (2006)
14028s *tolC*::*cat*	14028s *tolC*	14028s with inactivated *tolC* efflux pump gene, Cm^{15}	Nishino, et al. (2006)
$14028s^{Rif}$ *acrAB*::*cat*	-	Transductant of $14028s^{Rif}$ with *acrAB*::*cat*, Rif^{100} & Cm^{15}	This study
$14028s^{Rif}$ *tolC*::*cat*	-	Transductant of $14028s^{Rif}$ with *tolC*::*cat*, Rif^{100} & Cm^{15}	This study
$14028s^{Rif}$/pKpQIL-UK	-	$14028s^{Rif}$ transconjugant carrying pKpQIL-UK, Rif^{100} & $Dor^{0.25}$	This study
$14028s^{Rif}$ *acrAB*/pKpQIL-UK	-	$14028s^{Rif}$ *acrAB* transconjugant carrying pKpQIL-UK, Cm^{15} & $Dor^{0.25}$	This study
$14028s^{Rif}$ *tolC*/pKpQIL-UK	-	$14028s^{Rif}$ *tolC* transconjugant carrying pKpQIL-UK, Cm^{15} & $Dor^{0.25}$	This study
$14028s^{Rif}$ /pKpQIL-D2	-	$14028s^{Rif}$ transconjugant carrying pKpQIL-D2, Rif^{100} & $Dor^{0.25}$	This study
$14028s^{Rif}$ *acrAB*/pKpQIL-D2	-	$14028s^{Rif}$ *acrAB* transconjugant carrying pKpQIL-D2, Cm^{15} & $Dor^{0.25}$	This study
$14028s^{Rif}$ *tolC*/pKpQIL- D2	-	$14028s^{Rif}$ *tolC* transconjugant carrying pKpQIL-D2, Cm^{15} & $Dor^{0.25}$	This study

Where necessary growth media were supplemented with Rif^{100} (100 µg/ml rifampicin), $Dori^{0.25}$ (0.25 µg/ml doripenem), Amp^{100} (100 µg/ml ampicillin)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
***S.* Typhimurium**			
14028s^{Rif} /pNDM-HK	-	14028s^{Rif} transconjugant carrying pNDM-HK, Rif^{100} & Dor$^{0.25}$	This study
14028s^{Rif} *acrAB*/pNDM-HK	-	14028s^{Rif} *acrAB* transconjugant carrying pNDM-HK, Cm^{15} & Dor$^{0.25}$	This study
14028s^{Rif} *tolC*/pNDM-HK	-	14028s^{Rif} *tolC* transconjugant carrying pNDM-HK, Cm^{15} & Dor$^{0.25}$	This study
14028s^{Rif}/pCT	-	14028s^{Rif} transconjugant carrying pCT, Rif^{100} & Ctx^{8}	This study
14028s^{Rif} *acrAB*/pCT	-	14028s^{Rif} *acrAB* transconjugant carrying pCT, Cm^{15} & Ctx^{8}	This study
14028s^{Rif} *tolC*/pCT	-	14028s^{Rif} *tolC* transconjugant carrying pCT, Cm^{15} & Ctx^{8}	This study
14028s^{Rif}/pCT *bla*$_{CTX}$::*aph*	-	14028s^{Rif} transconjugant carrying pCT *bla*$_{CTX}$::*aph*, Rif^{100} & Kan^{50}	This study
14028s^{Rif} *acrAB*/pCT *bla*$_{CTX}$::*aph*	-	14028s^{Rif} *acrAB* transconjugant carrying pCT *bla*$_{CTX}$::*aph*, Cm^{15} & Kan^{50}	This study
14028s^{Rif} *tolC*/pCT *bla*$_{CTX}$::*aph*	-	14028s^{Rif} *tolC* transconjugant carrying pCT *bla*$_{CTX}$::*aph*, Cm^{15} & Kan^{50}	This study

Where necessary growth media were supplemented with Rif^{100} (100 μg/ml rifampicin), Kan^{50} (50 μg/ml kanamycin), Dor$^{0.25}$ (0.25 μg/ml doripenem), Cm^{15} (15 μg/ml chloramphenicol), Ctx^{8} (8 μg/ml cefotaxime)

Table 2.3 Bacterial strains (Continued)

Strains	Code	Description	Source
E. cloacae			
E. cloacae NCTC10005	Ecloacae	*E. cloacae* isolated from cerebrospinal fluid	NCTC
Ecloacae^{Rif}	-	Rifampicin-resistant *E. cloacae*, Ser328Ala, Rif^{100}	This study
Ecloacae^{Rif}/pKpQIL-UK	-	Ecloacae^{Rif} transconjugant carrying pKpQIL-UK, Rif^{100} & Dor$^{0.25}$	This study
Ecloacae^{Rif}/pKpQIL-D2	-	Ecloacae^{Rif} transconjugant carrying pKpQIL-D2, Rif^{100} & Dor$^{0.25}$	This study
S. marcescens			
S. marcescens NCTC10211	Serratia	*S. marcescens* isolated from pond water	NCTC
Serratia^{Rif}	-	Rifampicin-resistant *S. marcescens*, Asp516Gly, Rif^{100}	This study
Serratia^{Rif}/pKpQIL-UK	-	Serratia^{Rif} transconjugant carrying pKpQIL-UK, Rif^{100} & Dor$^{0.25}$	This study
Serratia^{Rif}/pKpQIL-D2	-	Serratia^{Rif} transconjugant carrying pKpQIL-D2, Rif^{100} & Dor$^{0.25}$	This study

Where necessary growth media were supplemented with Rif^{100} (100 µg/ml rifampicin), Dor$^{0.25}$ (0.25 µg/ml doripenem)

Table 2.4 Morphological appearance on differential and selective agar

(a) Xylose-Lysine-Deoxycholate (XLD)

Bacteria	Growth	Colony
E. coli	Partial to complete inhibition of some strains	Yellow and opaque colonies
S. Typhimurium	Good growth	Red colonies with black centers
K. pneumoniae	Good growth	Yellow, mucoid and opaque
E. cloacae	Good growth	Yellow and opaque
S. marcescens	Good growth	Yellow and opaque
Gram-positive	Partial to complete inhibition	-
E. coli ATCC25922	Inhibited	-

Source: Adapted from product sheets of XLD agar of Oxoid (CM0469), Sigma-Aldrich (95586) and Becton Dickinson GmbH (PA-254055.06)

(b) MacConkey Agar

Bacteria	Growth	Colony
E. coli	Good growth	Red colonies with hazy (pink halo) surrounding due to bile precipitate
S. Typhimurium	Good growth	Colourless (White/pale yellow)
K. pneumoniae	Good growth	Red and mucoid with hazy (pink halo) surrounding due to bile precipitate
E. cloacae	Good growth	Red colonies with hazy (pink halo) surrounding due to bile precipitate
S. marcescens	Good growth	Pale pink
Gram-positive	Weak to good growth	*Staphylococcus*: pale pink and opaque

Source: Adapted from product sheets of XLD agar of Oxoid (CM0007) and Sigma-Aldrich (70143)

strain has been constructed, the strain was checked using API® 20E Gram-negative Identification Strip (Biomérieux, France) according to manufacturer's recommendation. This strip is used to distinguish the different species of Enterobacteriaceae using results from a panel of biochemical assays.

2.2 Plasmid Extraction

In order to extract plasmids, bacterial strains carrying the plasmids were inoculated into 10 ml of LB broth supplemented with the necessary antibiotics. Cultures were then incubated overnight at 37°C, with shaking at 200 rpm. Plasmids were extracted using the GeneJET™ Plasmid Miniprep Kit (Fermentas, Ontario, Canada, Catalogue No.: K0503) according to manufacturer's instructions.

2.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to routinely amplify various DNA targets with MyTaq™ Red Mix (Bioline, London, UK, Catalogue No.: BIO-25044) according to the manufacturer's instructions.

The reactions used the following thermal cycling conditions:

Initial denaturation		95°C for 5 min
Cycling conditions (30 cycles)		
i)	Denaturation	95°C for 15 sec
ii)	Annealing (T_a)	15 sec (Temperature used is primer dependent)
iii)	Extension	72°C (10 sec per 1 kb)
Storage		4°C (Indefinite)

Where the amplicon was larger than 2 kb, a different polymerase, MyFi™ Mix (Bioline, London, UK, Catalogue No.: BIO-25050) was used with the same thermal cycling conditions with a modification in the extension time. The extension time using MyFi™ Mix was estimated as 60 sec per 1 kb amplicon. All primers (Table 2.5) used in this study were designed using Vector NTI Advance™ 11.5 (Life Technologies, USA).

2.4 DNA Gel Electrophoresis

Whenever DNA (plasmid or PCR amplicons) needed to be visualised, the DNA was separated using electrophoresis in 1% (w/v) agarose gels (Sigma-Aldrich, Catalogue No.: A9539). Midori Green nucleic acid stain (Labgene Scientific, Fribourg, Switzerland, Catalogue No.: MG02) was added into molten agarose (ca. 60°C) to give a final concentration of 1X (Stock concentration: 50,000X) before pouring the molten agarose into the electrophoresis gel tray. The DNA sample mixed with loading buffer (1 µl of 5X buffer per 4 µl of sample) was separated at 150 volts until the indicator dye has migrated 75% of the length of the gel. The separated DNA was visualised and images captured using G:Box Gel Documentation System (Syngene, Cambridge, UK).

2.5 DNA Sequencing

Where a nucleotide sequence was required, PCR amplicons were purified using the QIAquick® PCR purification Kit (Qiagen, Maryland, USA, Catalogue No.: 28104) or QIAquick® Gel Extraction Kit (Qiagen, Maryland, USA, Catalogue No.: 28704) according to manufacturer's instructions. The purified amplicons were quantified using the Qubit® dsDNA HS Assay Kit (Life Technologies, USA, Catalogue No.: Q32854). Then, the sequencing reaction was prepared as instructed by and sent to the Genomics Laboratory, School of Biosciences, College of Life and Environmental Sciences, University of Birmingham. The

Table 2.5 Primers used in this study

Primer Name	Amplicon (bp)	T_a (°C)	Sequence (5' to 3')	Description	Source
RpoB-F1	4482	56	ACACTGTTTGACTACTGCTGTGC	To amplify *S.* Typhimurium	This study
RpoB-R1	4482	56	CTCAGGTTTGAACGTACGGTAGT	SL1344/ATCC14028s *rpoB*	This study
RpoB-R2	-	-	GGCATTGCGGAAAGTGT	For sequencing of *S.*	This study
RpoB-F3	-	-	GCGAAGCCAGAGCAATT	Typhimurium *rpoB*	This study
RpoB-R3	-	-	TCGCCAATACCTTTCGCAG	For sequencing of *S.*	This study
RpoB-F4	-	-	ACGCCAAGCCGATTTCC	Typhimurium *rpoB*	This study
RpoB-F5	-	-	TTCCACGGCATGAACGC	For sequencing of *S.*	This study
RpoB-R4	-	-	ATGTGTGTCTCTGGGCGAG	Typhimurium *rpoB*	This study
RpoB-R5	-	-	AAACGCCGTATCCAGCC	For sequencing of *S.*	This study
RpoB-R2	-	-	TGAGGCATCAGGGTATCCA	Typhimurium *rpoB*	This study
RpoB-Ecoli-F1	4288	56	GGCATTGCGGAAAGTGT	To amplify *E. coli rpoB*	This study
RpoB-Ecoli-R1	4288	56	GCGAAGCCAGAGCAATT		This study
RpoB-Ecoli-F2	-	-	CGCATCTTTTGACATCGA	For sequencing of *E. coli rpoB*	This study
RpoB-Ecoli-R2	-	-	GTAAGGCATATCTTCGATCG		This study
RpoB-Ecoli-F3	-	-	ACGCACAAACGTCGTATC	For sequencing of *E. coli rpoB*	This study
RpoB-Ecoli-R3	-	-	GTTGTAACCATTCCACGG		This study

T_a: Annealing temperature

Table 2.5 Primers used in this study (Continued)

Primer Name	Amplicon (bp)	Ta (°C)	Sequence (5' to 3')	Description	Source
RpoB-Ecoli-F4	-	-	TCAGAACATGCGCGTAG	For sequencing of *E. coli rpoB*	This study
RpoB-Ecoli-F5	-	-	CGGTAACAAGGGTGTAATTT		This study
RpoB-Ecoli-R4	-	-	TTGTGCGTAATCTCAGACAG	For sequencing of *E. coli rpoB*	This study
RpoB-Ecoli-R5	-	-	CGATGTACTCAACCGGG		This study
KPCg-colpcrF	785	56	ATGTCACTGTATCGCCGTCT	Internal primers for bla_{KPC} gene	This study
KPCg-colpcrR	785	56	TAGACGGCCAACACAATAGG		This study
Chlor-Fwd	500	60	TTATACGCAAGGCGACAAGG	To verify inactivation of *Salmonella*	Nishino et al. (2006)
AcrA-Up	500	60	GATTGCGCCGCGCGTCACGCCAGCA	*acrAB*, no amplicon for wildtype	
AcrB-Down	415	60	TGCCCGATTTACGCCATTTTTGC	To verify inactivation of *Salmonella*	Nishino et al. (2006)
Chlor-Rev	415	60	GATCTTCCGTCACAGGTAGG	*acrAB*, no amplicon for wildtype	
TolC-Check-F	2489	56	CTTCTATCATGCCGGCGACC	To verify inactivation of *Salmonella*	Buckley et al.
TolC-Check-R	2489	56	CGCTTGCTGGCACTGACCTT	*tolC*, mutant will yield 1812 bp	(2006)
pQIL-F	383	56	CAGCATGACAGAATAGCGAGGCTT	To differentiate pKpQIL-UK from -	This study
pQIL-R	383	56	TACAAGGAGATGTGCCATGACCGT	D2	This study
pMan-F	600	56	CTTACTGGCAAACTGTTGA	To differentiate pKpQIL-D2 from -	This study
pMan-R	600	56	ATCCCGTGTGTTCAAAA	UK	This study

T_a: Annealing temperature

Table 2.5 Primers used in this study (Continued)

Primer Name	Amplicon (bp)	Ta (°C)	Sequence (5' to 3')	Description	Source
NDM1-colpcrF	578	56	TTGATGCTGAGCGGGTG	To verify the presence of *bla*$_{NDM}$	This study
NDM1-colpcrR	578	56	CTGTCCTTGATCAGGCAGC	gene	This study
pHK-F	1407	56	TTTTCATCGCGTATGCTCCC	To verify the presence of pNDM-HK	This study
pHK-R	1407	56	AATACCGCTAAACCGCAACG	plasmid	This study
acrB fwd	3482	51	CGGATGACAAAGTGGAAACC	To verify inactivation of *E. coli*	Morris, F
acrB rvs	3482	51	CGTATGAGATCCTGAGTTGG	*acrB*, mutant would yield 1659 bp	Morris, F
tolC fwd	2131	51	CCGCGATAAAGTGTTTCTCG	To verify inactivation of *E. coli tolC*,	Morris, F
tolC rvs	2131	51	TTCAGCGCATTGTTGTACGC	mutant would yield 1976 bp	Morris, F
OmpC-F	1927	53	TCGATACCAACCACGCTCAC	To verify inactivation of *ompC*, 2207	Lawler, A
OmpC-R	1927	53	TCTTACGAACGGTGCAGCAG	bp (+*aph*), 753 bp (Δ*ompC*)	Lawler, A
OmpF-F	780	53	CCGTCAATGCCGAGATAGTT	To verify inactivation of *ompF*, no	Lawler, A
OmpF-R	780	53	CAGTCATAGCCGAATAGCCT	amplicon for wildtype	Lawler, A
gyrB-F	273	45	GTACCTGGTGGAAGGGGACT	To check for DNA contamination in	Blair, J
gyrB-R	273	45	AACAGCAGCGTACGGATG	RNA samples	Blair, J
KpQIL-KPC-F	1580	56	AGCTACCGCTTGAAGGACAA	To sequence the entire *bla*$_{KPC}$ gene	This study
KpQIL-KPC-R	1580	56	GGATTGCGTCAGTTCAGCAT		This study

T_a: Annealing temperature

Table 2.5 Primers used in this study (Continued)

Primer Name	Amplicon (bp)	Ta (°C)	Sequence (5' to 3')	Description	Source
KPCg-KO-F	1494	51	CAACCTCGTCGCGGAACCATTCGCTAAACTCGAA CAGGACTTTG(GTGTAGGCTGGAGCTGCTTC)	For inactivation of *bla*$_{KPC-2}$ gene on pKpQIL-UK & -D2 plasmid. Nucleotide sequence in parenthesis is homologous to the *aph* gene	This study
KPCg-KO-R			GCCAGTGCAGAGCCCAGTGTCAGTTTTTGTAAGC TTTCCG(GGGAATTAGCCATGGTCCAT)		

T_a: Annealing temperature

sequencing services provided used BigDye terminator chemistry. The obtained nucleotide sequence was analysed using Vector NTI Advance™ 11.5 (Life Technologies, USA).

2.6 Insertional Inactivation of Plasmidic Genes

The method used to inactivate plasmidic genes by insertion of an antibiotic resistance marker in the target gene and was done as previously described (Sharan et al., 2009, Cottell et al., 2014, Cottell et al., 2012) with some modifications as listed below.

2.6.1 Primer design

The primers used to amplify the aminoglycoside phosphotransferase (*aph*) or chloramphenicol acetyltransferase (*cat*) antibiotic resistance marker conferring kanamycin and chloramphenicol resistance, respectively, consisted of 40 bp of sequence homologous to the target plasmid gene either side of 20 bp of sequence homologous to the antibiotic resistance marker i.e. *aph* or *cat* gene.

2.6.2 Electrocompetent Cell Preparation and Electroporation

Where plasmids needed to be transferred into *E. coli* SW105 which contained a chromosomally encoded λ-Red recombinase gene for inactivating plasmidic genes (Warming et al., 2005), electrocompetent cells were prepared by inoculating an overnight culture of the bacteria into 50 ml fresh LB broth at a 1:50 dilution and incubated at 30°C (to avoid expression of the recombinase genes) with shaking at 200 rpm. When the culture achieved mid-logarithmic phase i.e. an optical density at a wavelength of 600 nm (OD_{600}) of approximately 0.6, the culture was chilled in ice slurry for 15 min. Then, the culture was centrifuged at 4°C, 2,500 x *g*, 15 min. The cell pellet was resuspended in 50 ml of sterile 15% (v/v) ice-cold glycerol (Sigma-Aldrich, Missouri, USA, Catalogue No.: G5516), followed by centrifugation at 4°C, 2,500 x *g*, 15 min. This washing step was repeated twice and the cell pellet was resuspended in 100 µl ice-cold 15% glycerol. A volume of 50 µl of

electrocompetent cells was used for each electroporation. About 20 – 100 ng plasmid DNA was mixed with the electrocompetent cells and the cell suspension was left on ice for 15 min. The electroporation parameters used were 13 kV/cm, 25 μF, 200 Ω and 5 ms. Immediately after electroporation, 1 ml of pre-warmed (30°C) LB broth was added to the cells and mixed gently by pipetting. The transformants were recovered at 30°C with shaking at 200 rpm for 3 hours. Then, 100 μl of the transformants were spread on the surface of LB agar supplemented with appropriate antibiotics (Table 2.2) and incubated overnight at 30°C. The transformants were verified by PCR to carry the plasmid of interest.

2.6.3 Gene Inactivation by Homologous Recombination

Plasmids harbouring target genes were transferred into *E. coli* SW105 (Warming et al., 2005) by electroporation (Section 2.6.2). The amplicons generated using the chimeric primers were purified by the QIAquick® PCR Purification Kit or QIAquick® Gel Extraction Kit. Two 50 ml LB broths were inoculated with overnight cultures of *E. coli* SW105 carrying the plasmid of interest at a dilution of 1 in 50 and were allowed to grow at 30°C until an OD_{600} of 0.6. One of the cultures was then incubated at 42°C with shaking for 15 min to activate the λ-Red Recombinase. The other culture was left at 30°C as the non-activated control. After the incubation, both cultures were immediately cooled in ice-slurry for 15 min. The cells were made electrocompetent and electroporation was also done as in Section 2.6.2. The amplicons were electroporated into *E. coli* SW105 carrying the plasmid of interest. Depending on the resistance cassette use for gene inactivation, the transformants were selected on LB agar supplemented with 50 μg/ml kanamycin or 25 μg/ml chloramphenicol. Successful inactivation of the target gene was further verified by PCR and sequencing using appropriate primers (Table 2.5). The recombinant plasmid was then extracted and transferred into

Invitrogen MegaX DH10B^{TM} T1R Electrocomp^{TM} cells (Invitrogen, California, USA, Catalogue No.: C6400-03) by electroporation following manufacturer's instructions.

2.7 β-lactamase Assay

β-lactamase activity was studied by measuring the hydrolysis of nitrocefin evident as a colour change. An overnight culture was diluted 1 in 50 in 100 ml of fresh LB broth and allowed to grow until an OD_{600} of 0.6 at 37°C with shaking (200 rpm). The culture was centrifuged at 2,500 x *g* for 10 min at 4°C. The cell pellet was resuspended in 3 ml of ice-cold sodium phosphate buffer pH 7.0. The cell suspension was sonicated in ice slurry (Soniprep 150, MSE, UK) at an amplitude of 5 micron for 30 seconds four times with 30-second intervals to avoid overheating of the cell lysates. The cell lysate was centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant which contains the β-lactamase was transferred into a new microfuge tube. The amount of total protein was determined using the Bradford Reagent (Sigma-Aldrich, Missouri, USA, Catalogue No.: B6916) by mixing 10 µl of the samples with 990 µl of the reagent and measuring the absorbance at a wavelength of 595 nm. The values were compared against a series of bovine serum albumin (Sigma-Aldrich, Missouri, USA, Catalogue No.: P0914) of known concentrations (0.05 – 0.5 mg/ml). A total of 15 µg of total protein in 50 µl sodium phosphate buffer was transferred into wells of 96-well plate (Appleton Woods, Birmingham, UK, Catalogue No.: CC653) in triplicates. Freshly prepared nitrocefin (Oxoid, Massachusetts, USA, Catalogue No.: SR0112) was added into the reservoir of the injector of a FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany). The injector was set to inject 10 µl of nitrocefin after 10 seconds incubation and the absorbance was monitored at a wavelength of 482 nm every minute for 5 minutes. The changes in absorbance were then plotted against time. The slopes of the initial reaction (where the graph was linear) were

compared against the *S.* Typhimurium 14028s crude lysate control. The experiment was repeated three times and significant difference was analysed using Student's *t*-test ($p<0.05$).

2.8 Hoechst 33342 Accumulation Assay

The activity of efflux pumps was inferred by measuring accumulation of Hoechst 33342 efflux substrate in the absence and presence of efflux inhibitors as previously described (Webber and Coldham, 2010). An overnight culture of the strain of interest was diluted with fresh LB broth to give a 4% inoculum in 10 ml. The culture was allowed to grow at 37°C with shaking (200 rpm) until it achieved an OD_{600} of 0.6. The cultures were centrifuged at 2,500 x *g* for 10 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 3 ml sterile phosphate saline buffer (PBS) (Life Technologies, California, USA, Catalogue No.: 14190-094). The OD_{600} of the suspension was determined again and each cell suspension was diluted to an OD_{600} of 0.1 with PBS. Then, 180 µl of the diluted cell suspension was transferred into three wells in a black 96-well plate (Appleton Woods, Birmingham, UK, Catalogue No.: CC708). Three replicates (176 µl) of the same cell suspension were added into three more wells and 4 µl of 50X efflux inhibitor was added (e.g. if a final concentration of 25 µg/ml PAβN was needed, then 4 µl of 1250 µg/ml PAβN was added into the wells). For positive control, 200 µl of boiled overnight culture was transferred into three wells. For negative control, 200 µl PBS was transferred into three wells and 180 µl PBS was added into another three wells. The fluorescence of each well was monitored at 37°C using FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany). The reservoir of the injector was added with 25 µM Hoechst 33342 (Sigma-Aldrich, Missouri, USA, Catalogue No.: B2261). The injector was set to inject 20 µl of Hoechst 33342 into wells containing 180 µl of cell suspension. The fluorescence was measured at an excitation and emission wavelengths of 355 and 465 nm, respectively for 30 cycles (1 min per cycle). The

fluorescence of the samples at the 30th cycle were compared using Student's *t*-test with $p<0.05$ considered as significant.

2.9 Outer Membrane Protein Extraction

Bacterial outer membrane proteins were purified as previously described (Piddock et al., 1990). Briefly, an overnight culture of the bacteria of interest was diluted 1 in 50 in 90 ml of fresh LB broth and allowed to grow until an OD_{600} of 0.6. The cultures were centrifuged at 2,500 x *g* and 4°C for 15 min. The supernatant was discarded and the cell pellet was resuspended in 30 ml of 50 mM sodium phosphate buffer pH 7.0 to wash the cells. The cell suspension was centrifuged again at 2,500 x *g* and 4°C for 15 min. The washed cell pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer pH 7.0 and transferred into a sterile microfuge tube and froze overnight at -80°C. After freezing, the cell suspension was thawed on ice then homogenised by sonication in ice slurry (Soniprep 150, MSE, UK) at 5 micron amplitude for 30 seconds for four times with 30-second intervals to avoid overheating of the cell lysates. The cell lysates were centrifuged at 6,000 x *g* for 1 min at 4°C to discard larger cell debris. The supernatant was transferred into a microfuge tube and centrifuged again at 12,000 x *g* for 30 min at 4°C. Then, the supernatant was discarded. The pellet was resuspended vigorously in 200 µl 2% sarcosyl (Sigma-Aldrich, Missouri, USA, Catalogue No.: L5125) and incubated at room temperature for 30 min. The samples were centrifuged again at 12,000 x *g* for 30 min at 10°C. All the supernatant was carefully removed to ensure most of the detergent containing solution had been removed. The protein pellet was resuspended in 100 µl of 50 mM sodium phosphate buffer pH 7.0 and centrifuged at 12,000 x *g* for 10 min at 4°C to remove residual detergent from the outer membrane protein preparations. The supernatant was discarded and the extracted outer membrane proteins were resuspended in 50 µl of 50 mM sodium phosphate buffer pH 7.0. The amount of total protein in each samples

were quantified using Bradford Reagent (Sigma-Aldrich, Missouri, USA, Catalogue No.: B6916) against a series of bovine serum albumin standards (Sigma-Aldrich, Missouri, USA, Catalogue No.: P0914) of known concentrations (0.05 – 0.5 mg/ml).

2.10 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The extracted outer membrane proteins were separated by SDS-PAGE using a PROTEAN® II xi Electrophoresis Cell (Bio-Rad, California, USA). A 16 x 20 x 0.1 cm discontinuous gel (4% stacking gel and 10 % resolving gel) was prepared according to the manufacturer's instruction for the separation of protein samples. Sample loading buffer (Sigma-Aldrich, Missouri, USA, Catalogue No.: 161-0747) was added to the protein samples and the protein samples were heated at 95°C for 10 min. A total of 2 µg of total protein was loaded per lane for separation. The electrophoresis was done using 1X Tris/Glycine/SDS buffer (Sigma-Aldrich, Missouri, USA, Catalogue No.: 161-0732). Once the protein samples had been loaded into the wells, electrophoresis was started using 100 volts (constant) until the samples migrated into the resolving gel where the voltage was increased to 200 volts. The protein samples were allowed to separate until the dye front reached the end of the gel. The gel was then carefully removed from the electrophoresis tank and stained with PhastGel® Blue R (Sigma-Aldrich, Missouri, USA, Catalogue No.: B4921) for 1 hour with gentle shaking. Then, the protein gel was de-stained using de-staining buffer [10% (v/v) methanol and 5% (v/v) acetic acid in water] overnight with gentle shaking. The de-stained protein gel was visualised and image taken using G:Box Gel Documentation System (Syngene, Cambridge, UK).

2.11 Selection of Rifampicin Resistant Bacteria

To determine the conjugation frequency of plasmids or to transfer the plasmids into different bacterial strains, recipient strains required a selection marker to differentiate the transconjugants from the donor strain. Selection of rifampicin resistant mutants was used to

gain strains suitable for selection. A single colony of the desired recipient strain was inoculated into 10 ml of Iso-sensitest broth (Oxoid, Massachusetts, USA, Catalogue No.: CM0473) and incubated overnight at 37°C, 200 rpm. The overnight bacterial culture was sub-cultured into 100 ml of Iso-sensitest broth at a dilution of 1:100 and incubated overnight at 37°C (shaken at 200 rpm). A volume of 100 µl of the culture at various concentrations and dilutions (100X, 10X, 1X, 0.1X and 0.01X) was then spread on Iso-sensitest agar (Oxoid, Massachusetts, USA, Catalogue No.: CM0471) supplemented with 100 µg/ml rifampicin. The culture was also serially diluted and spread on antibiotic-free Iso-sensitest agar to determine the viable count. The mutation frequency of the rifampicin resistant strain was determined as the number of rifampicin-resistant mutants [colony forming unit (cfu)/ml] divided by the total number of colonies (cfu/ml) of the strain determined by viable counting.

2.12 P22 Transduction

Bacteriophage P22 was used to transfer genes inactivated by insertion of antibiotic resistance genes (e.g. *acrAB*::*cat* and *tolC*::*cat*) into different *S.* Typhimurium strains. An overnight culture of the strain carrying the genes to be transferred by P22 was diluted 1 in 100 in 5 ml LB broth supplemented with filter-sterilised 10 mM magnesium (II) sulphate ($MgSO_4$) (Sigma-Aldrich, Missouri, USA, Catalogue No.: M7506) and 5 mM calcium (II) chloride ($CaCl_2$) (Sigma-Aldrich, Missouri, USA, Catalogue No.: C1016). The culture was incubated for 30 min at 37°C with shaking, 200 rpm. Then, 5 µl of P22 stock was added to this culture and left to incubate overnight at 37°C with shaking. After the overnight incubation, 1 ml of chloroform (Sigma-Aldrich, Missouri, USA, Catalogue No.: C2432) was added to the culture and mixed with vigorous shaking. The lysed cells were centrifuged at 2,500 x *g* for 15 min at 4°C. The supernatant was transferred into a sterile glass bottle and 200 µl of chloroform was added. This phage stock was stored at 4°C until further use.

In order to transfer genes containing markers into the strain of interest, 5 ml overnight culture of the recipient strain was centrifuged at 2,500 x *g* for 15 min at room temperature. The cell pellet was resuspended in 1 ml LB broth supplemented with 10 mM $MgSO_4$ and 5 mM $CaCl_2$. Then, various volumes (5, 10, 50 and 100 µl) of the prepared phage stock were added to 100 µl aliquots of the resuspended cell pellet. The cell suspension was incubated at 37°C for 15 min. To stop the transduction, 1 ml of LB broth and 100 µl of filter-sterilised 1 M sodium citrate (Sigma-Aldrich, Missouri, USA, Catalogue No.: C7254) were added to the cell suspensions (transductants). The mixtures were incubated at 37°C for 45 min. The transductants were spread on LB agar supplemented with the relevant antibiotics (Table 2.3) and incubated at 37°C for 24 – 48 hours.

2.13 Conjugation

The plasmids used in this study are large. Hence, filter conjugation was frequently used to transfer the plasmids between bacterial strains and species. A volume of 1 ml of overnight liquid culture of donor and recipient strains grown with the appropriate antibiotics were centrifuged at 12,000 x *g* for 1 min. The cell pellets were washed with 1 ml of fresh LB broth and centrifuged again to remove traces of antibiotic from the overnight culture. The donor and recipient cell pellets were each resuspended in 50 µl of LB broth and mixed together. The cell suspension was transferred on to a sterile 0.45 µm 25 mm nylon membrane filter (Millipore, Massachusetts, USA, Catalogue No.: HNWP02500) placed on LB agar and incubated overnight at 37°C to ensure the plasmids have been successfully transferred. Then, the membrane filter was transferred into a sterile microfuge tube and 1 ml of LB broth was added. The cells were detached from the membrane by vigorous agitation. The filter membrane was removed after the cell suspension was centrifuged at 12,000 x *g* for 1 min. The supernatant was removed and the cell pellet was resuspended in 1 ml of LB broth. The cell suspension

was serially diluted and spread on LB agar supplemented with the necessary antibiotics (Table 2.3) to obtain single colonies.

2.14 Growth Kinetics

Bacterial growth rate at the logarithmic phase was used to determine the fitness of the strain when a particular mutation or plasmid had been introduced into the strain (Cottell et al., 2014). To do this, a single colony was inoculated into LB broth supplemented with the appropriate antibiotic and incubated overnight at 37°C, 200 rpm. The overnight culture was diluted with fresh LB broth to give a final inoculum of 4% (v/v). A volume of 200 µl of this diluted culture was transferred into wells of 96-well microtitre plate (Sterilin, Catalogue No.: 612U96). Three biological replicates and four technical replicates were used per experiment. The growth of the cultures was monitored at OD_{600} at 10 min intervals per cycle for 100 cycles using the FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany). The growth rates (generation time) for the bacteria were calculated from the logarithmic phase of growth using the following formula:

$$\text{Generation time } = \frac{(t_1 - t_0)}{\left\{3.3 \times \left[log_{10}\left(\frac{n_1}{n_0}\right)\right]\right\}}$$

Where, $t_1 - t_0$ is the difference in minutes between the estimated time when the logarithmic growth phase ends and starts. The variables, n_1 and n_0 are the estimated number of cells at the end and the beginning of the logarithmic growth phase obtained by multiplication of the OD_{600} reading with a constant of 10^6. The experiment was repeated three times. The generation times were compared using a Student's *t*-test, *p* values less than 0.05 were considered statistically significant.

2.15 Determination of Conjugation Frequency

The conjugation frequency of plasmids from their original clinical isolates was determined as previously described (Cottell et al., 2014). Overnight cultures of the donor and recipient bacterial strains were prepared from single colonies. Overnight cultures of the donor and recipient strains were then used to inoculate fresh 20 ml LB broth cultures at a dilution of 1 in 50. This fresh culture was allowed to incubate at 37°C with shaking (200 rpm) until they reached an OD_{600} of 0.6. Then, 1 ml of the culture was transferred into sterile microfuge tubes. The donor and recipient strains were centrifuged at 12,000 x *g*, 1 min and the supernatants were discarded. The donor was resuspended in 100 µl LB broth while the recipient strain was resuspended in 50 µl LB broth. Then, 50 µl of the donor strain was mixed with 50 µl of the recipient strain so that the donor:recipient ratio was 1:2. This was done to ensure that all donor cells were in contact with at least one recipient cell. The suspension was then transferred on to sterile 0.45 µm nylon filters placed on LB agar. The conjugation mixture was then left to incubate at 37°C for 3 hours. After the incubation period, the filter was aseptically transferred into a sterile microfuge tube and 1 ml of LB broth was added. The transconjugants were liberated from the filters by vigorous agitation. The transconjugants were serially diluted and plated on LB agar supplemented with relevant antibiotics. The transconjugants were left to incubate overnight at 37°C. The conjugation frequency of the plasmids was calculated according to the formula below. Conjugation frequencies were determined on three separate occasions and differences were deemed significant when $p<0.05$ by Student's *t*-test.

$$\text{Conjugation Frequency} = \frac{\text{Number of transconjugants (cfu/ml)}}{\frac{1}{2} \times \text{Number of donors (cfu/ml)}}$$

2.16 Minimum Inhibitory Concentration (MIC) Determination

The MIC of various antibiotics against the strains used in this study was routinely determined. The values were determined using the guidelines recommended by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2001). *E. coli* NCTC10418 was used as a control strain for susceptibility testing. Where the MIC values of the control strain were not within 2-fold dilution of those reported in the BSAC guidelines, all the MIC values for that particular experiment were disregarded. Similarly, a difference in MIC value of more than 2-fold dilution was considered significant according to BSAC guidelines.

2.17 Biofilm Assay

2.17.1 Biofilm Formation on Plastic in Microtitre Tray

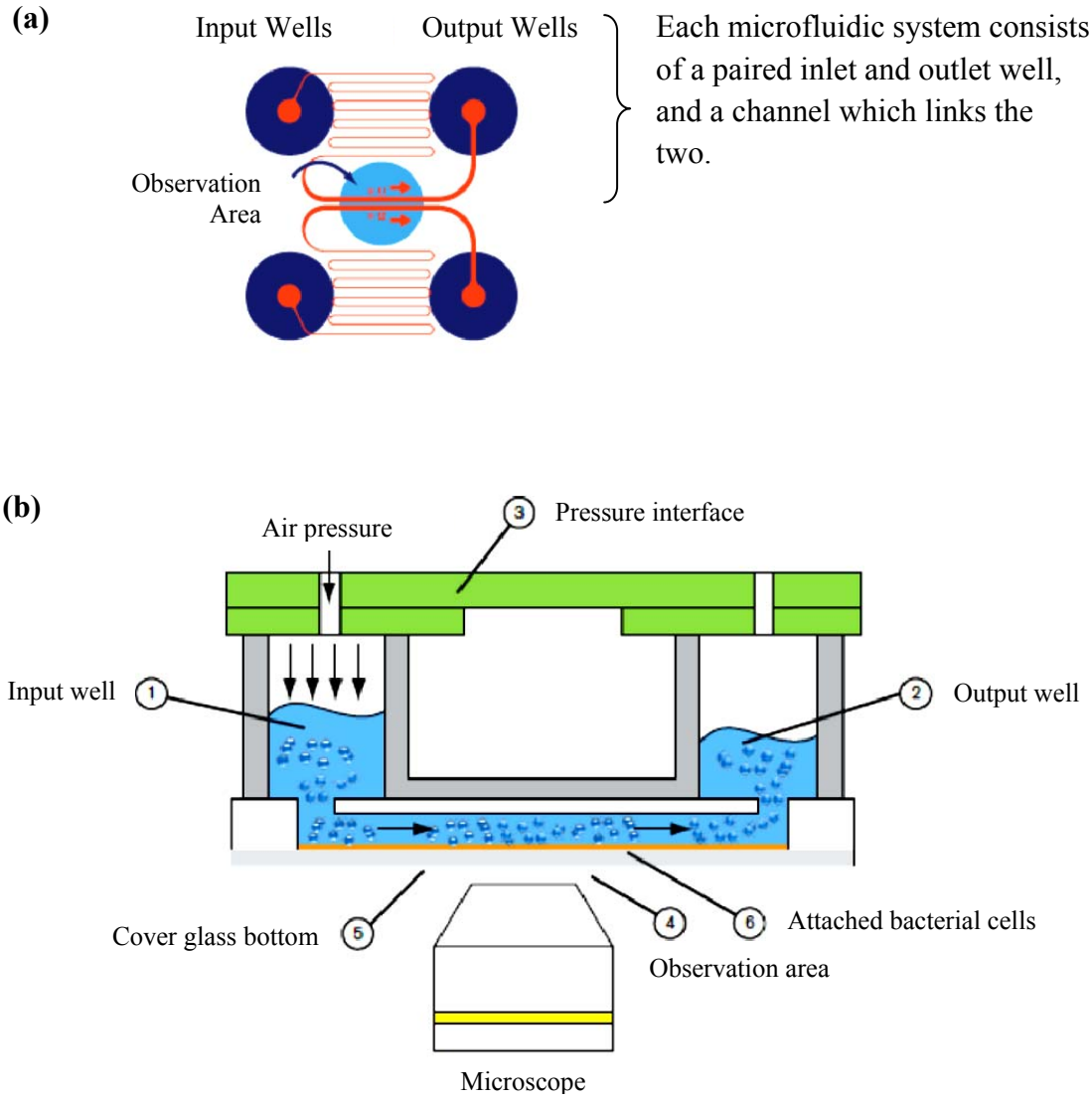
The impact of plasmid carriage on biofilm formation by bacterial strains was assessed in 96-well microtitre trays as previously described (Baugh et al., 2012). Briefly, overnight cultures of the bacterial strains were diluted in fresh LB broth without sodium chloride [10 g/L tryptone (Oxoid, Catalogue No.: LP0042) and 5 g/L yeast extract (Oxoid, Catalogue No.: LP0021)] to give a final inoculum of 4% (v/v). It has been shown that *Salmonella* strains are able to form biofilm more efficiently in LB broth without salt (Jain and Chen, 2007). Hence, this condition was chosen and used throughout the biofilm assays. A total of 200 µl of the diluted liquid culture was transferred into the wells of the microtitre tray. The microtitre tray was incubated at 30°C with gentle shaking for 48 hours. After incubation, the cultures were decanted from the tray and the biofilm formed in the wells was stained with 0.1% (w/v) crystal violet solution (Sigma Aldrich, Missouri, USA, Catalogue No.: C6158) for 15 min. Then, the dye was removed and the microtitre tray was washed with sterile water to remove the excess dye. The crystal violet stain was solubilised in 200 µl of 70% (v/v) ethanol (Analar Normapur, Pennsylvania, USA, Catalogue No.: 20821.330) with gentle shaking for 15 min.

The intensity of the solubilised crystal violet which relates to the amount of biofilm formed was measured at OD_{600}. In each experiment, three biological and four technical replicates were included for each strains tested. The biofilm formation of each strain was determined in three independent experiments. The data were analysed using Student's *t*-test and $p<0.05$ was considered as significant.

2.17.2 Biofilm Formation on Glass Under Flow

The ability of the plasmid carrying bacteria to form biofilms was also assessed under constant flow of liquid media in glass microfluidic flow cells as previously described (Baugh et al., 2014) with some modifications. In order to prime the BioFlux^{TM} 200 48-well low shear plate (0-20 dynes/cm^2) (Fluxion Biosciences, California, USA), 200 µl of LB broth (without salt) was added into the output well (used for waste collection) (Figure 2.1a). The BioFlux^{TM} interface was tightly fastened on to the 48-well plate. Flow tubes were attached to the BioFlux^{TM} interface and the settings (LB broth at 19°C, flow rate = 5 dynes/min) were selected on the BioFlux^{TM} system software. The flow of LB (without salt) was started by applying air pressure at the output well to allow the flow channel to be primed with broth for 5 min. Overnight cultures of the strains were diluted into an OD_{600} of 0.1. The excess broth was removed from the input and output wells (Dark blue) (Figure 2.1a). This left a small amount of broth in the inner region (Orange) of the input and output wells. About 50 µl of LB broth (without salt) was added to the input well. An equal volume of diluted bacteria was added into the output well. Then, the BioFlux^{TM} interface was attached and the air pressure was applied on the output well again at a flow rate of 3 dynes/min for 3 seconds. The flow was then stopped immediately. The presence of bacteria in the flow cell (Light blue) (Figure 2.1a) was verified with a LabtechScope LTSi-1000 inverted microscope (Labnet, New Jersey, USA) at 400X magnification. The 48-well plate was then incubated on a heating block

Figure 2.1 BioFlux system



(a) The general layout of the BioFlux system (Top view). (b) The functions of the individual components of the BioFlux system (Side view). The air pressure will force the LB broth (no salt) in the input well (1) to slowly flow through the microfluidic channel into the output well (2) at a constant flow rate. This will provide constant nutrients to the bacteria and mimic the conditions within blood vessels. The Bioflux interface (3) creates an airtight environment between the wells and the environment. The cover glass bottom (5) allows bacterial cells (6) to attach and form the biofilm. An inverted microscope can be used to observe the biofilm in the observation area (4).

Source: Adapted from BioFlux™ instruction manual.

(AccuBlock^{TM}, Labnet) set at 30°C for 2 hours. This is to allow the bacteria to attach to the surface of the microfluidic channel (Figure 2.1b). After incubation, the excess broth was removed from the output well and 1 ml of fresh LB broth (without salt) was added into the input well. The flow rate was set to a constant 0.3 dynes/min by applying air pressure on the input well and the plate was incubated on the heating block for 48 hours. The biofilms were observed at 6, 12, 24 and 48-hour time points at 400X magnification. The area of coverage by the biofilm in the microfluidic channel was determined using image analysis software, ImageJ (http://imagej.nih.gov/ij/). The student's *t*-test was used to determined whether a significant difference ($p<0.05$) was observed in the area of coverage.

2.18 Plasmid Persistence

The proportion of the bacterial population which retains a plasmid was determined over a period of 20 days as previously described (Cottell et al., 2014). A single colony was inoculated in 10 ml LB broth supplemented with 0.25 µg/ml doripenem. The culture was incubated overnight at 37°C with agitation, 200 rpm. On the next day, the culture was sub-cultured into 10 ml of fresh LB broth without antibiotics at a dilution of 1 in 100. This step was repeated daily for 20 days. At appropriate intervals (e.g. day 5, 10, 15 and 20), the culture was serially diluted and plated on LB agar and incubated overnight at 37°C. On the following day, the colonies were replica plated using sterile velveteen squares on to LB agar supplemented with 0.25 µg/ml doripenem. The retention of the plasmid in a particular bacterial strain was calculated as a percentage of doripenem resistant colonies over the total number of colonies observed on the antibiotic-free LB agar. The experiment was repeated on three separate occasions. To determine the number of generations the bacterial strains had achieved after 24 hours of incubation, viable counting was done to determine the number of cfu/ml of bacteria after the sub-culturing step of 1 in 100 and after the 24-hour incubation

period. The number of generations the bacteria had achieved after 24 hours were calculated using the following formula:

$$\text{Number of generations} = \log_2 \mathrm{P} - \log_2 \mathrm{Q}$$

P is the number of cfu/ml of the culture after 24 hours of incubation whereas Q is the number of cfu/ml after the initial dilution of the overnight culture.

2.19 Pairwise Competition Assay

Single colonies of pKpQIL-UK and pKpQIL-D2 *bla*$_{KPC}$::*aph* carrying *K. pneumoniae* Ecl8^{Rif} strains were inoculated into 10 ml LB broth supplemented with 0.25 µg/ml doripenem and the cultures were incubated overnight at 37°C (shaken at 200 rpm). The overnight cultures were diluted with fresh LB broth into an OD_{600} of 0.1. Then, 50 µl of each bacterial culture was inoculated into 10 ml of fresh LB broth without antibiotic selection. The cultures were incubated overnight at 37°C with agitation (200 rpm). On the following day, the culture was sub-cultured into 10 ml of fresh LB broth to give a 1 in 100 dilution. This step was repeated 20 days to allow both bacterial strains to compete against each other over the duration of the experiment. To determine the ratio of both of the plasmid carrying strains within the population, the culture was serially diluted and plated on LB agar and incubated overnight at 37°C at appropriate intervals (e.g. day 5, 10, 15 and 20). On the following day, the colonies were replica plated using sterile velveteen squares on to two LB agar each supplemented with 0.25 µg/ml doripenem and 50 µg/ml kanamycin. The competition experiment was repeated on three separate occasions. The competition index of the bacterial strains was calculated using the following formula (Luo et al., 2005):

$$\text{Competition Index} = \frac{\mathrm{X} - \mathrm{Y}}{\mathrm{X} + \mathrm{Y}}$$

X is the average proportion of pKpQIL-D2 carrying *K. pneumoniae* Ecl8^{Rif} in the total bacterial population whereas Y is the equivalent for the pKpQIL-UK carrying strain at the end of the experiment. A positive value indicates that the pKpQIL-D2 carrying strain is fitter than the pKpQIL-UK carrying strain. A negative value indicates the opposite.

2.20 *Galleria mellonella* Infection Model

Overnight cultures of *K. pneumoniae* ST258 and its pKpQIL-UK and -D2 carrying strains were prepared by inoculating a single colony of the bacterial strain into 10 ml LB broth supplemented with 0.25 μg/ml doripenem. Viable counting was done to determine the cfu/ml of the overnight cultures of the *K. pneumoniae* ST258 and its pKpQIL-UK and -D2 carrying strains. The *Galleria mellonella* Wax Moth larvae were purchased from Livefood UK Limited (www.livefoods.co.uk). The larvae were placed in groups of 10 into sterile petri dishes. The larvae used were about 2 cm in length. Using the data from the viable counting, the overnight cultures of the bacterial strains were diluted in sterile PBS to obtain an inoculum of 10^7 cfu/ml. A total of 10^5 cfu in 10 μl was injected into each larva at the last right proleg. The larvae were incubated in the dark at 37°C. The percentage of survival of the larvae was recorded every 24 hours over a period of five days. The larva was considered dead when it was black in colour and no movement was observed when the larva was gently agitated. The experiment was repeated on two separate occasions. Log-rank (Mantel-Cox) test was used to determine whether a significant difference ($p<0.05$) was observed between the groups of larvae infected with the different strains.

2.21 RNA Sequencing

2.21.1 RNA Extraction

In order to understand the effects of plasmids on host bacterium gene expression, RNA sequencing was used. Five overnight cultures (biological replicates) of the strains of interest

(*K. pneumoniae* ST258, *K. pneumoniae* ST258/pKpQIL-UK and *K. pneumoniae* ST258/pKpQIL-D2) were grown in MOPS minimal medium (Teknova, California, USA, Catalogue No.: M2101) at 37°C with shaking, 200 rpm. The strains were then sub-cultured into fresh MOPS medium the next day at a dilution of 1 in 50 and allowed to grow until the OD_{600} reached 0.6. Then, three OD units of the culture were centrifuged at 12,000 x *g*, 1 min. RNA was extracted from the cell pellets using the RiboPure™ RNA Purification Kit (Ambion, California, USA, Catalogue No.: AM1925) according to the manufacturer's instructions. Instead of using a vortex mixer as instructed by the manual, bacterial cells were homogenised three times using a RiboLyser (Hybaid, Basingstoke, UK) at 6.5 meters/sec for 45 sec (with 1 min interval to avoid overheating) to disrupt the capsular cells. The extracted RNA was diluted 1 in 10 and quantified using a Qubit® RNA HS Assay Kit (Life Technologies, Catalogue No.: Q32852). Undiluted RNA was also quantified for the presence of contaminating DNA using a Qubit® dsDNA HS Assay Kit (Life Technologies, Catalogue No.: Q32854). The percentage of DNA over the total amount of nucleic acid (DNA+RNA) was calculated. Where the percentage of DNA exceeded 5%, RNA samples were treated with TURBO™ DNase (Ambion, Catalogue No.: AM1907). DNase treatment was repeated until the percentage of DNA was less than 5%. Contamination with genomic DNA will reduce the efficiency of ribosomal RNA (rRNA) removal and affect RNA library preparation. The presence of DNA was also confirmed using PCR (40 cycles) to amplify the gyrase gene (*gyrB*). The quality of the DNAse-treated RNA samples was analysed using a 2100 Bioanalyzer (Agilent, California, USA) with the RNA 6000 Nano Kit (Agilent, California, USA, Catalogue No.: 5067-1511).

2.21.2 Ribosomal RNA (rRNA) Reduction

To avoid rRNA saturation of the RNA sequencing experiment, rRNA depletion is needed. The efficiency of the rRNA removal can be affected by the presence of contaminating salts and solvents from the reagents of the RNA extraction and DNAse treatment kits. Hence, samples with no detectable DNA contamination were purified to remove salts or solvents using the RNA Clean & Concentrator^{TM}-5 Kit (Zymo Research, California, USA, Catalogue No.: R1015). The RNA was eluted with RNAse-free water in the final step of the clean-up process. The ribosomal RNA in the purified total RNA samples was depleted using the Ribo-Zero^{TM} rRNA Removal Kit (Gram-negative bacteria) (Epicentre, Wisconsin, USA, Catalogue No.: MRZGN126) according to the manufacturer's instructions. Although the kit's manual stated a maximum of 5 µg total RNA can be used per reaction, a lesser amount ca. 2 – 4 µg was used to avoid overloading of the reaction which would result in reduced rRNA removal efficiency. The rRNA-depleted RNA samples were concentrated into 7 µl total volume using the RNA Clean & Concentrator^{TM}-5 Kit in RNAse-free water. Then, the samples (1 µl each) were analysed using 2100 Bioanalyzer (Agilent, California, USA) with the RNA 6000 Pico Kit (Agilent, California, USA, Catalogue No.: 5067-1513) which can detect a lower concentration of RNA compared to the RNA 6000 Nano Kit.

2.21.3 RNA Library Preparation

Four RNA samples from each of the three strains (*K. pneumoniae* ST258, *K. pneumoniae* ST258/pKpQIL-UK and *K. pneumoniae* ST258/pKpQIL-D2) which possessed the correct quantity (10 – 400 ng) and quality (no detectable rRNA, DNA contamination and RNA degradation) were chosen for library preparation. The library preparation used the TruSeq® Stranded mRNA Sample Preparation Kit (Illumina, California, USA, Catalogue No.: RS-122-2102). The kit is designed for library preparation with eukaryotic mRNA. Therefore, the

initial stages of mRNA purification stated in the manufacturer's manual were replaced with rRNA-depletion (Section 2.21.2). For each sample, a volume of 5 µl of concentrated rRNA-depleted RNA (10 – 400 ng) was used for library preparation. The process began with fragmentation of the RNA transcripts to smaller fragments by heating and priming with random hexamers at 94°C. The fragmented RNA was then converted to cDNA (complementary DNA) using reverse transcriptase and then into double stranded cDNA. Each of the 12 samples (cDNA) was then ligated with different adapters which possessed unique nucleotide sequences. The adapters were needed to differentiate all the 12 samples during sequencing. The double stranded cDNA linked with adapters were selectively enriched by PCR and purified as instructed in the manufacturer's manual. The quality of the prepared libraries for each sample were determined using the 2200 TapeStation (Agilent, California, USA) with the D1000 ScreenTape Kit (Agilent, California, USA, Catalogue No.: 5067-5582). A successful library preparation yielded a single major peak representing a majority of 260 bp amplicons on the electropherogram. The libraries were also diluted 1000 times in nuclease-free water and quantified using the KAPA Library Quantification Kit for Next-Generation Sequencing (KAPA Biosystems, Massachusetts, USA, Catalogue No.: KK4824). This kit quantifies the library by measuring their amplification signals using qRT-PCR against a standard curve. All the libraries were then normalised to 10 nM and pooled together.

2.21.4 Sequencing

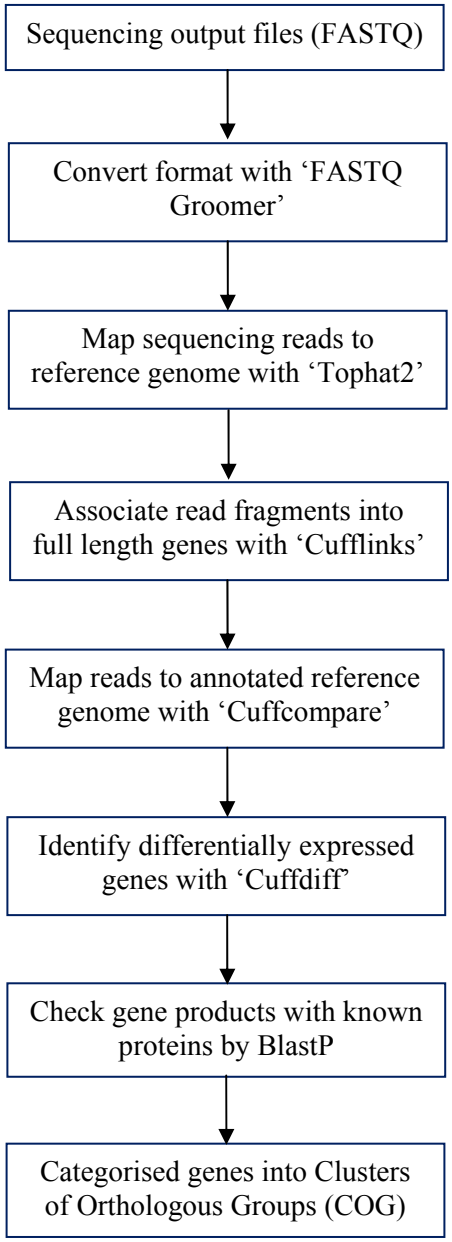
Before the sequencing step, the pooled library was diluted to 1 nM in nuclease-free water. A volume of 20 µl of the 1 nM library was mixed with 20 µl sodium hydroxide (0.2 N) (Sigma-Aldrich, Missouri, USA, Catalogue No.: 72082) to denature the library into single stranded cDNA. The mixture was incubated at room temperature for 5 min. Then, 40 µl of 200 mM Tris pH 7.0 was added to stop the denaturation process. The library was further diluted 10X in

HT1 (Hybridisation) buffer (Illumina, California, USA) to obtain a library at a final concentration of 25 pM for sequencing. The sequencing was done using a MiSeq® Sequencer (Illumina, California, USA) and MiSeq® Reagent Kit v3 (Illumina, California, USA, Catalogue No.: MS-102-3003). The sequencing parameters were set at 200 bp paired-end for 400 cycles.

2.21.5 Data Analyses

The RNA sequencing data was analysed using a variety of programmes hosted in Galaxy (https://usegalaxy.org). The general workflow is summarised in Figure 2.2. Briefly, the format of the sequencing files was converted into Sanger FASTQ format which was compatible with the analysis software on the website using 'FASTQ Groomer'. The converted forward and reverse reads of each sequence were then mapped on to a reference sequence (*K. pneumoniae* MGH78578 Accession No.: CP000647, pKpQIL-UK or pKpQIL-D2) using 'Tophat2'. As the sequences were of fragmented transcripts, the 'Cufflinks' programme was used to associate reads (sequences) to genes. The output from the 'Cufflinks' programme was then used to map the sequences on to known annotated genes on the reference sequence using 'Cuffcompare'. Finally, 'Cuffdiff' was used to detect the genes which were differentially expressed between the three different strains. The 'Cuffdiff' programme provides a list of genes which were the expression was significantly different between the three strains (*K. pneumoniae* ST258, ST258/pKpQIL-UK and ST258/pKpQIL-D2). The protein sequences of the genes were checked for known homology using the BlastP database. Then, the genes were categorised into their respective Clusters of Orthologous Groups (COG) (http://www.microbesonline.org/).

Figure 2.2 RNA sequencing data analyses workflow



CHAPTER THREE: THE CELL ENVELOPE AND CARBAPENEM RESISTANCE

3.1 Background

Detection of carbapenemase-producing Enterobacteriaceae (CPE) is usually carried out by susceptibility testing. However, this phenotypic test is not always accurate. Some CPE isolates are inhibited by a carbapenem MIC value in the susceptible range even in the presence of carbapenemases, resulting in false susceptible reports (Jain et al., 2014, Voulgari et al., 2013, Moland et al., 2003, Psichogiou et al., 2008, Scoulica et al., 2004, Bratu et al., 2005, Landman et al., 2010, Landman et al., 2011, Hrabák et al., 2013, Nahid et al., 2013, Tijet et al., 2013). Although the Clinical and Laboratory Standards Institute (CLSI) carbapenem recommended breakpoint concentration values were revised in 2010, so that isolates which were previously classified as carbapenem susceptible are now classified as carbapenem non-susceptible, there is the still possibility of false negative reports arising from the detection methods used (Doern et al., 2011, Zavascki et al., 2014). BSAC (http://bsac.org.uk/) and EUCAST (http://www.eucast.org/) which recommend higher MIC breakpoint concentrations also do not address this problem. The heterogeneity in carbapenem MIC values ranging from susceptible to resistant observed in these isolates may be associated with a combination of resistance mechanisms present in each isolate. Porins have been shown to affect the susceptibility of carbapenemase-producing *K. pneumoniae* to carbapenems via the OmpK35 (OmpF homologue) and OmpK36 (OmpC homologue), where the latter has been reported to play a more significant role (Tsai et al., 2011, Landman et al., 2009). Not much is known about the contribution of the AcrAB-TolC efflux system to carbapenem resistance in the presence of carbapenemases. However, this system is required for resistance to fluoroquinolones conferred by mutations in the DNA gyrase genes, and also to various β-lactam antibiotics (Piddock, 2006b).

3.2 Hypothesis and Aims

It was hypothesised that a functional AcrAB-TolC efflux system is required for the elaboration of resistance to clinically important carbapenem antibiotics in carbapenemase-producing Enterobacteriaceae. Therefore, the aims of this study were:

1. To transfer carbapenemase-encoding plasmids into AcrAB and TolC efflux pump component deletion mutants of *K. pneumoniae*, *E. coli* and *Salmonella*.

2. To determine the susceptibility of the plasmid carrying strains in the presence and absence of AcrAB or TolC to clinically relevant carbapenem antibiotics.

3.3 Carbapenem Resistance by bla_{KPC} is Seen Even in the Absence of the Tripartite AcrAB-TolC Multidrug Resistance Efflux Pump

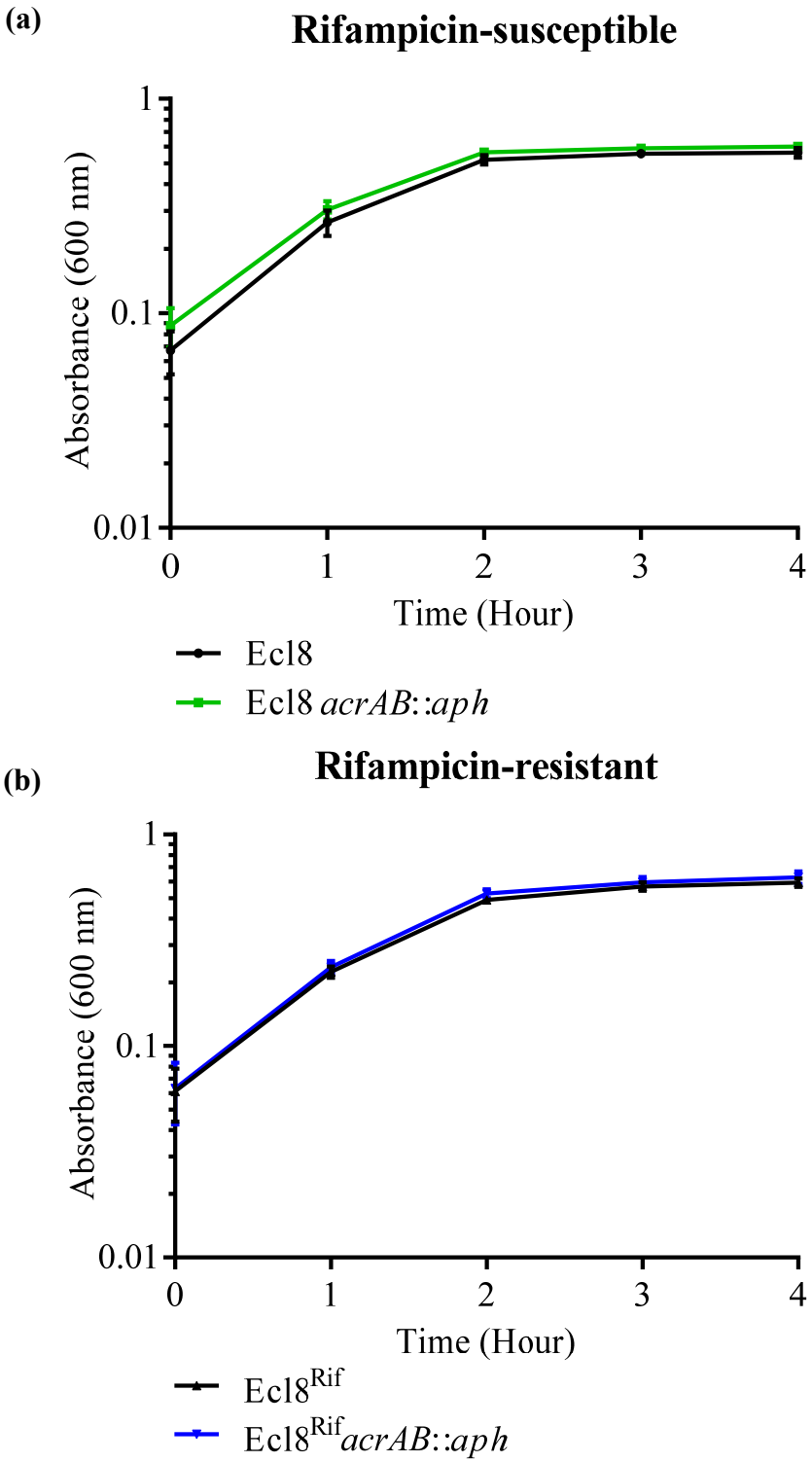
3.3.1 Strain Constructions

To investigate the function of the AcrAB-TolC efflux system in carbapenem resistance, a set of isogenic efflux mutants (AcrAB and TolC) were used. The carbapenemase gene encoding plasmids pKpQIL-UK and pKpQIL-D2 are large (ca. 114 kb) and transfer to new bacterial host strains via transformation is difficult (Hanahan, 1983, Ohse et al., 1995). Therefore, conjugation was used as the mode of transfer. To do this, the host bacterium *K. pneumoniae* Ecl8 and its isogenic AcrAB mutant needed a resistance marker so that the transconjugants carrying the plasmids could be selected from the donor bacterium. Unfortunately, despite repeated requests for a *K. pneumoniae* TolC mutant from T. Schneiders, the strain was not provided due to not being confirmed as the correct strain in her laboratory. Rifampicin-resistance was the chosen marker for the recipient strains as this is easily selected *in vitro* (Miller et al., 2002). The rifampicin-resistant *K. pneumoniae* Ecl8 and AcrAB mutants were selected at a frequency of 8.6×10^{-9} and 8.5×10^{-10}, respectively. This suggested a single point mutation during the selection of the mutants (Miller et al., 2002). No difference in generation

times was observed between the parental and the isogenic efflux mutant strain (Figure 3.1 & Table 3.1). The *rpoB* gene which encodes for the subunit of bacterial RNA polymerase is a known target for rifampicin and mutation in this gene can lead to resistance to this antibiotic (Miller et al., 2002).

A set of efflux deletion mutants was also constructed in *E. coli* BW25113. Rifampicin-resistant mutants were selected from the *E. coli* strain and its isogenic efflux mutants concurrently. The *E. coli* BW25113 and its isogenic efflux mutants (*acrB*::*aph* and *tolC*::*aph*) from the Keio Collection were used for this experiment (Baba et al., 2006). Rifampicin-resistant mutants of *E. coli* BW25113 (to give *E. coli* BW25113^{Rif}), *E. coli* BW25113 *acrB*::*aph* (to give BW25113^{Rif} *acrB*) and *E. coli* BW25113 *tolC*::*aph* (to give BW25113^{Rif} *tolC*) were selected at a frequency of 2.3 x 10^{-8}, 1.3 x 10^{-8} and 1.1 x 10^{-8}, respectively. Sequencing of the purified *rpoB* gene PCR amplicon (Figure 3.2) showed a single point mutation in all the three strains. *E. coli* BW25113^{Rif} and its AcrB mutant possess a single C1691T (Pro574Leu) substitution, whereas the *E. coli* BW25113^{Rif} *tolC* mutant contained a single C1576T (His526Tyr) substitution. The rifampicin-resistant mutants of the *E. coli* strain set were then verified by PCR to ensure that the correct efflux pump component genes were inactivated (Figure 3.3). Due to the difference in the mutation found in the *rpoB* gene, the generation time of the three strains were investigated. Without the rifampicin-resistant marker, there was no difference in generation time of the Keio Collection strains with/without AcrB or TolC (Figure 3.4a, Table 3.2). Compared to the *E. coli* BW25113^{Rif}, the rifampicin-resistant mutants with the inactivated efflux pump component genes had the same significant, but slower generation time ($p<0.05$) (Figure 3.4b). Despite this, the slower growth rate of the rifampicin-resistant mutants is unlikely to have a significant impact on carbapenem activity as

Figure 3.1 **Growth kinetics of rifampicin-susceptible/resistant mutants of *K. pneumoniae* Ecl8 and its efflux mutants**



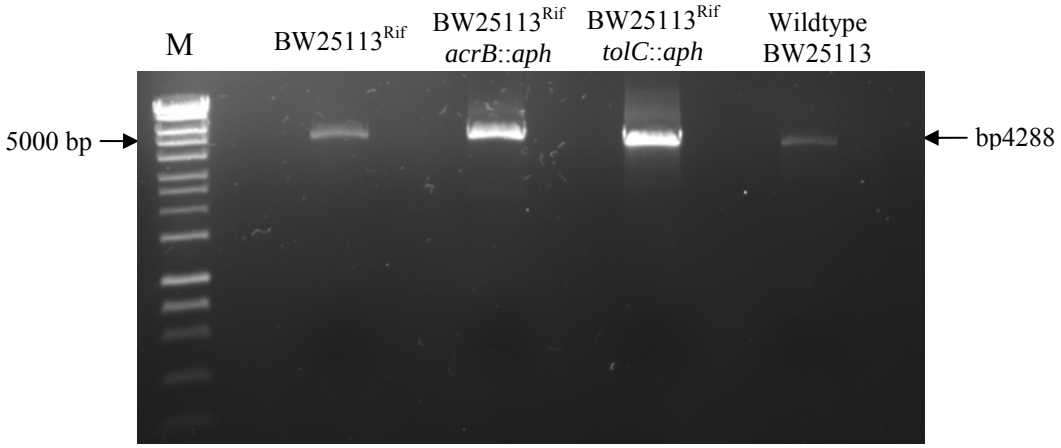
Growth kinetics was done to compare the growth of the rifampicin-susceptible and -resistant *K. pneumoniae* Ecl8 and its isogenic *acrAB* mutant. Absorbance values were recorded as mean ± standard deviation of three independent experiments. Generation times were recorded in Table 3.1.

Table 3.1 Generation times of rifampicin-susceptible/resistant *K. pneumoniae* Ecl8 and its isogenic *acrAB* mutant

***K. pneumoniae* Strain**	**Generation Time (min)**	**Student's *t*-test**
Ecl8	27.4 ± 3.8	-
Ecl8 *acrAB*::*aph*	29.1 ± 2.3	0.54
$Ecl8^{Rif}$	28.7 ± 1.0	-
$Ecl8^{Rif}$ *acrAB*::*aph*	27.2 ± 1.3	0.25

Generation time was determined to assess the fitness impact of the rifampicin resistant marker in the *K. pneumoniae* Ecl8 and its isogenic *acrAB* mutant. Generation time is shown as a mean ± standard deviation of three independent experiments. Student's *t*-test was used to analyse the generation times ($p<0.05$).

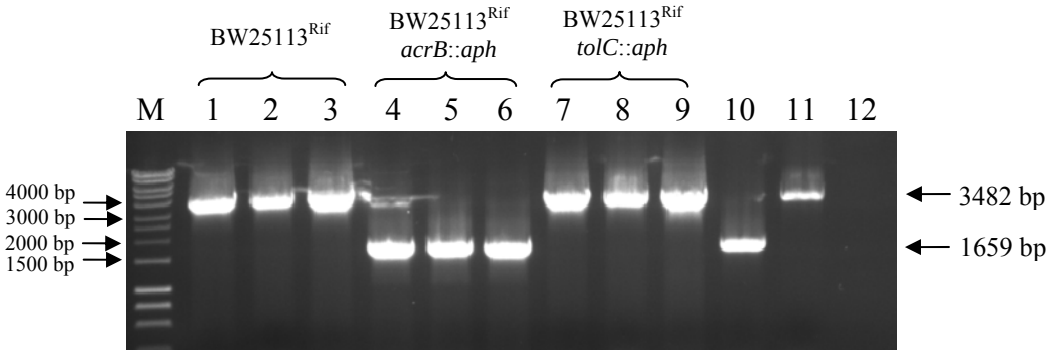
Figure 3.2 PCR amplification of *rpoB* of rifampicin-resistant *E. coli* BW25113 for DNA sequencing



The *rpoB* genes of the various *E. coli* BW25113 strains were amplified. The amplicons were purified for sequencing to determine the mutation in the *rpoB* gene. M: Hyperladder I (Bioline); Primers: RpoB-Ecoli-F1/R1; Expected amplicon: 4288 bp.

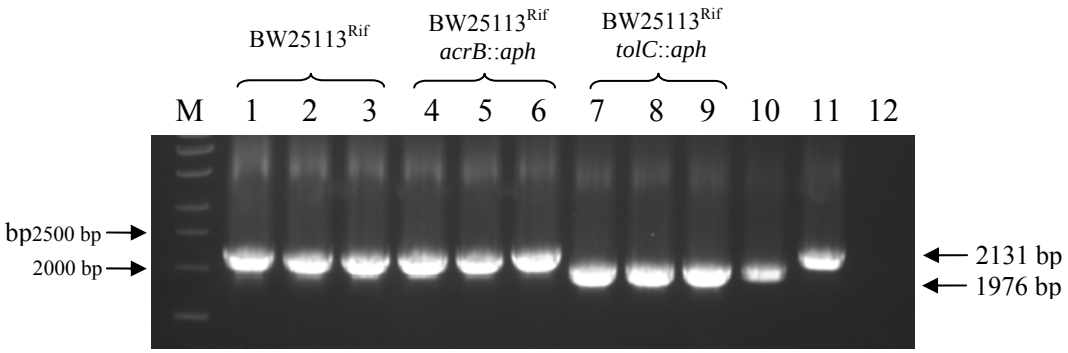
Figure 3.3 PCR verification of inactivated *acrB* and *tolC* genes in rifampicin-resistant mutants of Keio Collection strains

(a) PCR to check for inactivated *acrB* gene



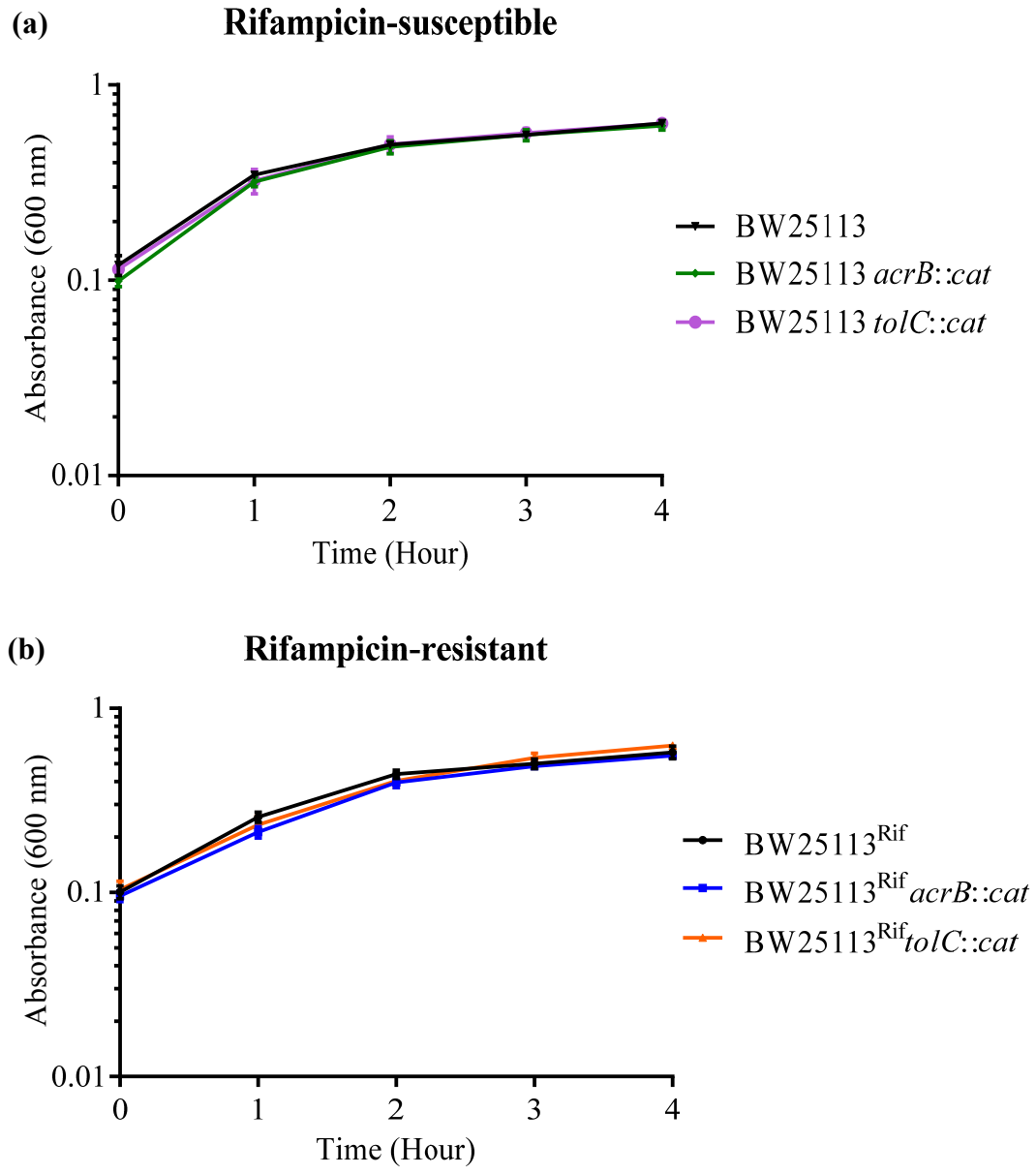
The various rifampicin-resistant mutants of *E. coli* BW25113 were verified by PCR for the presence of the inactivated *acrB* gene. M: Hyperladder I (Bioline); Lane 10: BW25113 *acrB*::*aph* genomic DNA (Positive control); Lane 11: BW25113 genomic DNA (Negative control); Lane 12: Water (Contamination control). Primers: acrB fwd/rev; Expected amplicon: 1659 bp (mutant), 3482 bp (wildtype).

(b) PCR to check for inactivated *tolC* gene



The various rifampicin-resistant mutants of *E. coli* BW25113 were verified by PCR for the presence of the inactivated *tolC* gene. M: Hyperladder I (Bioline); Lane 10: BW25113 *tolC*::*aph* genomic DNA (Positive control); Lane 11: BW25113 genomic DNA (Negative control); Lane 12: Water (Contamination control). Primers: tolC fwd/rev; Expected amplicon: 1976 bp (mutant), 2131 bp (wildtype).

Figure 3.4 **Growth kinetics of rifampicin-susceptible/resistant mutants of *E. coli* BW25113 and its efflux mutants**



Growth kinetics was done to compare the growth of the rifampicin-susceptible and -resistant *E. coli* BW25113 and its isogenic efflux mutants. Absorbance values were recorded as mean ± standard deviation of three independent experiments. Generation times were recorded in Table 3.2.

Table 3.2 Generation times of rifampicin-susceptible/resistant *E. coli* BW25113 and its isogenic efflux pump mutants

***E. coli* Strain**	**Generation Time (min)**	**Student's *t*-test**
BW25113	35.7 ± 4.4	-
BW25113 *acrB*::*cat*	31.9 ± 2.3	0.07
BW25113 *tolC*::*cat*	35.3 ± 2.4	0.86
$BW25113^{Rif}$	34.6 ± 2.0	-
$BW25113^{Rif}$ *acrB*::*cat*	39.2 ± 3.3	**0.04***
$BW25113^{Rif}$ *tolC*::*cat*	40.6 ± 2.9	**0.002***

Asterisk (*) denotes statistically significant difference ($p<0.05$) between the generation time of the mutants relative to the parental strain (BW25113/$BW25113^{Rif}$). Generation time is shown as a mean ± standard deviation of three independent experiments.

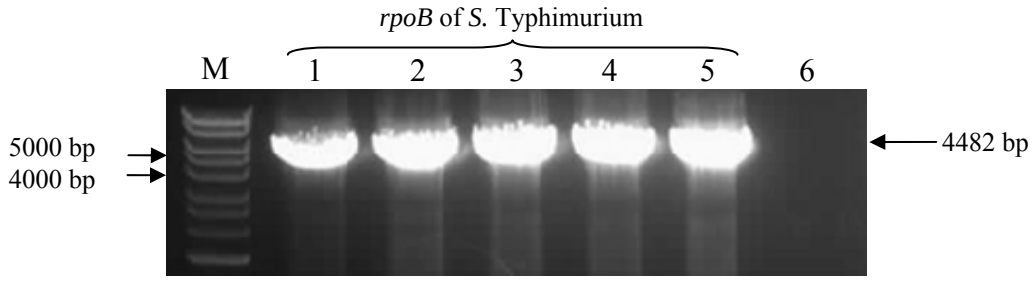
the killing effect of carbapenem antibiotics is growth independent (Cozens et al., 1989, Eng et al., 1991).

Rifampicin-resistant *S*. Typhimurium ATCC14028s mutants (henceforth call 14028s^{Rif}) were selected at a frequency of 7.3 x 10^{-10}, suggesting a single mutation had occurred during selection (Miller et al., 2002). The *rpoB* gene was amplified by PCR, and the 4482 bp amplicon, which contained the *rpoB* gene, was purified for DNA sequencing (Figure 3.5a). The sequence data revealed a single C1565A nucleotide substitution (single Ser522Tyr amino acid substitution) in the *rpoB* gene.

In order to ensure that efflux pump component mutants (*acrAB*::*cat* and *tolC*::*cat*) were in the same genetic background, the inactivated efflux pump genes from insertion mutants of EG16564 (*tolC*::*cat*) and EG16566 (*acrAB*::*cat*) (Nishino et al., 2006) were transferred into 14028s^{Rif} by P22 phage transduction. The *S.* Typhimurium 14028s^{Rif} *acrAB*::*cat* (henceforth call 14028s^{Rif} *acrAB*) and 14028s^{Rif} *tolC*::*cat* (henceforth call 14028s^{Rif} *tolC*) transductants were then verified by PCR to carry the correct inactivated efflux pump component genes (Figure 3.5b). PCR was also used to verify the presence of the chloramphenicol resistance gene (*cat*) which had been inserted to inactivate the *acrAB* genes. Only the mutant with the inactivated genes would yield the amplicons of 500 bp (Primers: Chlor-Fwd/AcrA-Up) and 415 bp (Primers: AcrB-Down/Chlor-Rev). The inactivated *tolC* gene yielded a 1812 bp amplicon. The strains carrying the wildtype *tolC* gene gave a larger amplicon of 2489 bp. All strains were correct by PCR. The generation time of 14028s^{Rif} *tolC*::*cat* was significantly slower than 14028s^{Rif} ($p<0.05$) (Figure 3.6, Table 3.3). The efflux mutant series were also checked by API® 20E Gram-negative identification strips to confirm they were *K. pneumoniae*, *E. coli* and *S.* Typhimurium.

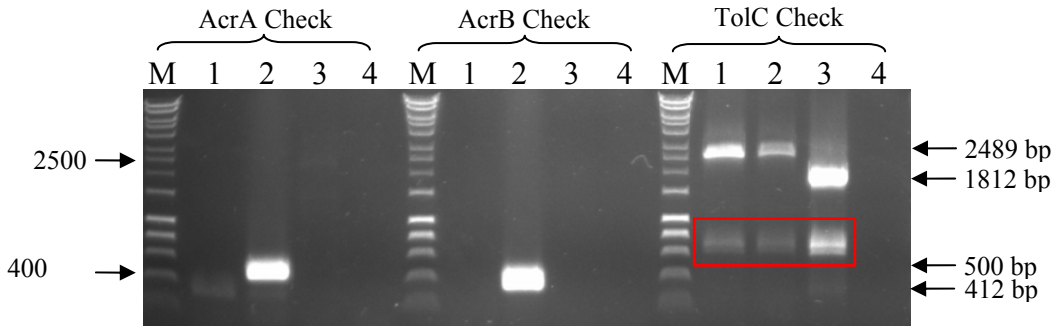
Figure 3.5 PCR amplification of *rpoB* of rifampicin-resistant *S.* Typhimurium ATCC14028s for sequencing and mutant verification

(a) Amplification of *rpoB* gene from rifampicin-resistant *Salmonella*



The *rpoB* gene of the rifampicin-resistant *S.* Typhimurium 14028s mutants was amplified. The amplicons were purified for sequencing to determine the mutation in the *rpoB* gene. M: Hyperladder I (Bioline); Lane 6: Water (Contamination control); Primers: RpoB-F1/R1; Expected amplicon: 4482 bp.

(b) PCR check for inactivated efflux genes



The various rifampicin-resistant mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the respective inactivated efflux component genes. M: Hyperladder I (Bioline); Lane 1: 14028s^{Rif}; Lane 2: 14028s^{Rif} *acrAB*::*cat*; Lane 3: 14028s^{Rif} *tolC*::*cat*; Lane 4: Water (Contamination control).

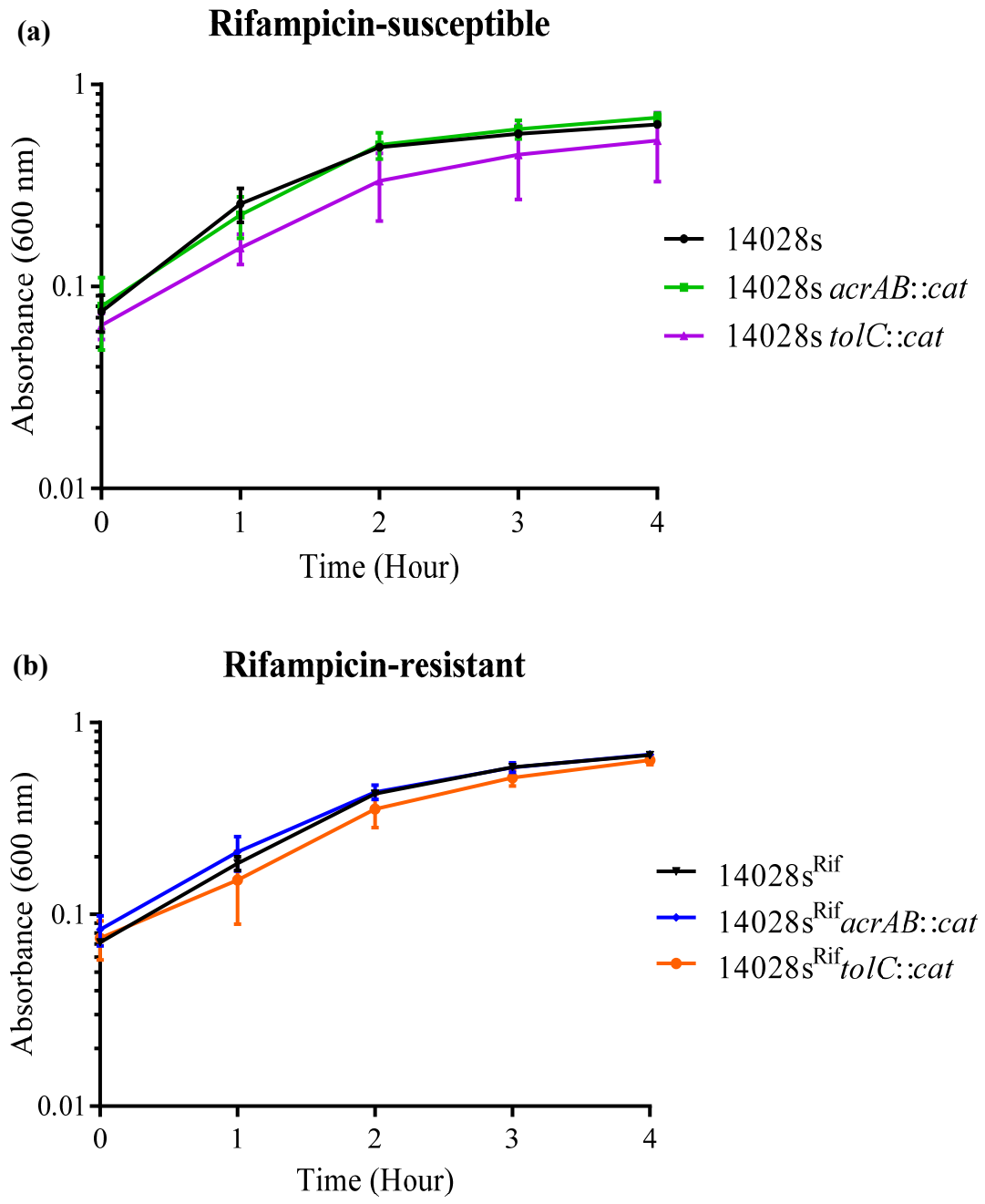
To check for the inactivated AcrA, Primers: Chlor-Fwd & AcrA-Up; Expected amplicon: 500 bp (mutant), no amplicon (wildtype).

To check for the inactivated AcrB, Primers: AcrB-Down & Chlor-Rev; Expected amplicon: 415 bp (mutant), no amplicon (wildtype).

To check for the inactivated TolC, Primers: TolC-Check-F/R; Expected amplicon: 1812 bp (mutant), 2489 bp (wildtype).

Red box: Amplification due to false priming.

Figure 3.6 Growth kinetics of rifampicin-susceptible/resistant mutants of *S.* Typhimurium 14028s and its efflux mutants



Growth kinetics was done to compare the growth of the rifampicin-susceptible and -resistant *S.* Typhimurium 14028s and its isogenic efflux mutants. Absorbance values were recorded as mean ± standard deviation of three independent experiments. Generation times were recorded in Table 3.3.

Table 3.3 Generation times of rifampicin-susceptible/resistant *S.* Typhimurium 14028s and its isogenic efflux pump mutants

***S.* Typhimurium Strain**	**Generation Time (min)**	**Student's *t*-test**
14028s	30.9 ± 3.1	-
14028s *acrB*::*cat*	35.1 ± 4.6	0.05
14028s *tolC*::*cat*	42.8 ± 4.6	**<0.001***
$14028s^{Rif}$	39.8 ± 4.0	-
$14028s^{Rif}$ *acrB*::*cat*	40.7 ± 3.6	0.61
$14028s^{Rif}$ *tolC*::*cat*	53.7 ± 9.2	**0.002***

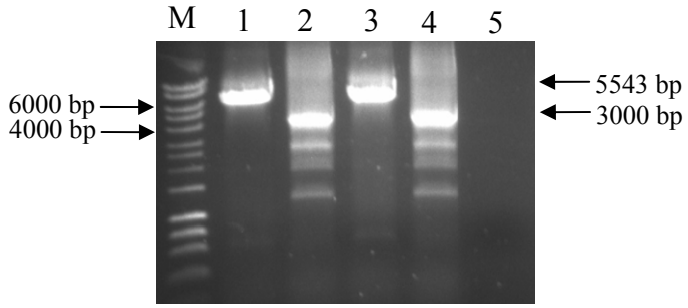
Asterisk (*) denotes statistically significant difference ($p<0.05$) between the generation time of the mutants relative to the parental strain (14028s/$14028s^{Rif}$). Generation time is shown as a mean ± standard deviation of three independent experiments.

In order to assess the contribution of a functional AcrAB-TolC efflux pump in carbapenem resistance, the *bla*$_{KPC}$-encoding plasmid pKpQIL-UK was transferred from *E. coli* DH10B into the various parental and isogenic efflux mutant strains by filter conjugation. The *K. pneumoniae* Ecl8^{Rif} and Ecl8^{Rif} *acrAB* mutant with/without pKpQIL-UK were verified by PCR for the inactivation of the *acrAB* genes (Figure 3.7a). The transconjugants were also checked by PCR for the presence of the *bla*$_{KPC}$ (Figure 3.7b) and pKpQIL-UK plasmid (Figure 3.7c). All transconjugants were verified by PCR for the presence of the expected amplicons which represent the *bla*$_{KPC}$ gene (Primers: KPCg-colpcrF/R), a plasmid backbone fragment which differentiates pKpQIL-UK from -D2 (Primers: pQIL-F/R), and the respective inactivated efflux pump component genes (Primers: AcrABdelchkF/R). All transconjugants contained the expected amplicons i.e. *bla*$_{KPC}$ gene (785 bp), pKpQIL-UK backbone fragment (383 bp) and inactivated *acrAB* genes (3000 bp). The series of *E. coli* BW25113^{Rif} transconjugants carrying pKpQIL-UK were also verified by PCR for the presence of the *bla*$_{KPC}$ gene (Figure 3.8a) and pKpQIL-UK plasmid (Figure 3.8b).

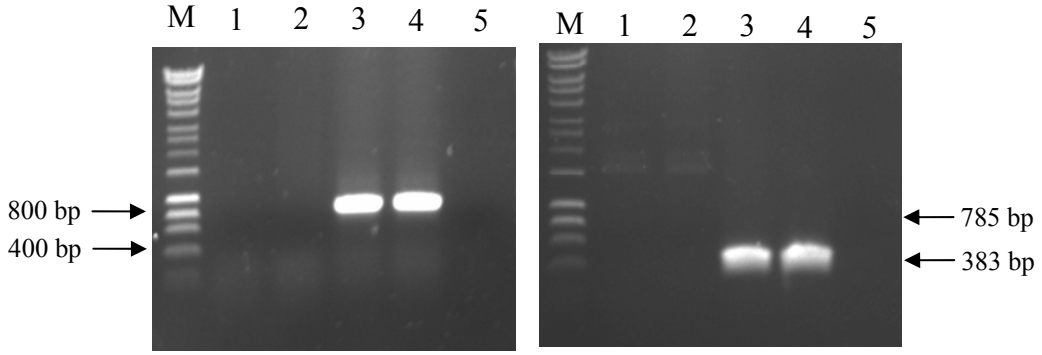
PCR verifications were also done for the various *S.* Typhimurium 14028s^{Rif} strains carrying the pKpQIL-UK plasmids: 14028s^{Rif}/pKpQIL-UK (Figure 3.9a & b), 14028s^{Rif} *acrAB*/pKpQIL-UK and 14028s^{Rif} *tolC*/pKpQIL-UK (Figure 3.10a & b). In order to investigate whether the plasmid variant region had an impact on the carbapenem resistance of the host bacterium, pKpQIL-D2 was also transferred into the *Salmonella* strains using the same method. All transconjugants were confirmed by PCR to carry the *bla*$_{KPC}$ gene and the respective plasmids (Figure 3.10a, 3.11 & 3.12). In order to differentiate the highly similar plasmids pKpQIL-UK and pKpQIL-D2, a primer pair (pMan-F/R) which only amplifies a 600 bp fragment of the pKpQIL-D2 backbone was also included in the PCR tests.

Figure 3.7 PCR verification of *K. pneumoniae* Ecl8, *acrAB*, Ecl8^{Rif}/pKpQIL-UK and Ecl8^{Rif} *acrAB*/pKpQIL-UK

(a) PCR check for the inactivation of *acrAB*



(b) PCR check for the presence of *bla*$_{KPC}$ **(c) PCR check for the presence of pKpQIL-UK**



The various rifampicin-resistant mutants of *K. pneumoniae* Ecl8 were verified by PCR for the presence of the inactivated *acrB* gene and the pKpQIL-UK plasmid. M: Hyperladder I (Bioline); Lane 1: Ecl8^{Rif}; Lane 2: Ecl8^{Rif} *acrAB*; Lane 3: Ecl8^{Rif}/pKpQIL-UK; Lane 4: Ecl8^{Rif} *acrAB*/pKpQIL-UK; Lane 5: Water (Contamination control).

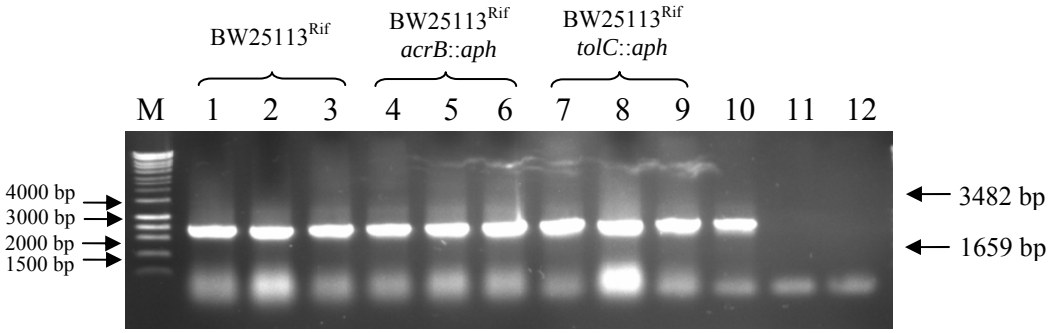
(a) Check for inactivation of *acrAB*
Primers: AcrABdelchkF/R
Expected amplicon: 5543 bp (wildtype), 3000 bp (mutant)

(b) Check for *bla*$_{KPC}$
Primers: KPCg-colpcrF/R
Expected amplicon: 785 bp

(c) Check for pKpQIL-UK
Primers: pQIL-F/R
Expected Amplicon: 383 bp

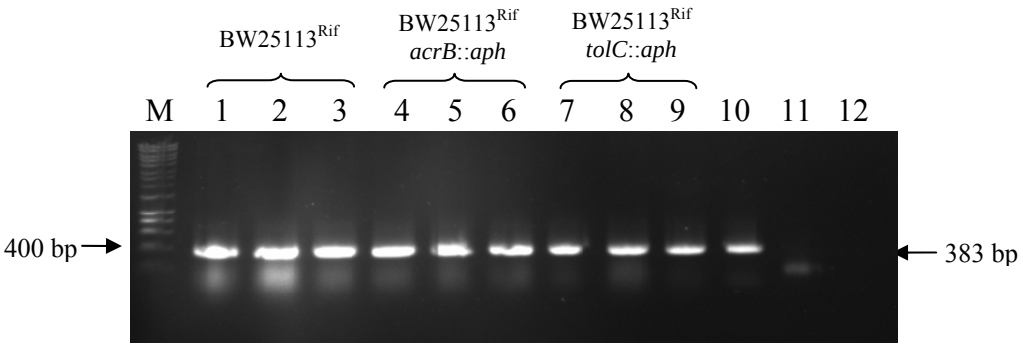
Figure 3.8 PCR verification for the presence of pKpQIL-UK in the *E. coli* BW25113^{Rif} and isogenic efflux mutants

(a) PCR to check for presence of *bla*$_{KPC}$



The various rifampicin-resistant mutants of *E. coli* BW25113 were verified by PCR for the presence of the pKpQIL-UK plasmid's *bla*$_{KPC}$ gene. M: Hyperladder I (Bioline); Lane 10: pKpQIL-UK plasmid (Positive control); Lane 11: BW25113^{Rif} genomic DNA (Negative control); Lane 12: Water (Contamination control); Primers: KPCg-colpcrF/R; Expected amplicon: 785 bp.

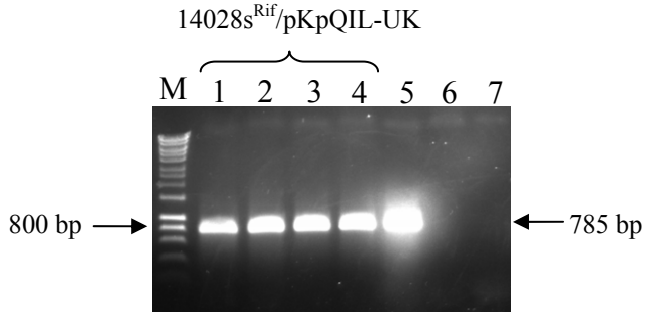
(b) PCR to check for presence of pKpQIL-UK



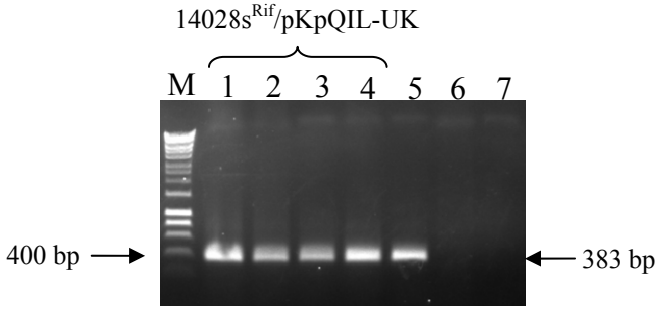
The various rifampicin-resistant mutants of *E. coli* BW25113 were verified by PCR for the presence of the pKpQIL-UK plasmid's backbone gene. M: Hyperladder I (Bioline); Lane 10: pKpQIL-UK plasmid (Positive control); Lane 11: BW25113^{Rif} genomic DNA (Negative control); Lane 12: Water (Contamination control); Primers: pQIL-F/R; Expected Amplicon: 383 bp.

Figure 3.9 PCR verification of 14028s^{Rif}/pKpQIL-UK transconjugants

(a) PCR check for the presence of *bla*$_{KPC}$



(b) PCR check for the presence of pKpQIL-UK



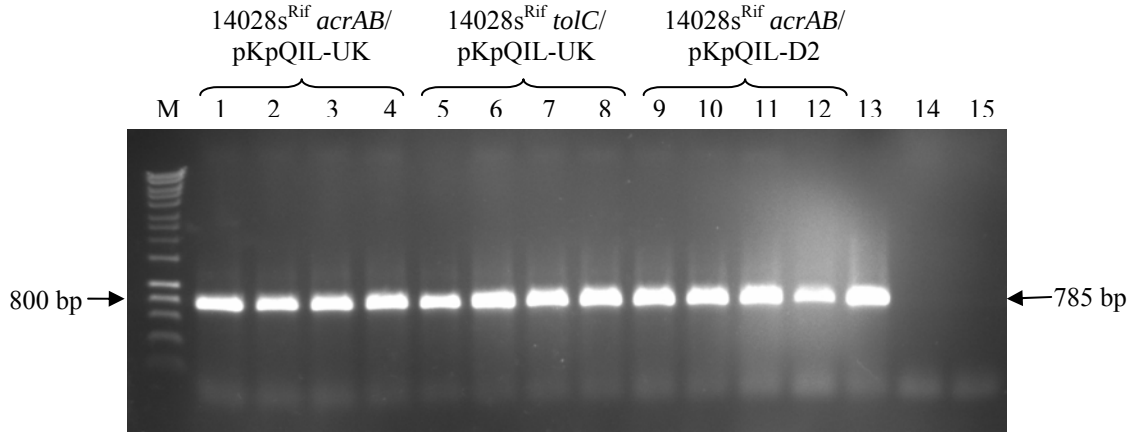
The various rifampicin-resistant mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pKpQIL-UK plasmid's (a) *bla*$_{KPC}$ and (b) backbone genes. M: Hyperladder I (Bioline); Lane 5: Plasmid pKpQIL-UK (Positive control); Lane 6: 14028s^{Rif} genomic DNA (Negative control); Lane 7: Water (Contamination control).

(a) Check for *bla*$_{KPC}$
Primers: KPCg-colpcrF/R
Expected amplicon: 785 bp

(b) Check for pKpQIL-UK
Primers: pQIL-F/R
Expected Amplicon: 383 bp

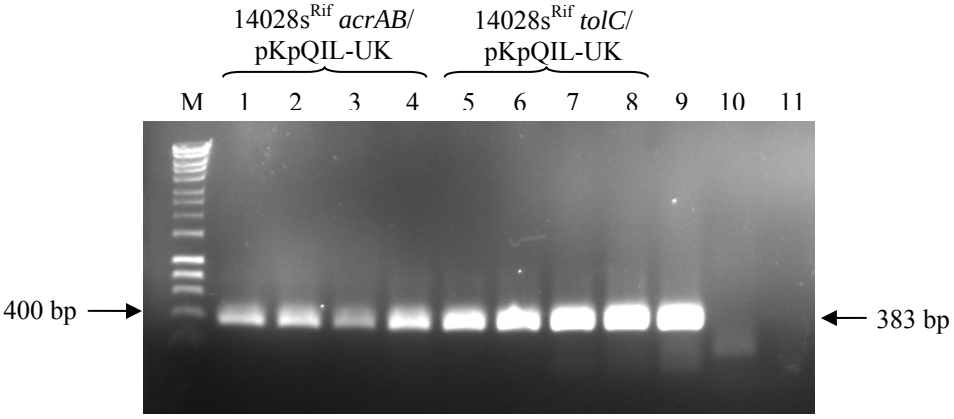
Figure 3.10 PCR verification of 14028s^{Rif} *acrAB*/pKpQIL-UK, 14028s^{Rif} *tolC*/pKpQIL-UK and 14028s^{Rif} *acrAB*/pKpQIL-D2

(a) PCR check for the presence of *bla*$_{KPC}$



The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pKpQIL-UK and -D2 plasmid's *bla*$_{KPC}$ genes. M: Hyperladder I (Bioline); Lane 13: Plasmid pKpQIL-UK (Positive control); Lane 14: 14028s^{Rif} genomic DNA (Negative control); Lane 15: Water (Contamination control); Primers: KPCg-colpcrF/R; Expected amplicon: 785 bp.

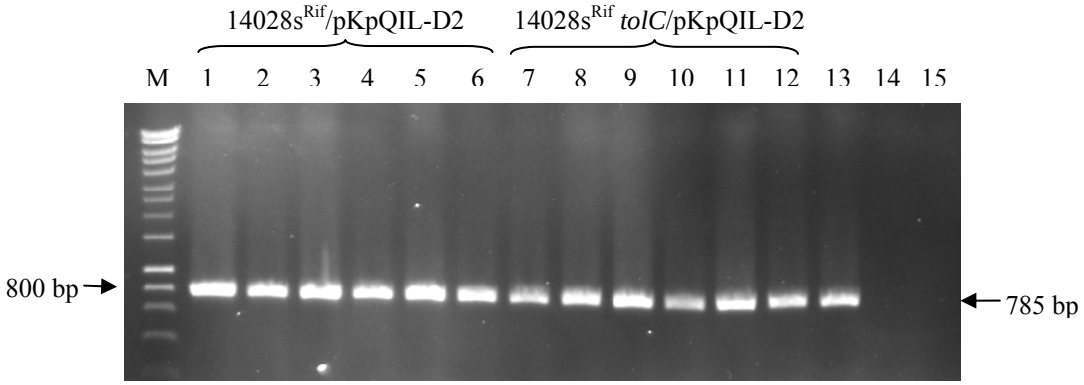
(b) PCR check for the presence of pKpQIL-UK



The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pKpQIL-UK plasmid's backbone gene. M: Hyperladder I (Bioline); Lane 9: Plasmid pKpQIL-UK (Positive control); Lane 10: Plasmid pKpQIL-D2 (Negative control); Lane 11: Water (Contamination control); Primers: pQIL-F/R; Expected amplicon: 383 bp.

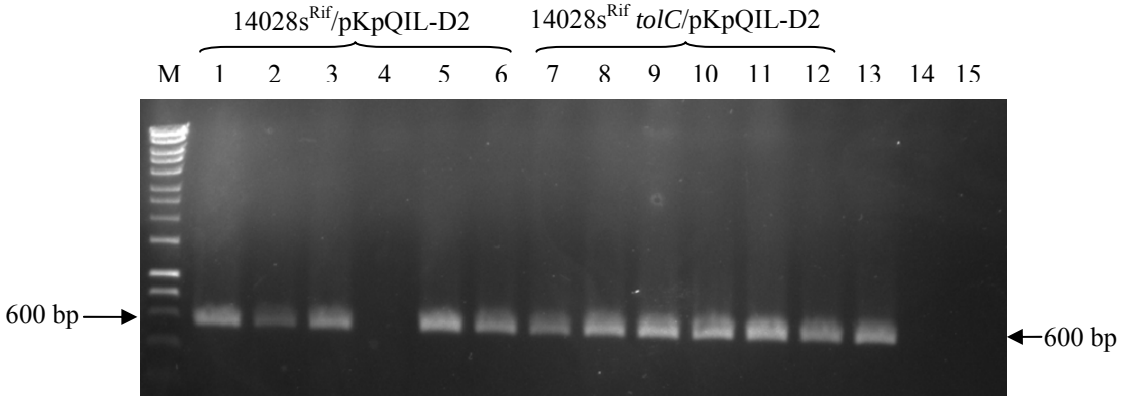
Figure 3.11 PCR verification of 14028s^{Rif}/pKpQIL-D2 and 14028s^{Rif} *tolC*/pKpQIL-D2

(a) PCR check for the presence of *bla*$_{KPC}$



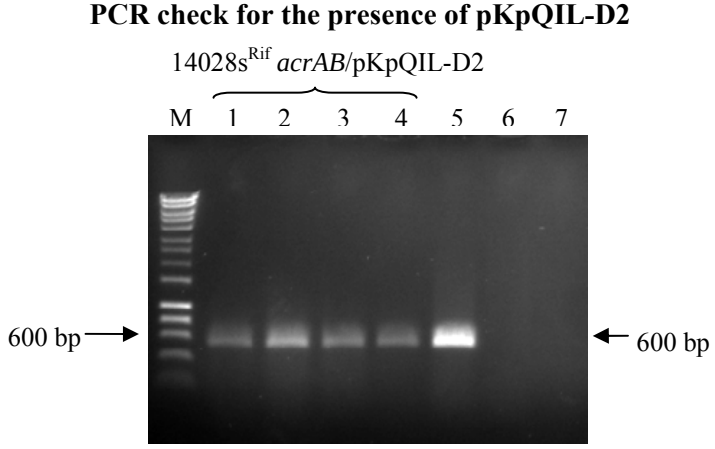
The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pKpQIL-D2 plasmid's *bla*$_{KPC}$ gene. M: Hyperladder I (Bioline); Lane 13: Plasmid pKpQIL-D2 (Positive control); Lane 14: 14028s^{Rif} genomic DNA (Negative control); Lane 15: Water (Contamination control); Primers: KPCg-colpcrF/R; Expected amplicon: 785 bp.

(b) PCR check for the presence of pKpQIL-D2



The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pKpQIL-D2 plasmid's backbone gene. M: Hyperladder I (Bioline); Lane 13: Plasmid pKpQIL-D2 (Positive control); Lane 14: Plasmid pKpQIL-UK (Negative control); Lane 15: Water (Contamination control); Primers: pMan-F/R; Expected amplicon: 600 bp.

Figure 3.12 PCR verification of 14028s^{Rif} *acrAB*/pKpQIL-D2 transconjugants



The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pKpQIL-D2 plasmid's backbone gene. M: Hyperladder I (Bioline); Lane 5: Plasmid pKpQIL-D2 (Positive control); Lane 6: Plasmid pKpQIL-UK (Negative control); Lane 7: Water (Contamination control); Primers: pMan-F/R; Expected Amplicon: 600 bp.

3.3.2 The MICs of Antibiotics in Strains Lacking a Component of the AcrAB-TolC Efflux Pump

The susceptibility of *K. pneumoniae* Ecl8 and isogenic AcrAB mutant carrying the pKpQIL-UK plasmid was tested (Table 3.4). No difference in β-lactam antibiotic MIC values was observed between the plasmid-free strains. However, when pKpQIL-UK was present, the AcrAB mutant strain showed an 8- and 4-fold decrease in susceptibility to ertapenem and meropenem, respectively. Similarly the *E. coli* BW25113 *acrB*::*aph* carrying the plasmid showed a 4-fold decrease in susceptibility to ertapenem. However, the *E. coli* TolC mutant was significantly more susceptible to meropenem, doripenem, biapenem and ceftazidime. Other antibiotics such as chloramphenicol, erythromycin, ciprofloxacin and tetracycline were included as controls to verify the phenotype of the efflux mutants. As expected, antibiotics which are known substrates to the AcrAB-TolC efflux system had lower MIC values for the mutant strains.

The susceptibility of the *Salmonella* efflux mutants in the presence and absence of the pKpQIL-UK plasmid was also determined. With the exception of ertapenem in the absence of the plasmid, there was no difference in susceptibility observed between the parental strain (14028s^{Rif}) and the respective efflux pump component mutants (14028s^{Rif} *acrAB* and 14028s^{Rif} *tolC*) for the β-lactam antibiotics tested (Table 3.4). However, compared to 14028s^{Rif}, the TolC mutant showed a 4-fold decrease in ertapenem susceptibility (0.008 μg/ml to 0.03 μg/ml). Although chloramphenicol is a substrate of this efflux pump, the MIC value for chloramphenicol was higher in the mutants as a chloramphenicol resistance gene (*cat*) was inserted in the efflux pump genes to construct the mutants (Nishino et al., 2006).

In the presence of the bla_{KPC} carbapenemase-encoding pKpQIL-UK, compared to the parental *Salmonella* strain (14028s^{Rif}), the TolC mutant was 4- to 8-fold less susceptible to

Table 3.4 MIC values of various antibiotics for efflux pump component mutants carrying the pKpQIL-UK plasmid

Strains	Genotype	Plasmid Introduced	Antibiotics (µg/ml)									
			ETP	**IMI**	**MER**	**DOR**	**BIA**	**CAZ**	**CHL**	**CIP**	**EM**	**TET**
E. coli NCTC10418	Wildtype	-	0.015	0.25	0.03	0.03	0.06	0.25	2	0.008	32	1
***K. pneumoniae* Ecl8**												
Ecl8^{Rif}	Rif^{R}	-	0.015	0.12	0.015	0.03	0.25	0.03	2	0.008	64	0.5
Ecl8^{Rif}	*acrAB*::*aph*	-	0.015	0.12	0.03	0.03	0.25	0.06	1	0.004	**4**	0.5
Ecl8^{Rif}	Rif^{R}	pKpQIL-UK	4	4	1	2	4	16	4	0.008	64	0.5
Ecl8^{Rif}	*acrAB*::*aph*	pKpQIL-UK	**32**	8	**4**	4	8	16	**1**	0.004	**4**	0.5
***E. coli* BW25113**												
BW25113^{Rif}	Rif^{R}	-	0.015	0.25	0.03	0.06	0.06	0.06	8	0.008	32	2
BW25113^{Rif}	*acrB*::*aph*	-	0.015	0.5	0.03	0.06	0.06	0.06	**1**	0.004	**4**	**0.5**
BW25113^{Rif}	*tolC*::*aph*	-	0.008	0.12	0.015	**0.015**	**0.015**	0.03	**1**	**0.002**	**2**	**0.5**
BW25113^{Rif}	Rif^{R}	pKpQIL-UK	0.12	2	0.25	0.5	2	2	8	0.008	32	1
BW25113^{Rif}	*acrB*::*aph*	pKpQIL-UK	**0.5**	2	0.25	1	4	2	**1**	0.004	**4**	0.5
BW25113^{Rif}	*tolC*::*aph*	pKpQIL-UK	0.06	1	**0.03**	**0.12**	**0.12**	**0.5**	**1**	**0.002**	**2**	0.5

Continued overleaf.

Table 3.4 (Continued) **MIC values of various antibiotics for efflux pump component mutants carrying the pKpQIL-UK plasmid**

Strains	Genotype	Plasmid Introduced	Antibiotics (µg/ml)									
			ETP	**IMI**	**MER**	**DOR**	**BIA**	**CAZ**	**CHL**	**CIP**	**EM**	**TET**
***S.* Typhimurium ATCC14028s**												
14028s	Wildtype	-	0.015	0.25	0.03	0.03	0.06	0.25	4	0.015	64	2
14028s^{Rif}	Rif^{R}	-	0.008	0.5	0.03	0.03	0.03	0.25	8	0.015	128	2
14028s^{Rif}	*acrAB*::*cat*	-	0.008	0.12	0.015	0.015	0.03	0.12	>16	**0.004**	**4**	**0.5**
14028s^{Rif}	*tolC*::*cat*	-	**0.03**	0.25	0.03	0.03	0.06	0.5	>16	**0.004**	**2**	**0.5**
14028s^{Rif}	Rif^{R}	pKpQIL-UK	2	4	1	1	4	8	8	0.015	64	2
14028s^{Rif}	*acrAB*::*cat*	pKpQIL-UK	2	4	2	1	4	8	**>16**	**0.004**	**4**	**0.5**
14028s^{Rif}	*tolC*::*cat*	pKpQIL-UK	**16**	8	**8**	**4**	4	**32**	**>16**	**0.004**	**2**	**0.5**

ETP: Ertapenem; IMI: Imipenem; MER: Meropenem; DOR: Doripenem; BIA: Biapenem; CAZ: Ceftazidime; CHL: Chloramphenicol; CIP: Ciprofloxacin; EM: Erythromycin; TET: Tetracycline; Bolded fonts denote significant increase/decrease in MIC values; Changes of 4-fold or more in MIC values are considered significant.

three carbapenem antibiotics (ertapenem, meropenem and doripenem). No significant difference in MIC value was observed for biapenem between the strains. A similar increase in the MIC value of ceftazidime was also observed. Compared to the parental strain (14028s^{Rif}), there was no significant difference in MIC values between the 14028s^{Rif} AcrAB mutant carrying the plasmid.

3.4 The *Salmonella* TolC Mutant is Less Susceptible to β-lactam Antibiotics and This is Not Specific to Carbapenemase

The AcrAB-TolC efflux system is one of the most well characterised RND efflux pump which is highly conserved among Gram-negative bacteria (Piddock, 2006a, Nikaido and Zgurskaya, 2001). It has been reported that the amino acid identity of AcrA and AcrB proteins among *Proteus mirabilis*, *E. coli*, *K. pneumoniae* and *Enterobacter aerogenes* is 75%. The *acrB* gene shares about 85-88% identity among the latter three bacteria (Visalli et al., 2003). The *S.* Typhimurium AcrAB-TolC has also been shown to have high DNA sequence identity (>70%) and amino acid similarity (>80%) with its *E. coli* counterpart (Piddock, 2006a). The AcrAB-TolC protein sequence identities of the various strains used in this PhD study were compared to *K. pneumoniae* MGH78578 by BlastP (Table 3.5). It is well established that *S.* Typhimurium is a model organism for studying Gram-negative bacteria as there are tractable infection models (Garai et al., 2012). Therefore, the AcrAB-TolC efflux system in this bacterium has been used in this PhD study as a model to investigate its contribution towards carbapenem resistance in other Gram-negative bacteria.

Although it was observed that the carriage of the pKpQIL-UK plasmid in the TolC mutant conferred higher resistance to β-lactam antibiotics compared to the 14028s^{Rif}/pKpQIL-UK, it was hypothesised that this observation was specific to this plasmid. Hence, in order to investigate whether the increase in MIC values of the various β-lactam antibiotics tested was a

Table 3.5 Protein sequence homology of AcrAB-TolC efflux system

Species	Genbank Accession No.	Percentage Protein Sequence Identity (Coverage)		
		AcrA	AcrB	TolC
K. pneumoniae MGH78578	CP000647	-	-	-
K. pneumoniae Ecl8	HF536482	100 (100)	100 (100)	99 (100)
E. coli K12 MG1655	U00096	85 (100)	92 (100)	84 (99)
E. coli BW25113	CP009273	85 (100)	92 (100)	84 (99)
S. Typhimurium 14028s	CP001363	85 (99)	92 (100)	86 (100)
S. Typhimurium SL1344	FQ312003	85 (99)	92 (100)	86 (100)

AcrAB-TolC efflux pump protein sequence identity of the various strains was compared to *K. pneumoniae* MGH78578

result specific to pKpQIL-UK, its variant (pKpQIL-D2) and a *bla*$_{NDM}$ carbapenemase-encoding plasmid (pNDM-HK) were tested. The plasmid pNDM-HK was transferred into *S.* Typhimurium 14028s^{Rif}, 14028s^{Rif} *acrAB* and 14028s^{Rif} *tolC* strains by filter conjugation. All transconjugants were verified by PCR for the presence of the carbapenemase gene (Figure 3.13a) and a backbone fragment of the plasmid (Primers: pHK-F/R; 1407 bp) (Figure 3.13b). A similar increase in MIC values of ertapenem, meropenem, doripenem and ceftazidime was observed for the plasmid pKpQIL-D2 and the unrelated pNDM-HK in the TolC mutant (Table 3.6). Together, these data suggest that the increase in carbapenem MIC value was not due to the presence of the *bla*$_{KPC}$ gene or the plasmids used, but instead due to a different mechanism of resistance.

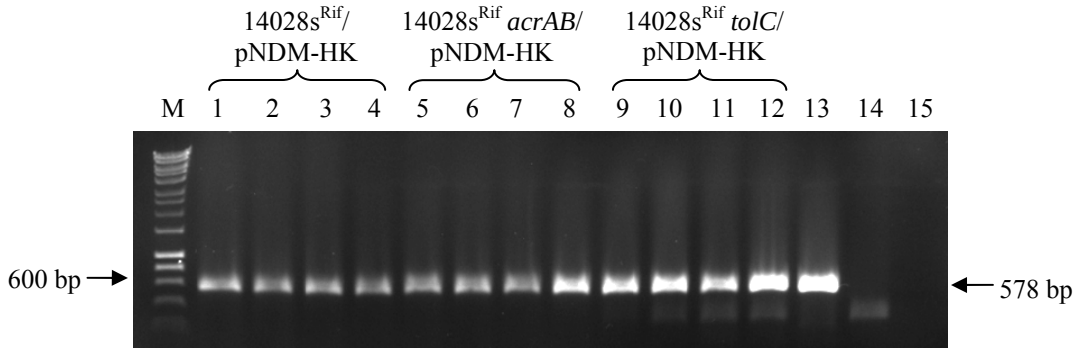
To determine whether the reduction in carbapenem susceptibility was also observed when a non-carbapenemase β-lactamase was present. The MICs of antibiotics for *S.* Typhimurium SL1344 and its isogenic efflux mutants (Δ*acrA*, Δ*acrB* and Δ*tolC*) carrying a *bla*$_{TEM-1}$ β-lactamase-encoding plasmid (pUC18) were also determined. Except for ertapenem, no significant difference was observed in the MIC values for the various β-lactam antibiotics tested. Compared to *S.* Typhimurium SL1344/pUC18, SL1344 Δ*tolC*/pUC18 was 4-fold less susceptible to ertapenem (Table 3.6). These data further suggest that the reduced susceptibility to β-lactam antibiotics observed in the *S.* Typhimurium TolC mutant is not specific to the plasmids or β-lactamase genes it carries.

3.5 Increased Carbapenem Resistance is Not Associated with Passive Release of Carbapenemase from the Periplasmic Space of the *Salmonella* Efflux Mutant

Previous study has suggested that the membrane of an *E. coli* TolC mutant is under stress as there was an increase in the expression of the Tol-Pal system which is essential for maintaining membrane integrity (Cascales et al., 2002, Dhamdhere and Zgurskaya, 2010).

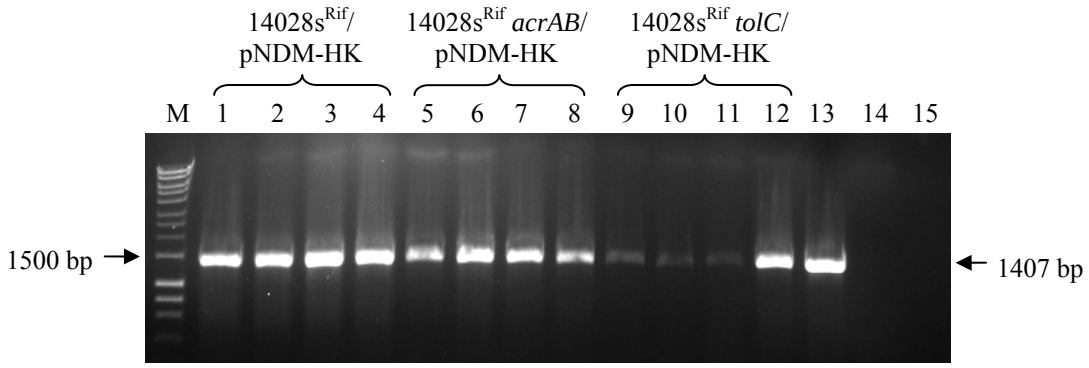
Figure 3.13 PCR verification of transconjugants of 14028s^{Rif} and its mutants carrying the plasmid pNDM-HK

(a) PCR check for the presence of *bla*$_{NDM-1}$



The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pNDM-HK plasmid's *bla*$_{NDM}$ gene. M: Hyperladder I (Bioline); Lane 13: Plasmid pNDM-HK (Positive control); Lane 14: 14028s^{Rif} genomic DNA (Negative control); Lane 15: Water (Contamination control); Primers: NDM1-colpcrF/R; Expected amplicon: 578 bp.

(b) PCR check for the presence of pNDM-HK



The various rifampicin-resistant efflux component mutants of *S.* Typhimurium 14028s were verified by PCR for the presence of the pNDM-HK plasmid's backbone gene. M: Hyperladder I (Bioline); Lane 13: Plasmid pNDM-HK (Positive control); Lane 14: 14028s^{Rif} genomic DNA (Negative control); Lane 15: Water (Contamination control); Primers: pHK-F/R; Expected amplicon: 1407 bp.

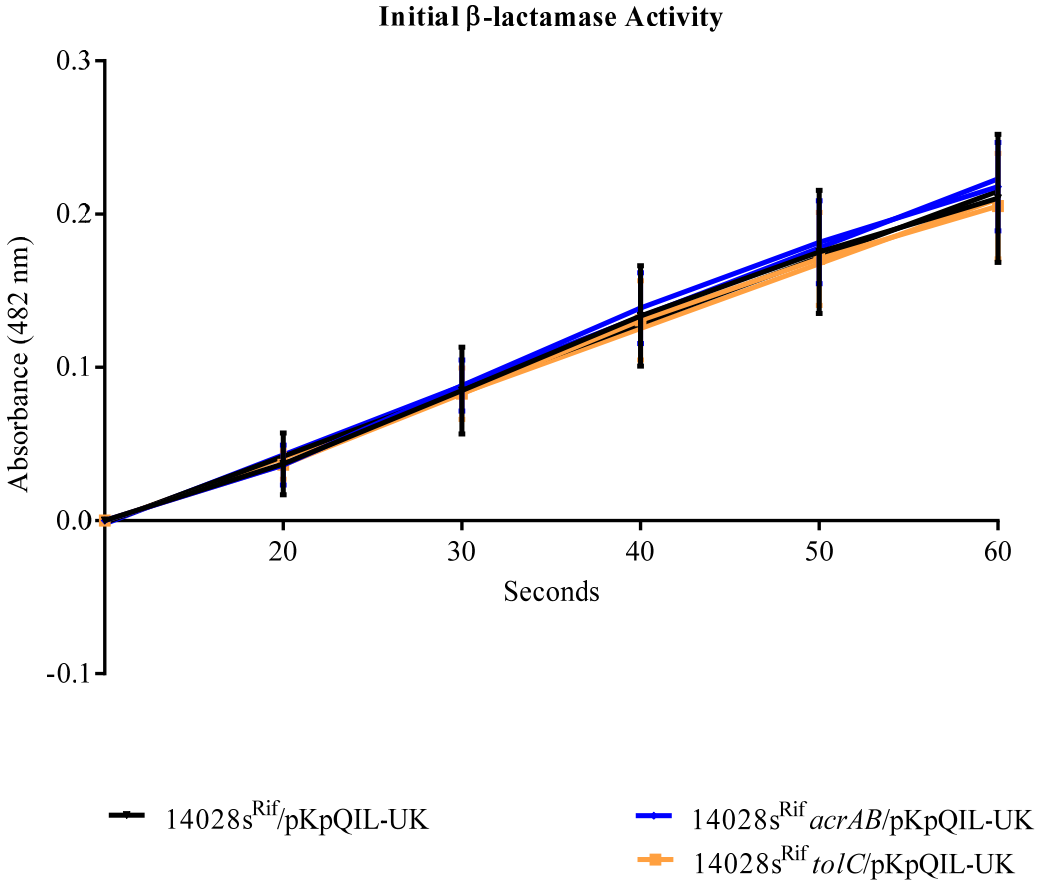
Table 3.6 MIC values of various antibiotics for efflux pump component mutants carrying various plasmids

Strains	Genotype	Plasmid Introduced	Antibiotics (μg/ml)									
			ETP	IMI	MER	DOR	BIA	CAZ	CHL	CIP	EM	TET
***S.* Typhimurium ATCC14028s**												
14028s^{Rif}	Rif^{R}	pKpQIL-D2	1	4	1	1	4	8	8	0.016	64	2
14028s^{Rif}	*acrAB*::*cat*	pKpQIL-D2	2	4	1	1	4	8	>16	**0.004**	**4**	**0.5**
14028s^{Rif}	*tolC*::*cat*	pKpQIL-D2	**16**	8	**4**	**4**	4	**32**	>16	**0.004**	**2**	**0.5**
14028s^{Rif}	Rif^{R}	pNDM-HK	8	8	4	8	2	>512	8	0.016	256	2
14028s^{Rif}	*acrAB*::*cat*	pNDM-HK	8	8	4	4	2	>512	>16	**0.004**	**512**	**0.5**
14028s^{Rif}	*tolC*::*cat*	pNDM-HK	**32**	16	**16**	16	1	>512	>16	**0.004**	**128**	**0.5**
***S.* Typhimurium SL1344**												
SL1344	Wildtype	-	0.03	0.5	0.06	0.125	0.06	2	8	0.016	64	2
SL1344	Δ*acrA*	pUC18	0.03	0.5	0.06	0.125	0.125	2	**1**	**0.004**	**2**	**0.5**
SL1344	Δ*acrB*	pUC18	0.03	0.5	0.06	0.125	**0.25**	2	**1**	**0.004**	**2**	**0.5**
SL1344	Δ*tolC*	pUC18	**0.125**	1	0.125	0.25	0.125	2	**1**	**0.002**	**2**	**0.5**

ETP: Ertapenem; IMI: Imipenem; MER: Meropenem; DOR: Doripenem; BIA: Biapenem; CAZ: Ceftazidime; CHL: Chloramphenicol; CIP: Ciprofloxacin; EM: Erythromycin; TET: Tetracycline; Bolded fonts denote significant increase/decrease in MIC values; Changes of 4-fold or more in MIC values are considered significant.

E. coli TolC mutant is also known to have an alteration in its lipopolysaccharide which compromises its membrane integrity and permeability, resulting in passive release of enzymes including RNase and β-lactamase (Cascales et al., 2002, Li et al., 2012, Stone and Miller, 1995). As the increased carbapenem MIC values were only observed when the carbapenemase gene was associated with the *S.* Typhimurium TolC mutant, it was hypothesised that *S.* Typhimurium TolC mutant leaked carbapenemase into the external environment, thereby hydrolysing the carbapenem and preventing it from reaching its target. This results in the higher carbapenem MIC values in the TolC mutant. To explore this hypothesis, the hydrolytic activity of the crude cell lysates of the three strains (14028s^{Rif}, 14028s^{Rif} *acrAB* and 14028s^{Rif} *tolC*) carrying the plasmid pKpQIL-UK was tested using nitrocefin. The crude lysates of these three strains showed comparable hydrolytic activity against nitrocefin (Figure 3.14). As the amount of total protein in the supernatant was insufficient (15 μg of total protein was required for hydrolysis of nitrocefin), the carbapenemase activity in the supernatant (i.e. LB broth which the strains were grown in) could not be determined directly. A release of carbapenemase enzyme from the cell would cause the hydrolytic activity in the crude cell lysate to decrease as there would be lesser enzymes within the bacterial cell. If the bacterial cells with higher membrane permeability (i.e. compromised membrane integrity) were lysed and used for the β-lactamase assay, the slope of the graph showing the initial hydrolysis of nitrocefin would be reduced corresponding to the lower hydrolytic activity. However, this was not observed, suggesting no passive release of carbapenemase into the environment was detected.

Figure 3.14 β-lactamase activity assay for pKpQIL-UK carrying *Salmonella* and isogenic efflux pump component mutants



All values were mean ± standard deviation of three independent experiments. The carbapenemase activity in the crude lysate was tested with nitrocefin to determine the difference in the total amount of the carbapenemase in the three *S.* Typhimurium strains. The initial enzyme activity represented by the slope from the 10th to the 50th second was not significantly different (Analysed by GraphPad Prism).

3.6 Efflux Inhibitor Alters Susceptibility to Some β-lactam Antibiotics

3.6.1 PAβN Reduces β-lactam Antibiotic Susceptibility in *K. pneumoniae*, *E. coli* and *Salmonella*

As a complete set efflux mutant was available for *S.* Typhimurium, the effect of the efflux inhibitor (PAβN) was tested on this set of carbapenemase-producing strains. PAβN has been repeatedly shown to decrease the susceptibility of some antibiotics, including β-lactams and fluoroquinolones (Piddock, 2006a). The TolC mutant had the lowest MIC value for PAβN (50 μg/ml). Hence, the MIC values for the antibiotics were determined in the presence of a sub-inhibitory concentration, 25 μg/ml PAβN (Table 3.7). In the presence of PAβN, there was a 4- to 16-fold increase in the MIC of ertapenem for the wildtype and AcrAB mutant carrying the pKpQIL-UK, pNDM-HK or pUC18 plasmids. The increase in MIC was similar to the trend observed between the wildtype *S.* Typhimurium 14028s^{Rif} and its isogenic TolC mutant carrying the plasmids in the absence of PAβN. Apart from *S.* Typhimurium SL1344/pUC18, which showed a 4-fold increase in cephalothin susceptibility in the presence of PAβN, no other strain carrying any of the plasmids showed differences in cephalothin susceptibility in the presence and absence of PAβN.

To investigate the role of RND efflux pumps in the susceptibility of the strains to carbapenem antibiotics, CCCP, a proton-motive force uncoupler was used to remove the energy source required for RND efflux pumps (Richmond et al., 2013). Except for *S.* Typhimurium 14028s^{Rif}/pNDM-HK, which showed 4-fold decrease in the ertapenem MIC value (8 μg/ml to 2 μg/ml), generally no difference in the MIC values of ertapenem and cephalothin were observed in the presence of 25 μM CCCP. Tetracycline was included as a control to detect reduced efflux function in the presence of 25 μg/ml PAβN and 25 μM CCCP. The *S.* Typhimurium and *E. coli* TolC mutants were tested in the presence of 3 μM CCCP as

Table 3.7 Effects of PAβN and CCCP on MIC of β-lactam antibiotics

Strains	Genotype	Plasmid Introduced	ETP			CEP			TET			PAβN	CCCP
			-	+PAβN	+CCCP	-	+PAβN	+CCCP	-	+PAβN	+CCCP		
E. coli NCTC 10418	Wildtype	-	0.015	0.015	0.015	8	16	8	1	0.5	0.25	200	50
S. Typhimurium 14028s													
14028s^{Rif}	Rif^{R}	pKpQIL-UK	2	**32**	2	1024	2048	512	2	1	1	400	100
14028s^{Rif}	*acrAB*::*cat*	pKpQIL-UK	2	**16**	2	512	512	512	**0.5**	0.25	0.25	100	100
14028s^{Rif}	*tolC*::*cat*	pKpQIL-UK	**16**	8	32	512	128	1024	**0.5**	0.12	0.5	50	12.5
14028s^{Rif}	Rif^{R}	pNDM-HK	8	**64**	2	2048	2048	2048	2	1	1	400	100
14028s^{Rif}	*acrAB*::*cat*	pNDM-HK	8	**32**	4	1024	1024	1024	**0.5**	0.25	0.25	100	100
14028s^{Rif}	*tolC*::*cat*	pNDM-HK	**32**	4	64	**512**	32	512	**0.5**	0.12	0.5	50	12.5
S. Typhimurium SL1344													
SL1344	Wildtype	pUC18	0.03	**0.5**	0.03	512	**2048**	512	2	1	1	400	100
SL1344	Δ*acrA*	pUC18	0.03	0.25	0.03	512	512	256	**0.5**	0.25	0.25	50	200
SL1344	Δ*acrB*	pUC18	0.03	0.25	0.06	512	1024	512	**0.5**	0.25	0.25	100	100
SL1344	Δ*tolC*	pUC18	**0.12**	0.25	0.06	512	128	512	**0.5**	0.25	0.25	50	12.5

All values are in μg/ml. Unit for CCCP is in μM. ETP: Ertapenem; CEP: Cephalothin; TET: Tetracycline; PAβN: Phenylalanine-arginine-β-naphthylamide; CCCP: Carbonyl cyanide m-chlorophenyl hydrazone; Bolded fonts denote significant increase/decrease in MIC (without efflux inhibitors); Bolded and underlined fonts denote significant increase in MIC (with efflux inhibitors); TolC mutants were tested in the presence of 3 μM CCCP. All other strains were tested in the presence of 25 μM CCCP.

the MIC value for CCCP was 12.5 μM. *E. coli* DH10B carrying the plasmids also showed a similar decrease in ertapenem susceptibility in the presence of PAβN (Table 3.8). In the presence of PAβN, even without the carbapenemase-encoding plasmids, *E. coli* DH10B was less susceptible to ertapenem and cephalothin. In the absence of the plasmids, the *S.* Typhimurium 14028s^{Rif} and its isogenic AcrAB mutant were also less susceptible to ertapenem and cephalothin in the presence of PAβN.

Another known efflux inhibitor 1-(1-naphtylmethyl)-piperazine (NMP) and a novel AcrAB specific inhibitor (denoted as Compound A) were used to investigate whether the decrease in susceptibility towards ertapenem was specific to PAβN. In order to determine which concentration of the compounds would give a similar inhibitory effect to that of 25 μg/ml PAβN, Hoechst H33342 accumulation assays comparing 25 μg/ml of PAβN with varying concentrations of NMP and Compound A were carried out using the parental *S.* Typhimurium 14028s^{Rif} carrying pKpQIL-UK. The AcrAB mutant was included as a control. It was found that 2.5 μg/ml Compound A (Figure 3.15a) and 100 μg/ml NMP (Figure 3.15b) gave comparable intracellular accumulation of H33342 to that in the presence of 25 μg/ml PAβN.

No difference in susceptibility was observed in the presence of various efflux inhibitors for the *K. pneumoniae* Ecl8^{Rif} carrying the pKpQIL-UK plasmid. Only in the presence of PAβN, did the isogenic *K. pneumoniae* Ecl8^{Rif} AcrAB mutant carrying the pKpQIL-UK show a difference: a 16-fold increase in susceptibility to ertapenem. In contrast, and as found for *Salmonella*, the series of pKpQIL-UK carrying *E. coli* BW25113^{Rif} (wildtype, AcrB and TolC mutants) had decreased susceptibility to ertapenem in the presence of PAβN and NMP. The *E. coli* TolC mutant was less susceptible to ertapenem in the presence of

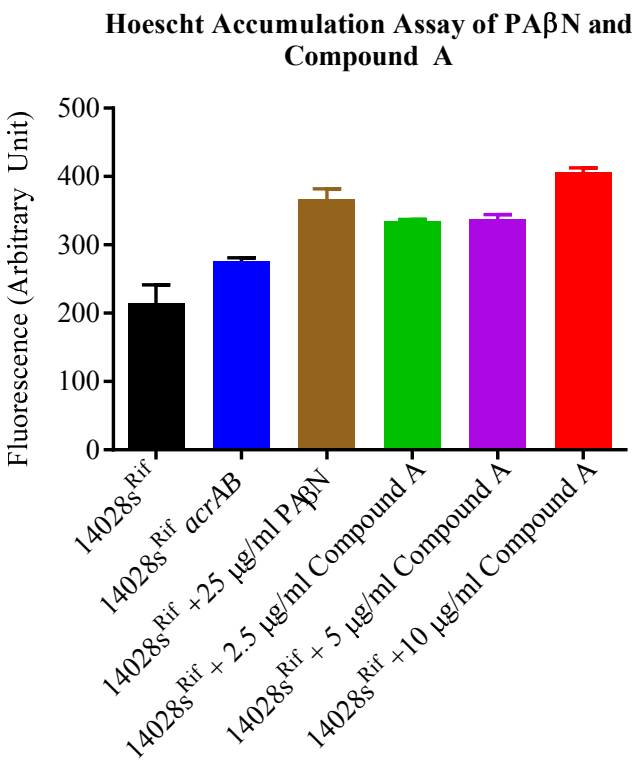
Table 3.8 Effects of PAβN and CCCP on MIC of β-lactam antibiotics

Strains	Genotype	Plasmid Introduced	ETP			CEP			TET			PAβN	CCCP
			-	+PAβN	+CCCP	-	+PAβN	+CCCP	-	+PAβN	+CCCP		
***S.* Typhimurium 14028s**													
14028s	Wildtype	-	0.008	**0.03**	0.015	4	**16**	4	2	1	1	>400	100
14028s^{Rif}	Rif^{R}	-	0.008	**0.03**	0.008	4	**16**	4	2	1	1	400	100
14028s^{Rif}	*acrAB*::*cat*	-	0.008	**0.03**	0.008	0.5	**4**	0.5	0.5	0.5	0.5	100	100
14028s^{Rif}	*tolC*::*cat*	-	**0.03**	0.015	0.03	0.5	0.12	1	0.5	0.25	0.5	50	12.5
***S.* Typhimurium SL1344**													
SL1344	Wildtype	-	0.015	0.015	0.015	4	**16**	4	2	1	1	>400	100
SL1344^{Rif}	Rif^{R}	-	0.008	**0.03**	0.008	4	**16**	4	2	1	1	>400	100
***E. coli* DH10B**													
DH10B	Wildtype	-	0.015	**0.06**	0.015	4	**16**	4	2	1	1	200	100
DH10B	Wildtype	pKpQIL-UK	1	**4**	2	256	512	256	2	1	1	200	100
DH10B	Wildtype	pKpQIL-D2	1	**4**	1	256	512	256	2	1	1	200	100
DH10B	Wildtype	pNDM-HK	8	**32**	16	1024	2048	1024	2	1	1	200	100

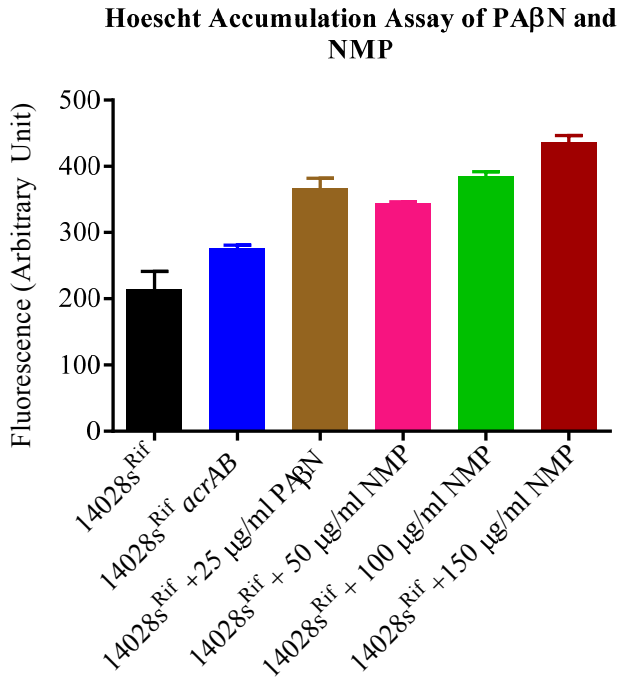
All values are in μg/ml. Unit for CCCP is in μM. ETP: Ertapenem; CEP: Cephalothin; TET: Tetracycline; PAβN: Phenylalanine-arginine-β-naphthylamide; CCCP: Carbonyl cyanide m-chlorophenyl hydrazone; Bolded fonts denote significant increase/decrease in MIC (without efflux inhibitors); Bolded and underlined fonts denote significant increase in MIC (with efflux inhibitors); TolC mutants were tested in the presence of 3 μM CCCP. All other strains were tested in the presence of 25 μM CCCP.

Figure 3.15 Hoechst 33342 accumulation assay for PAβN and varying concentrations of Compound A and NMP

(a)



(b)



Hoechst accumulation assay was done to determine the concentration needed for the Compound A and NMP to give comparable efflux inhibition to that of 25 µg/ml PAβN. All values were recorded as mean ± standard deviation of three independent experiments.

CCCP and Compound A. In the presence of NMP, the *Salmonella* and isogenic efflux mutants carrying pKpQIL-UK were 4- to 8-fold less susceptible to ertapenem (Table 3.9).

As it was found that *S.* Typhimurium TolC mutant carrying a $bla_{\text{TEM-1}}$ encoding plasmid had reduced susceptibility to ertapenem, the susceptibility of *S.* Typhimurium strains carrying a $bla_{\text{CTX-M-14}}$ (ESBL)-encoding plasmid (pCT) was also determined in the presence of the various efflux inhibitors. In the presence of PAβN, both *S.* Typhimurium 14028s$^{\text{Rif}}$/pCT and 14028s$^{\text{Rif}}$ *acrAB*/pCT had a 4-fold reduction in susceptibility to ertapenem. However, the 14028s$^{\text{Rif}}$ *tolC*/pCT strain was more susceptible to ertapenem in the presence of PAβN. Nonetheless it was 4- and 8-fold less susceptible to ertapenem in the presence of Compound A and NMP, respectively. Inactivation of the $bla_{\text{CTX-M-14}}$ gene on pCT showed that the ESBL enzyme played a minimal role in the susceptibility of the strains towards ertapenem (Table 3.9).

3.6.2 PAβN Increases Carbapenem Resistance in Clinical Isolates of Enterobacteriaceae

As data from this study suggested that PAβN conferred increased resistance to some β-lactam antibiotics, it was hypothesised that the PAβN-associated decrease in β-lactam antibiotic susceptibility would be observed among clinical isolates of Enterobacteriaceae. Hence, the effect of PAβN on the susceptibility of clinical isolates of carbapenemase producing Enterobacteriaceae was investigated. Eighty six non-replicate of clinical isolates from Public Health England were tested for their ertapenem susceptibility in the absence and presence of 25 μg/ml PAβN. The isolates included various species including *Klebsiella oxytoca* (n = 4), *K. pneumoniae* (n = 25), *Enterobacter asburiae* (n = 1), *E. aerogenes* (n = 3), *E. cloacae* (n = 22), *Enterobacter gergoviae* (n = 2) and *E. coli* (n = 29). Each of these strains carry one of the

Table 3.9 Ertapenem MIC in the presence of various efflux inhibitors

Strains	Genotype	Plasmid Introduced	MIC of Ertapenem (µg/ml)				
			-	+PABN	+CCCP	+ A	+NMP
E. coli NCTC10418	Wildtype	-	0.015	0.015	0.015	0.015	0.015
***K. pneumoniae* Ecl8**							
Ecl8^{Rif}	Rif^{R}	pKpQIL-UK	4	8	4	4	4
Ecl8^{Rif}	*acrAB*::*cat*	pKpQIL-UK	32	**2**	32	32	32
***E. coli* BW25113**							
BW25113^{Rif}	Rif^{R}	pKpQIL-UK	0.12	**1**	0.12	0.12	**0.5**
BW25113^{Rif}	*acrB*::*aph*	pKpQIL-UK	0.5	**4**	1	1	**4**
BW25113^{Rif}	*tolC*::*aph*	pKpQIL-UK	0.06	**0.25**	**0.5**	**0.25**	**0.5**
***S.* Typhimurium ATCC14028s**							
14028s^{Rif}	Rif^{R}	-	0.008	**0.03**	0.008	0.008	0.015
14028s^{Rif}	*acrAB*::*cat*	-	0.008	0.015	0.008	0.008	0.015
14028s^{Rif}	*tolC*::*cat*	-	0.015	**0.008**	0.03	0.03	**0.06**
14028s^{Rif}	Rif^{R}	pKpQIL-UK	2	**32**	2	2	**16**
14028s^{Rif}	*acrAB*::*cat*	pKpQIL-UK	1	**4**	2	2	**8**
14028s^{Rif}	*tolC*::*cat*	pKpQIL-UK	16	**0.008**	32	32	**64**
***S.* Typhimurium SL1344**							
SL1344^{Rif}	Rif^{R}	pKpQIL-UK	1	**8**	2	2	**8**
SL1344	*ompC*::*aph*	pKpQIL-UK	2	**32**	2	2	**8**
SL1344	*ompF*::*aph*	pKpQIL-UK	16	32	16	16	16
SL1344	Δ*ompC* *ompF*::*aph*	pKpQIL-UK	32	64	32	32	32

Continued overleaf.

Table 3.9 (Continued) Ertapenem MIC in the presence of various efflux inhibitors

Strains	Genotype	Plasmid Introduced	MIC of Ertapenem (µg/ml)				
			-	+PABN	+CCCP	+ A	+NMP
***S.* Typhimurium ATCC14028s**							
14028s^{Rif}	Rif^{R}	pCT	0.015	**0.06**	0.015	0.015	0.03
14028s^{Rif}	*acrAB*::*cat*	pCT	0.015	**0.06**	0.015	0.015	0.03
14028s^{Rif}	*tolC*::*cat*	pCT	0.03	**0.008**	0.06	**0.12**	**0.25**
14028s^{Rif}	Rif^{R}	pCT *bla*$_{CTX\text{-}M\text{-}14}$::*aph*	0.008	**0.03**	0.008	0.004	0.016
14028s^{Rif}	*acrAB*::*cat*	pCT *bla*$_{CTX\text{-}M\text{-}14}$::*aph*	0.008	0.015	0.008	0.004	0.008
14028s^{Rif}	*tolC*::*cat*	pCT *bla*$_{CTX\text{-}M\text{-}14}$::*aph*	0.015	0.008	0.03	0.03	**0.06**

All values are in µg/ml. PAβN: Phenylalanine-arginine-β-naphthylamide; CCCP: Carbonyl cyanide m-chlorophenyl hydrazone; Compound A: Novel AcrAB specific inhibitor; NMP: 1-(1-naphthylmethyl)-piperazine; Bolded fonts denote significant increase/decrease in MIC (when inhibitors are added); TolC mutants were tested in the presence of 3 µM CCCP. All other strains were tested in the presence of 25 µM CCCP.

major carbapenemase genes found in the UK i.e. *bla*$_{OXA-48}$ (n = 18), *bla*$_{IMP}$ (n = 12), *bla*$_{VIM}$ (n = 16), *bla*$_{NDM}$ (n = 20) and *bla*$_{KPC}$ (n = 20).

In the presence of 25 μg/ml PAβN, 42% (n = 36) of the Enterobacteriaceae isolates became 4-fold or more resistant to ertapenem (Table 3.10a). 26% (n = 22) and 30% (n = 26) of the isolates showed no change or a 2-fold increase in ertapenem MIC values, respectively. When compared to the ertapenem MIC values in the absence of the inhibitor, only two isolates were more susceptible to ertapenem in the presence of PAβN.

To investigate whether a higher concentration of PAβN would result in a larger number of clinical isolates showing greater resistance to ertapenem, 100 μg/ml PAβN was used. At this concentration and compared with 25 μg/ml PAβN, the number of isolates for which the ertapenem MIC increased 4-fold was reduced to seven (8.1%). A total of 26 (30.2%) and 33 (38.4%) isolates showed 2-fold increase or no changes in ertapenem MIC value, respectively. Twenty isolates (ca. 23%) showed more than 2-fold increase in ertapenem susceptibility. The 36 isolates for which 25 μg/ml PAβN conferred a 4-fold or more increase in the ertapenem MIC were affected differently when 100 μg/ml PAβN was used (Table 3.10b). At the higher PAβN concentration, only seven of the isolates were 4-fold less susceptible to ertapenem compared to when no PAβN was added. Of the remaining isolates, 23 were 2-fold less susceptible and 6 isolates had no change in ertapenem MIC value. In the presence of 25 μg/ml PAβN, the MIC_{50} and MIC_{90} values of the clinical isolates increased 4- and 2-fold, respectively. However, no changes in MIC_{50} and MIC_{90} values were observed in the presence of 100 μg/ml PAβN (Table 3.11).

Table 3.10 Changes in ertapenem resistance of clinical isolates in the presence of PAβN

(a) Comparison of the changes in ertapenem resistance between two PAβN concentrations

Changes in MIC	25 μg/ml PAβN		100 μg/ml PAβN	
	Number of isolates	Percentage (%)	Number of isolates	Percentage (%)
≥4-fold Reduction	-	-	6	7.0
2-fold Reduction	2	2.3	14	16.3
No Change	22	25.6	33	38.4
2-fold Increase	26	30.2	26	30.2
≥4-fold Increase	36[†]	41.9	7	8.1
Total	86	100.0	86	100.0

[†]The impact of 100 μg/ml PAβN on this 36 isolates were recorded in (b).

(b) The impact of 100 μg/ml PAβN on the isolates which showed 4-fold increase or more in ertapenem MIC in the presence of 25 μg/ml PAβN

Concentration	The number of isolates (%) with altered ertapenem MIC in the presence of PAβN*			Total number of isolates
	No Change	2-fold Increase	4-fold Increase	
100 μg/ml	6 (16.7)	23 (63.9)	7 (19.4)	36

*Changes in ertapenem MIC is relative to the MIC of ertapenem in the absence of PAβN

The MIC values of ertapenem were determined for a set of 86 non-replicate clinical isolates of various Enterobacteriaceae (*Klebsiella* spp., *E. coli* and *Enterobacter* spp.), each carrying one of the five major carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP} and bla_{OXA-48}) detected in the UK. (a) The changes in the susceptibility of the isolates in the presence of 25 and 100 μg/ml PAβN. (b) The effect of 100 μg/ml of PAβN on the susceptibility of the 36 isolates ([†]) which showed ≥4-fold increase in ertapenem MIC value in the presence of 25 μg/ml PAβN. The MIC values were determined on two separate occasions.

Table 3.11 The changes in MIC_{50} and MIC_{90} of ertapenem of the clinical isolates in the absence and presence of PAβN

Concentration of PAβN	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
0 μg/ml	16	128
25 μg/ml	64	256
100 μg/ml	32	128

*MIC_{50} and MIC_{90} are defined as the MIC values of which the growth of 50% and 90% of the bacterial isolates are inhibited (Turnidge et al., 2006). In the presence of various concentration of PAβN, the MIC_{50} and MIC_{90} of ertapenem were determined for a set of 86 non-replicate clinical isolates of various Enterobacteriaceae (*Klebsiella* spp., *E. coli* and *Enterobacter* spp.), each carrying one of the five major carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP} and bla_{OXA-48}) detected in the UK. The MIC values were determined on two separate occasions.

3.7 PAβN and Lack of TolC Alter the Porin Profile and Confers Decreased Susceptibility to β-lactam Antibiotics

3.7.1 Outer Membrane Protein Mutants Showed Reduced Susceptibility to Ertapenem

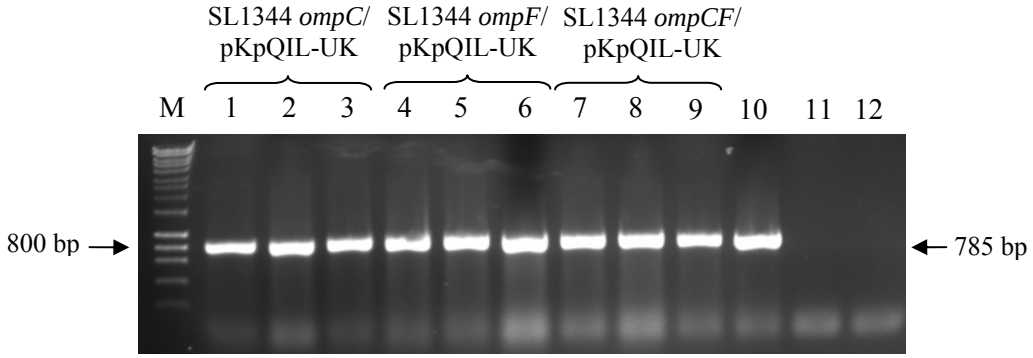
As there was no effect on the MICs in the absence of AcrB, it was hypothesised that the PAβN effect on the MICs was due to this compound altering expression of the outer membrane proteins. Furthermore, it was hypothesised that reduction in outer membrane protein expression, possibly porin proteins, caused the observed reduced susceptibility to ertapenem. Hence, plasmid pKpQIL-UK was transferred by filter conjugation into *S.* Typhimurium SL1344 in which the genes encoding major outer membrane proteins (OmpC and OmpF) have been inactivated (henceforth called SL1344 *ompC*, SL1344 *ompF* and SL1344 *ompCF* double mutant). The transconjugants were verified by PCR (Figure 3.16 & 3.17) and the lack of the respective outer membrane proteins were verified by SDS-PAGE (Figure 3.18) (Please also see Section 3.7.2).

Without the carbapenemase-producing plasmid, compared to the wildtype *S.* Typhimurium SL1344 there was a 2-fold increase in the ertapenem MIC value between the OmpC and OmpF double mutant (SL1344 *ompCF*) (Table 3.12). However, there was a larger difference (4-fold increase) in the cephalothin MIC value between these two strains. In the presence of the plasmid pKpQIL-UK, the increase in ertapenem MIC value was more apparent for the OmpF mutant (8-fold increase) and OmpC-OmpF double mutants (16-fold increase). An increase in MIC value was also observed for cephalothin.

There was also a 16-fold increased in the ertapenem MIC value for *S.* Typhimurium SL1344^{Rif}/pKpQIL-UK in the presence of 25 μg/ml PAβN. When PAβN was present, this increase in carbapenem resistance was greater for the *S.* Typhimurium OmpC mutant (16-fold), than the OmpF and OmpCF mutants (4-fold) (Table 3.12). The changes in the MIC of

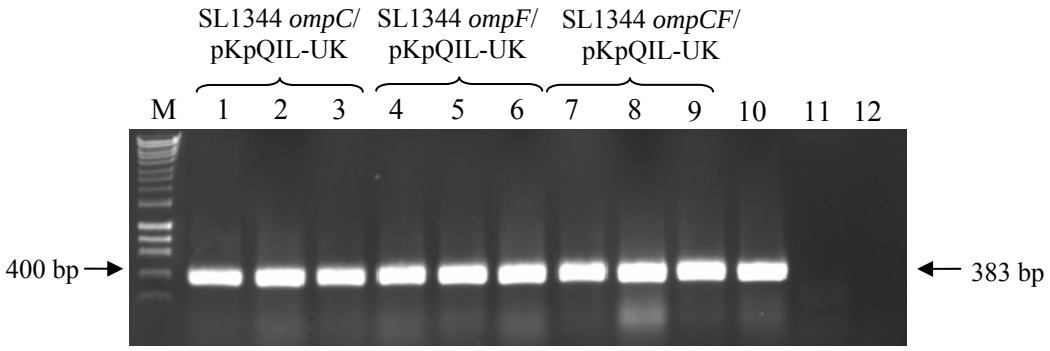
Figure 3.16 PCR verification of SL1344 porin mutants carrying pKpQIL-UK

(a) PCR check for the presence of *bla*$_{KPC}$



The various porin mutants of *S.* Typhimurium SL1344 were verified by PCR for the presence of the pKpQIL-UK plasmid's *bla*$_{KPC}$ gene. M: Hyperladder I (Bioline); Lane 10: Plasmid pKpQIL-UK (Positive control); Lane 11: SL1344 genomic DNA (Negative control); Lane 12: Water (Contamination control); Primers: KPCg-colpcrF/R; Expected amplicon: 785 bp.

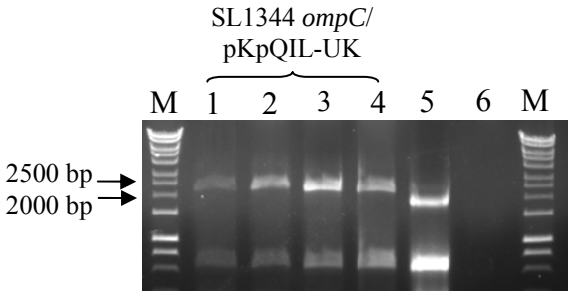
(b) PCR check for the presence of pKpQIL-UK



The various porin mutants of *S.* Typhimurium SL1344 were verified by PCR for the presence of the pKpQIL-UK plasmid's backbone gene. M: Hyperladder I (Bioline); Lane 10: Plasmid pKpQIL-UK (Positive control); Lane 11: Plasmid pKpQIL-D2 (Negative control); Lane 12: Water (Contamination control); Primers: pQIL-F/R; Expected amplicon: 383 bp.

Figure 3.17 PCR verification of SL1344 porin mutants carrying pKpQIL-UK

(a) PCR check for inactivated *ompC*

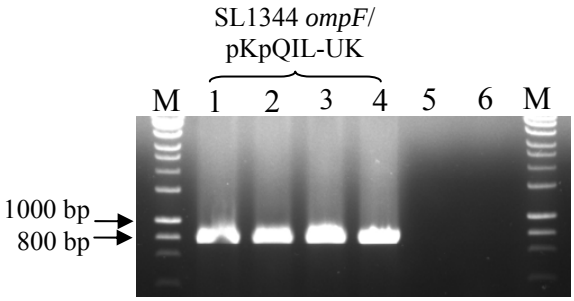


The *S.* Typhimurium porin mutants were verified by PCR for the presence of the inactivated *ompC*.

Key to lanes:
M: Hyperladder I (Bioline)
Lane 4: SL1344 *ompC* genomic DNA (Positive control)
Lane 5: SL1344 genomic DNA (Negative control)
Lane 6: Water (Contamination control)

Primers: OmpC-F/R
Expected amplicon: 2207 bp (mutant), 1927 (wildtype)

(b) PCR check for inactivated *ompF*

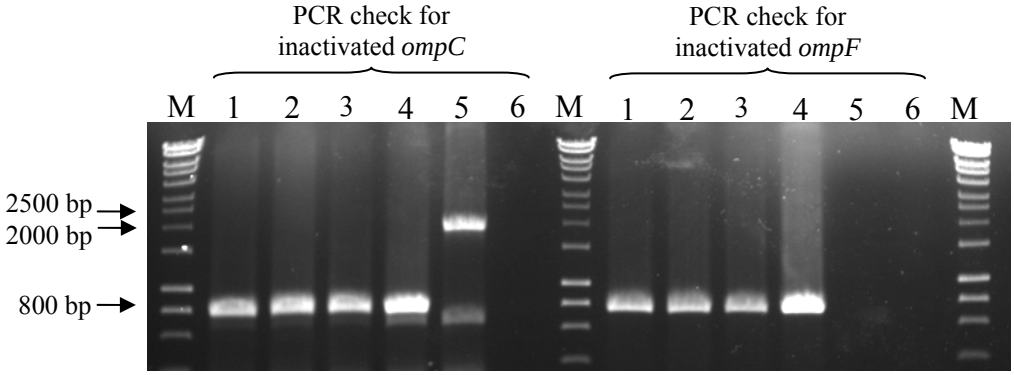


The *S.* Typhimurium porin mutants were verified by PCR for the presence of the inactivated *ompF*.

Key to lanes:
M: Hyperladder I (Bioline)
Lane 4: SL1344 *ompF* genomic DNA (Positive control)
Lane 5: SL1344 genomic DNA (Negative control)
Lane 6: Water (Contamination control)

Primers: OmpF-F/R
Expected amplicon: 780 bp (mutant), no amplicon for wildtype gene

(c) PCR check for inactivated *ompC* and *ompF* in the double mutant



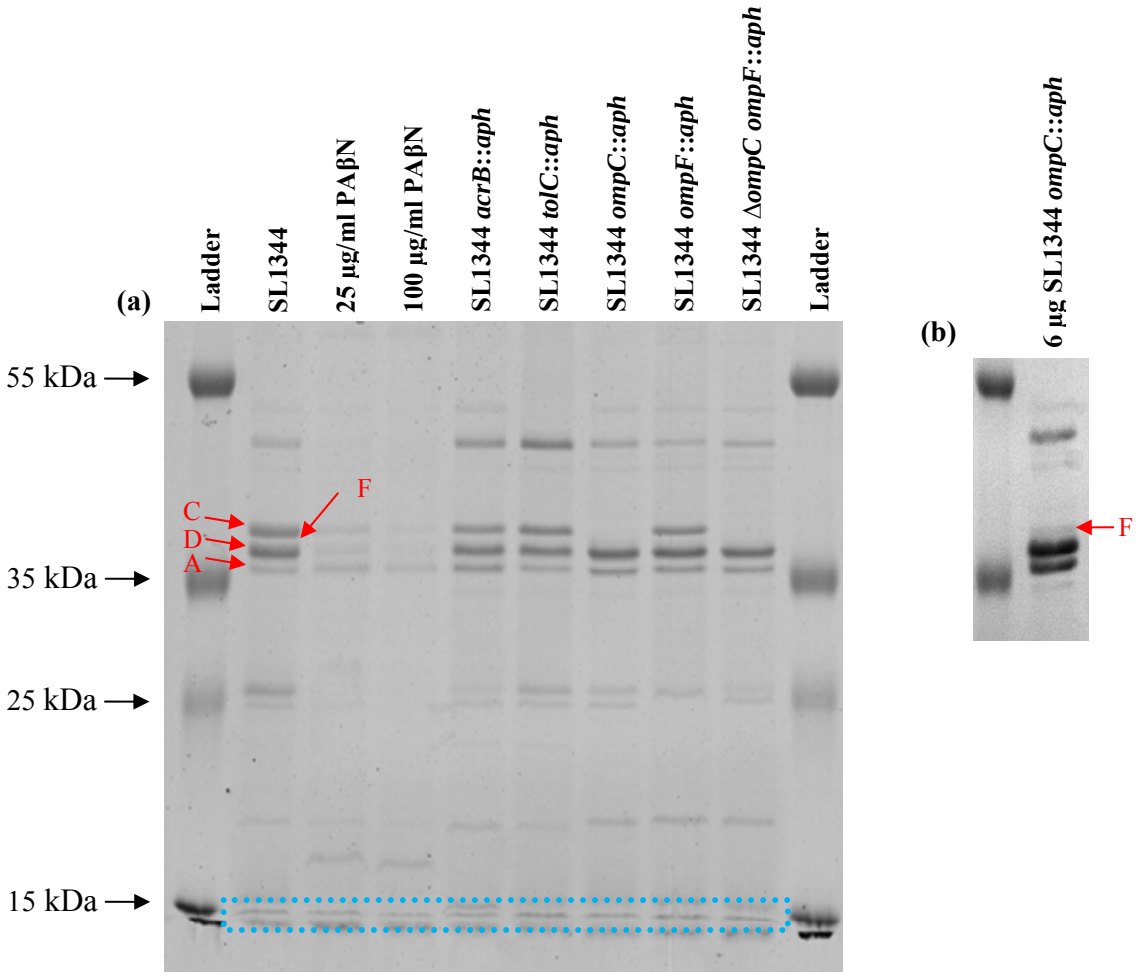
The *S.* Typhimurium double porin mutants were verified by PCR for the presence of the inactivated *ompC* and *ompF*.

Key to lanes:
M: Hyperladder I (Bioline)
Lane 1 – 3: SL1344 *ompCF*/pKpQIL-UK
Lane 4: SL1344 *ompCF* genomic DNA (Positive control)
Lane 5: SL1344 genomic DNA (Negative control)
Lane 6: Water (Contamination control)

PCR check for inactivated *ompC*
Primers: OmpC-F/R
Expected amplicon: 753 bp (Δ*ompC* mutant), 1927 (wildtype)

PCR check for inactivated *ompF*
Primers: OmpF-F/R
Expected amplicon: 780 bp (mutant), no amplicon for wildtype gene

Figure 3.18 Outer membrane protein profile of *Salmonella* Typhimurium SL1344 and its isogenic efflux pump mutants



(a) SDS-PAGE comparing the outer membrane porin profile of the various *S.* Typhimurium strains. (b) 6 µg of the total protein of SL1344 OmpC mutant was loaded to allow the faint band migrating slightly slower than the OmpD protein to be excised for mass spectrometry.

Ladder: PageRuler Plus Prestained Protein Ladder (Thermo Scientific)

Band C, D, A and F were determined as OmpC, OmpD, OmpA and OmpF, respectively by mass spectrometry. The amount of total protein was determined by Bradford assay and 2 µg of total protein was loaded per lane. The blue box indicates equal loading of each sample.

Table 3.12 Effects of PAβN and CCCP on MIC of β-lactam antibiotics

Strains	Genotype	Plasmid Introduced	ETP			CEP			TET			PAβN	CCCP
			-	+PAβN	+CCCP	-	+PAβN	+CCCP	-	+PAβN	+CCCP		
***S.* Typhimurium SL1344**													
SL1344^{Rif}	Rif^{R}	-	0.015	0.015	0.015	4	**16**	4	2	1	1	>400	100
SL1344	*ompC*::*aph*	-	0.015	0.03	0.015	8	**32**	8	2	1	1	>400	100
SL1344	*ompF*::*aph*	-	0.015	**0.06**	0.015	8	**32**	8	2	1	1	400	100
SL1344	Δ*ompC* *ompF*::*aph*	-	**0.03**	0.06	0.03	**16**	32	16	2	1	1	400	100
SL1344^{Rif}	Rif^{R}	pKpQIL-UK	1	**8**	2	512	**2048**	1024	2	1	1	400	100
SL1344	*ompC*::*aph*	pKpQIL-UK	2	**32**	2	**2048**	4096	2048	2	1	1	400	100
SL1344	*ompF*::*aph*	pKpQIL-UK	**8**	**32**	8	1024	2048	512	2	1	1	400	100
SL1344	Δ*ompC* *ompF*::*aph*	pKpQIL-UK	**16**	**64**	16	**2048**	4096	2048	2	1	1	400	100

All values are in μg/ml. Unit for CCCP is in μM. ETP: Ertapenem; CEP: Cephalothin; TET: Tetracycline; PAβN: Phenylalanine-arginine-β-naphthylamide; CCCP: Carbonyl cyanide m-chlorophenyl hydrazone; Bolded fonts denote significant increase in MIC (without efflux inhibitors); Bolded and underlined fonts denote significant increase in MIC (with efflux inhibitors); Changes of 4-fold or more in MIC values are considered significant.

cephalothin were less obvious for the porin mutants carrying the plasmid. Nonetheless, the susceptibility of *S.* Typhimurium SL1344-Rif/pKpQIL-UK to cephalothin decreased 4-fold in the presence of PAβN. However, there were no changes in β-lactam antibiotic susceptibility in the presence of CCCP. Tetracycline was used as a control and all strains showed reduced susceptibility to tetracycline in the presence of PAβN and CCCP.

The susceptibility to ertapenem of the porin mutants in the presence of Compound A and NMP was also determined (Table 3.13). The largest decrease in ertapenem susceptibility was only observed in the plasmid carrying *S.* Typhimurium SL1344 OmpC mutant when PAβN was present. In the presence of NMP, the decrease in susceptibility was also observed in *S.* Typhimurium SL1344 and its isogenic OmpC mutant. These data suggest that OmpF plays a more important role in the changes in susceptibility to ertapenem.

3.7.2 PAβN Reduces Outer Membrane Protein Expression in *Salmonella* Strains

The loss of outer membrane proteins (OmpC and OmpF) was associated with the reduction in susceptibility of the *S.* Typhimurium strains towards β-lactam antibiotics. Hence, it was hypothesised that the repression of outer membrane proteins (OmpC and OmpF) resulted in the reduced β-lactam antibiotic susceptibility in the *S.* Typhimurium TolC mutants. Therefore, the expression of the outer membrane protein of the *Salmonella* strains in the absence of the AcrAB/TolC efflux pump components and when treated with PAβN was investigated. The outer membrane proteins were prepared from wildtype *Salmonella* SL1344 and its isogenic efflux pump mutants (AcrB and TolC). From the SDS-PAGE gel, no apparent difference were observed for the efflux pump mutants compared to the wildtype *S.* Typhimurium SL1344 (Figure 3.18a; On page 127). Based on protein mobility, there is contradictory identification of outer membrane proteins of *Salmonella* in the literature (Piddock et al., 1993, Santiviago et al., 2003). As there was no specific outer membrane protein anti-serum available for this

Table 3.13 Ertapenem MIC in the presence of various efflux inhibitors

Strains	Genotype	Plasmid Introduced	MIC of Ertapenem (µg/ml)				
			-	+PABN	+CCCP	+ A	+NMP
***S.* Typhimurium SL1344**							
SL1344^{Rif}	Rif^{R}	pKpQIL-UK	1	**8**	2	2	**8**
SL1344	*ompC*::*aph*	pKpQIL-UK	2	**32**	2	2	**8**
SL1344	*ompF*::*aph*	pKpQIL-UK	8	**32**	8	16	16
SL1344	Δ*ompC* *ompF*::*aph*	pKpQIL-UK	16	**64**	16	32	32

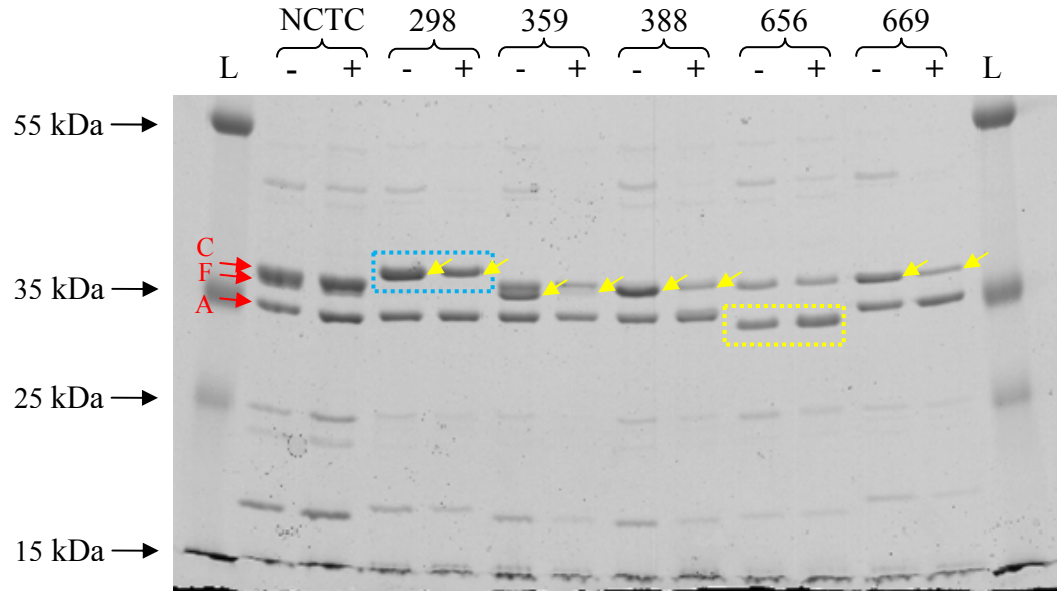
All values are in µg/ml. PAβN: Phenylalanine-arginine-β-naphthylamide; CCCP: Carbonyl cyanide m-chlorophenyl hydrazone; Compound A: Novel AcrAB specific inhibitor; NMP: 1-(1-naphthylmethyl)-piperazine; Bolded fonts denote significant increase in MIC (when inhibitors are added).

study, the three major protein bands around 35 kDa were excised for N-terminal sequencing to determine the identity of the proteins. After repeated attempts, N-terminal sequencing was only able to identify the protein band labelled as 'A'. Hence, mass spectrometry was used to identify the protein bands 'C', 'D' and a very faint protein band 'F' which was only observed in the OmpC mutant (Figure 3.18b). All the bands were identified as OmpC (Band C), OmpF (Band F), OmpD (Band D) and OmpA (Band A). From the SDS-PAGE gel, there was a concentration dependent down-regulation of outer membrane proteins when PAβN was added. In the presence of PAβN, there was a clear difference in the total intensity of protein bands visible on the gel even when 2 μg of total protein was used in each lane for electrophoresis. Although this may suggest unequal loading of protein samples, this phenomenon was repeatedly observed, despite equal loadings being used in all experiments indicated by the lower molecular mass protein (ca. 15 kDa) highlighted with the blue box.

3.7.3 Outer Membrane Protein Profile of Clinical Isolates of Enterobacteriaceae

Sixty-two Enterobacteriaceae clinical isolates had reduced susceptibility to ertapenem in the presence of PAβN. It was hypothesised that PAβN altered the outer membrane and/or porin expression in these isolates, resulting in reduced susceptibility to ertapenem. Hence, selected isolates of *E. coli* which were tested for their susceptibility in the presence and absence of PAβN (Section 3.6.2) were chosen to determine whether 25 μg/ml PAβN changed their outer membrane protein profile. The selected clinical isolates consisted of isolates which had increased ertapenem MIC values and one isolate which was not affected by the presence of PAβN. Based on previous studies of *E. coli* (Erdei et al., 1994, Ma et al., 2013, Oteo et al., 2008), protein bands C, F and A were identified as OmpC, OmpF and OmpA, respectively (Figure 3.19). After SDS-PAGE, it was observed that for isolates which showed an increase in ertapenem resistance in the presence of PAβN, also showed reduced expression of OmpF.

Figure 3.19 Outer membrane protein of *E. coli* clinical isolates in the presence and absence of 25 µg/ml PAβN



L: PageRuler Plus Prestained Protein Ladder (Thermo Scientific)

Strain	PAβN (25 µg/ml)	Ertapenem MIC (µg/ml)
E. coli NCTC10418	−	0.016
	+	0.016
Isolate 298	−	8
	+	**64**
Isolate 359	−	2
	+	**8**
Isolate 388	−	8
	+	**32**
Isolate 656	−	128
	+	128
Isolate 669	−	8
	+	16

−: Not added; +: Added

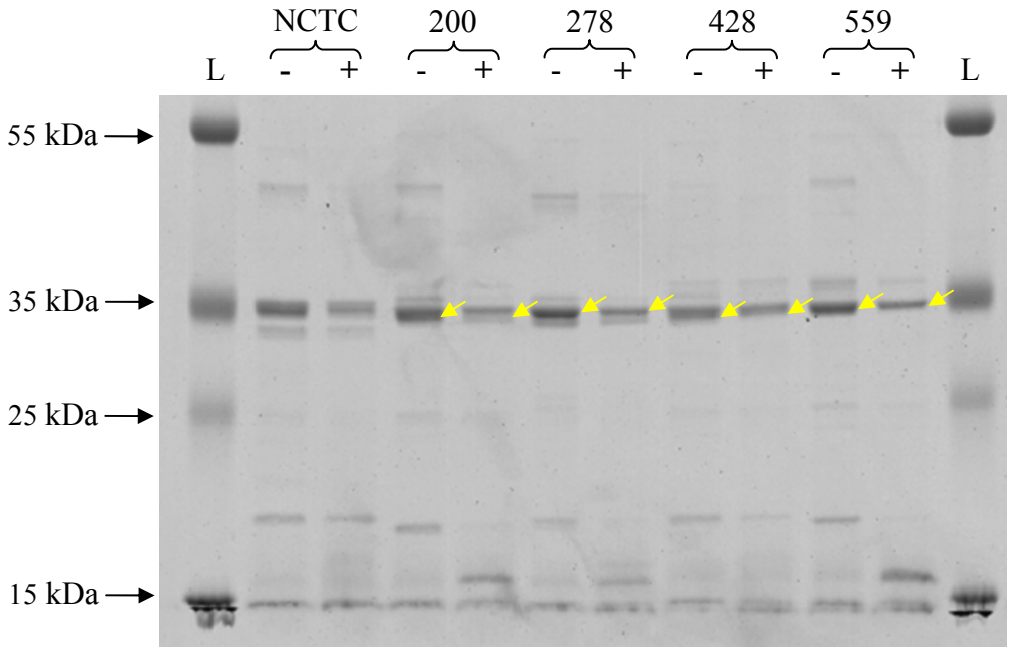
Band C, F and A are assumed as OmpC, OmpF and OmpA by previously published data. Blue and yellow box were determined by mass spectrometry as OmpC and OmpA, respectively. Bolded fonts denote significant increase in MIC values. Where the strains showed a change in the MIC values of ertapenem in the presence of 25 µg/ml PAβN, these strains also showed reduced expression of their porins when treated with the compound. Yellow arrows show the changes in the intensity of the porin bands.

Isolate 656, which showed no difference in ertapenem MIC value when PAβN was added, also had no detectable changes in OmpC and OmpF production. The outer membrane protein of higher molecular mass (Blue box) of isolate 298 was determined by mass spectrometry to be OmpC. The smaller outer membrane protein band (Yellow box) of isolate 656 was confirmed to be OmpA by mass spectrometry. Other isolates of *Enterobacter* spp. (n = 4) and *Klebsiella* spp. (n = 5) were also selected to assess the effects of PAβN on the outer membrane protein profiles. In both cases, the addition of PAβN changed the outer membrane protein profile of the isolates of these two species (Figure 3.20 and Figure 3.21). In conclusion, in many of the isolates where PAβN was found to have increased the ertapenem MIC values, the compound was also found to alter porin expression of these isolates. However, exceptions were observed in some cases (e.g. Figure 3.21 isolate 582 & 664).

3.8 Discussion

Previous studies have shown that in *E. coli* the AcrAB-TolC efflux system works synergistically with other mechanisms to confer a higher level of resistance to antibacterial compounds, such as triclosan and ciprofloxacin (McMurry et al., 1998, Oethinger et al., 2000). However, the data obtained from my study suggest that a functional AcrAB-TolC is not required for the full elaboration of carbapenem resistance i.e. not as expected in the hypothesis (Section 3.2). This is in disagreement with some reports that have often implicated increased efflux to contribute (at least in part) to carbapenem resistance among Enterobacteriaceae (Bornet et al., 2003, Lavigne et al., 2012, Woodford et al., 2007). Some authors have also suggested that the AcrAB efflux pump plays a role in carbapenem resistance (Bornet et al., 2003, Lavigne et al., 2012, Seecoomar et al., 2013). During the course of treatment with imipenem, susceptible isolates of *E. aerogenes* which evolved into imipenem intermediate and fully resistant strains had elevated AcrA and TolC expression

Figure 3.20 Outer membrane protein of *Enterobacter* spp. clinical isolates in the presence and absence of 25 µg/ml PAβN



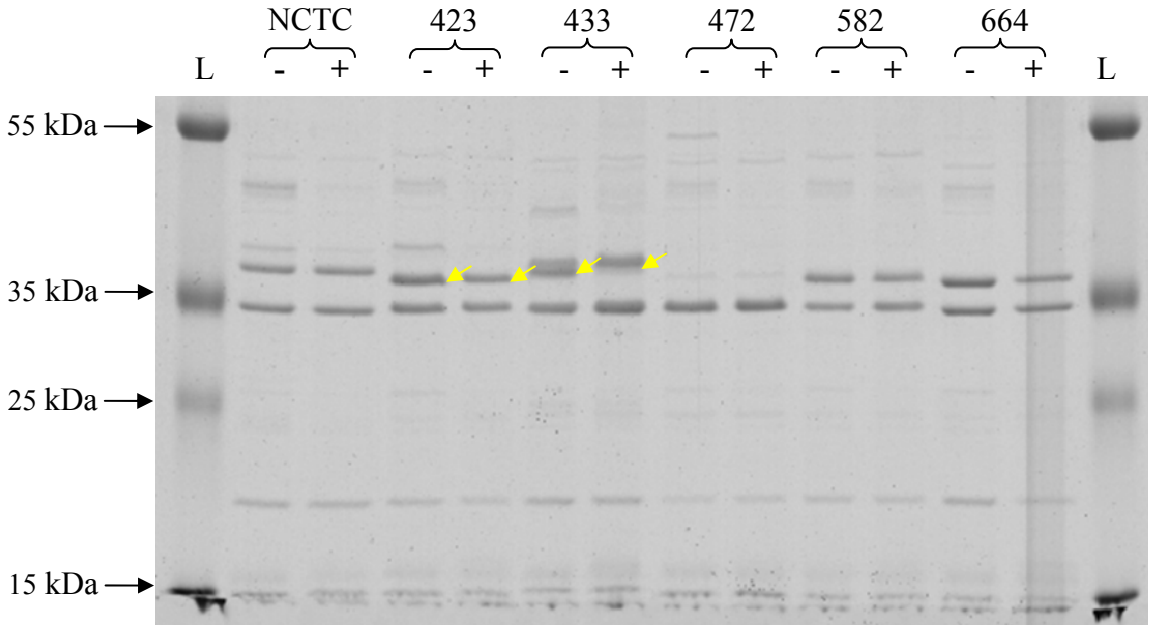
L: PageRuler Plus Prestained Protein Ladder (Thermo Scientific)

Strain	PAβN (25 µg/ml)	Ertapenem MIC (µg/ml)
E. cloacae	−	0.5
NCTC10005	+	0.5
Isolate 200	−	16
(*E. cloacae*)	+	32
Isolate 278	−	4
(*E. asburiae*)	+	4
Isolate 428	−	32
(*E. cloacae*)	+	64
Isolate 559	−	8
(*E. cloacae*)	+	**32**

−: Not added; +: Added; ND: Not determined; Bolded fonts denote significant increase in MIC values.

Where the strains showed a change in the MIC value of ertapenem in the presence of 25 µg/ml PAβN, changes in the porin profile were also observed. Yellow arrows show the changes in the intensity of the porin bands.

Figure 3.21 Outer membrane proteins of *Klebsiella* spp. clinical isolates in the presence and absence of 25 µg/ml PAβN



L: PageRuler Plus Prestained Protein Ladder (Thermo Scientific)

Strain	PAβN (25 µg/ml)	Ertapenem MIC (µg/ml)
K. pneumoniae	−	0.016
NCTC9633	+	0.016
Isolate 423	−	32
K. pneumoniae	+	64
Isolate 433	−	8
K. oxytoca	+	**64**
Isolate 472	−	64
K. pneumoniae	+	128
Isolate 582	−	2
K. pneumoniae	+	**8**
Isolate 664	−	8
K. pneumoniae	+	8

−: Not added; +: Added; ND: Not determined; Bolded fonts denote significant increase in MIC values. Bolded fonts denote significant increase in MIC values.

Where the strains showed a change in the MIC value of ertapenem in the presence of 25 µg/ml PAβN, changes in the porin profile were also observed. Yellow arrows show the changes in the intensity of the porin bands.

(Lavigne et al., 2012). However, the resistant isolate had no expression of OmpK35 and OmpK36 which are frequently associated with carbapenem resistance (Adams-Sapper et al., 2015, Clancy et al., 2013a, Clancy et al., 2013b, Hernandez-Alles et al., 1999, Shields et al., 2015). In another study, imipenem resistant mutants of *E. aerogenes* were selected by sequential sub-culturing of an ATCC13048 type strain in increasing concentration of imipenem. The mutants also showed increased expression of AcrA efflux protein (Bornet et al., 2003).

A more recent study cloned and expressed the bla_{KPC} gene on a plasmid in an *E. coli* N43 strain which does not produce the AcrA protein (Seecoomar et al., 2013). Compared to the parental strain, *E. coli* W4573 which carried the same carbapenemase-producing plasmid, the authors concluded that the AcrAB efflux pump is important for carbapenem resistance as there was a 32- and 16-fold increase in meropenem and imipenem MIC values, respectively in the parental strain (Seecoomar et al., 2013). Although the same bla_{KPC-2} in Tn*4401* isoform 'a' and similar K-12 derived *E. coli* strains were used (Leavitt et al., 2010a, Nakamura, 1968, Seecoomar et al., 2013, Wanner et al., 1983), the AcrAB associated reduction in carbapenem susceptibility was not observed in this PhD study.

The N43 strain carries a mutant allele of *acrA* termed *acrA1* due to the insertion of an insertion element (IS*2*) close to the 5' end of the *acrA* gene (Ma et al., 1993, Ma et al., 1995). Together with MIC data, it was assumed that N43 does not produce the functional AcrAB efflux pump as the susceptibility of the N43 strain could be restored to wildtype levels by introducing a wildtype *acrA* gene (Ma et al., 1993). It is important to note that this N43 strain is not an *acrA* null mutant and may carry other mutations with unknown effects on drug susceptibility (Ma et al., 1995). It is also not known whether there is any polar effect arising from the insertion element (Ma et al., 1993, Thanassi et al., 1997). If the AcrB protein is still

produced, the presence of other membrane fusion protein such as AcrE can complement the function of AcrA (Smith and Blair, 2014). Hence, the use of this N43 strain in the study does not provide a defined background to investigate the relationship of AcrAB efflux system with carbapenem resistance.

On the contrary, the *Salmonella* strain used in this PhD study is different: the function of AcrAB has been completely abolished due to the inactivation method used which replaced the *acrAB* genes with a chloramphenicol resistance gene (*cat*) (Nishino et al., 2006). Similarly, the *acrB* efflux pump component has been completely removed from *E. coli* BW25113 (Baba et al., 2006). Hence, the data gathered from this PhD suggest that the AcrAB-TolC efflux system does not play a role in carbapenem resistance. This finding also corroborates a previous study which found no increase expression of *acrB* mRNA transcripts among carbapenem resistant isolates of *Klebsiella* spp. and *Enterobacter* spp. (Doumith et al., 2009). Moreover, to date, there is no study which clearly associates carbapenems (in particular, ertapenem) as a substrate of the AcrAB-TolC efflux system. Crystallisation and molecular docking studies have frequently centred on substrates, such as penicillins, cephalosporins, doxorubicin and minocycline (Collu et al., 2012, Eicher et al., 2012, Lim and Nikaido, 2010, Murakami et al., 2006, Nagano and Nikaido, 2009).

Interestingly, although the functional AcrAB-TolC efflux system was not found to be required for the resistance of the carbapenemase-producing *Salmonella* strains, the loss of TolC function instead caused an increase in resistance towards a variety of carbapenem antibiotics (ertapenem, meropenem and doripenem) and a cephalosporin (ceftazidime). To investigate whether the plasmid (pKpQIL-UK) used was responsible for the reduced susceptibility observed in the TolC mutant, its variant (pKpQIL-D2), pNDM-HK and pUC18 plasmids were also included in the experiment. The plasmid ca. 89 kb pNDM-HK is an

IncL/M plasmid which encodes for a *bla*$_{NDM-1}$ carbapenemase (Ho et al., 2011). This plasmid shares no significant homology (≈4% Blastn alignment coverage) to the ca. 114 kb *bla*$_{KPC}$-encoding pKpQIL-UK and -D2 plasmids which belong to the IncF group of plasmids. Unlike the natural conjugative plasmids (pKpQIL-UK, -D2 and pNDM-HK), the much smaller (2.7 kb) pUC18 is a laboratory generated plasmid (Norrander et al., 1983). Although as expected that the *bla*$_{TEM-1}$-encoding pUC18 plasmid did not confer a clinically significant level of resistance to ertapenem in the TolC mutant, the fold increase in the ertapenem MIC value (when compared to the wildtype) was similar to those shown for strains containing the carbapenemase-producing plasmids. Taken together, these data suggest that the increase in β-lactam (especially carbapenem) resistance in the *Salmonella* TolC mutant was not an effect of expression of other vector genes in the plasmids or chromosome of the strains used. Neither was it an effect of the carbapenemase or β-lactamase genes involved. This finding is similar to those that have been reported where clinical isolates harbouring different carbapenemases showed heteroresistance towards various carbapenem antibiotics (Jain et al., 2014, Pournaras et al., 2010, Tijet et al., 2013, Zavascki et al., 2014). The decrease in carbapenem susceptibility was not observed in the *E. coli* BW25113 TolC mutant. Instead, the *E. coli* TolC mutant carrying the pKpQIL-UK plasmid was more susceptible to various carbapenem antibiotics.

It has been suggested previously that *E. coli* lacking TolC protein exhibit membrane stress (Dhamdhere and Zgurskaya, 2010). Hence, it was hypothesised that membrane damage from the loss of TolC might allow the diffusion of the carbapenemase into the extracellular environment allowing better tolerance to the carbapenem antibiotics tested. Possibly due to low expression of the carbapenemase (*bla*$_{KPC}$) and/or low plasmid copy number of pKpQIL-UK, a high amount of total protein was required to detect the hydrolysis of the nitrocefin dye.

This amount was not achievable using the supernatant of the culture medium. Hence, the passive release of the carbapenemase could only be assessed using the crude lysate of the bacterial pellet. As no significant difference in the initial hydrolysis of nitrocefin was observed between the wildtype *Salmonella* compared to the AcrAB and TolC mutant, it is unlikely that this hypothesis is correct.

The effect of the well established efflux inhibitor PAβN in carbapenem susceptibility was also investigated. Of note, the addition of this efflux inhibitor increased the ertapenem MIC values of the wildtype *S.* Typhimurium 14028s^{Rif} and AcrAB mutant strain. The fold increase is similar to that observed in the *Salmonella* TolC mutant carrying the plasmids (in the absence of PAβN). It has been suggested that a low concentration of PAβN (20 μM ≈ 10.4 μg/ml) will have efflux inhibitory effect, whereas a higher concentration (0.1 mM ≈ 51.9 μg/ml) will enhance the efflux rate of cephalosporins via AcrB (Kinana et al., 2013). However, this phenomenon does not explain the observations of this PhD study as the increase in ertapenem resistance was also observed in the AcrAB *Salmonella* and AcrB *E. coli* mutants when treated with PAβN. Other studies have used PAβN to associate the role of efflux in carbapenem resistance when a decrease in carbapenem MIC value was observed in the presence of the inhibitor (Baroud et al., 2013, Szabó et al., 2006, Yang et al., 2012). The concentration of PAβN used in these studies ranged from 25 – 100 μg/ml. Unlike the data obtained from this PhD study, where 72 out of 86 clinical isolates showed at least 2-fold increase in ertapenem resistance, the previous studies reported reduction in carbapenem MIC values when the isolates were tested with PAβN (Baroud et al., 2013, Szabó et al., 2006, Yang et al., 2012). In one study, one of the *E. coli* isolate (E44) showed a 8-fold decrease in ertapenem susceptibility in the presence of 100 μg/ml PAβN (Baroud et al., 2013). However, the authors did not discuss this isolate further.

Previous studies have shown that efflux pump expression and repression of porin are closely regulated (Barbosa and Levy, 2000, De Majumdar et al., 2013). Microarray studies have also shown that the disruption of the *acrB* or *tolC* gene resulted in decreased expression of *ompF* (Webber et al., 2009). Recently RNA-sequencing from the Piddock Group which studied the impact of PAβN on *S.* Typhimurium SL1344 also showed decreased *ompF* mRNA transcript (Blair, J. & Piddock, L., Unpublished data). The ertapenem MIC values of the porin mutants carrying the pKpQIL-UK plasmid showed increased resistance to ertapenem, but the fold-change in MIC value after addition of PAβN decreases in the following order: OmpC, OmpF and OmpC/F double mutants. Taken together, these data suggested that altered porin production was associated with the observed reduction in ertapenem susceptibility in the strains carrying the various plasmids. This was supported by the SDS-PAGE gels which showed a decreased in *Salmonella* porin expression with an increasing concentration of PAβN. Similar changes in outer membrane protein profile were observed with the Enterobacteriaceae clinical isolates harbouring a variety of carbapenemases. These findings support a previous study which showed OmpC and OmpF or their orthologues play a role in carbapenem resistance among *Enterobacter* spp. and *Klebsiella* spp. (Doumith et al., 2009).

To investigate whether the decrease in carbapenem susceptibility was specific to PAβN, MIC values were determined with two other efflux inhibitors (NMP and Compound A). The *S.* Typhimurium 14028s^{Rif} which carries the pKpQIL-UK plasmid was tested for its susceptibility towards ertapenem. This carbapenem was chosen as it showed the largest change in susceptibility. Independent of the presence of the functional AcrAB-TolC efflux system, PAβN and NMP reduced the susceptibility of the *Salmonella* and *E. coli* strains harbouring the pKpQIL-UK plasmid. Compound A had no impact on the carbapenem susceptibility of the strains. This may be due to the difference in the mechanism of action of

the efflux inhibitors. Since its discovery, PAβN has been known to have membrane permeabilising effects (Lomovskaya et al., 2001), but that the permeabilising effect was only apparent when efflux function was compromised. At up to 128 μg/ml PAβN, *P. aeruginosa* with a functional MexAB-OprM showed no significant membrane permeabilisation. However, more recently it has been suggested that the permeabilising effect of PAβN is stronger than previously reported (Lamers et al., 2013, Matsumoto et al., 2011). The membrane damaging effects occur also in wildtype *Pseudomonas* without efflux defects and the effects are stronger on the *E. coli* outer membrane compared to the former (Lamers et al., 2013, Matsumoto et al., 2011). It may be possible that the membrane stress caused by PAβN is similar to that of the loss of the TolC physical protein and/or its function. The permeabilising effect of PAβN also explains the observation that most of the clinical isolates which showed a 4-fold increase in ertapenem MIC values at 25 μg/ml PAβN had only a 2-fold increase when 100 μg/ml PAβN was used. A previous study comparing the membrane permeabilising effects of PAβN and NMP also suggested a possible membrane permeabilising effect by NMP albeit less prominent than PAβN (Pannek et al., 2006). This suggests that NMP may have reduced the susceptibility of the *Salmonella* and *E. coli* strains via the same mechanism as PAβN. The effect of NMP via down-regulation of OmpF is also supported by the observation that the ertapenem MIC values were only increased in the SL1344 OmpC mutant but not when OmpF was absent. The regulation of porin expression is known to be complex and may differ between species (De La Cruz and Calva, 2010, Martínez-Flores et al., 1999). This may explain the differences observed between the *K. pneumoniae*, *E. coli* and *S.* Typhimurium efflux mutants' susceptibility to the antibiotics tested.

Previous studies have associated ESBL with carbapenem resistance (Doumith et al., 2009, Woodford et al., 2007). Hence, the $bla_{\text{CTX-M-14}}$ encoding pCT plasmid and its ESBL

inactivated plasmids were introduced into the set of *Salmonella* strains to study the effects of an ESBL on carbapenem susceptibility in the presence/absence of a functional AcrAB-TolC efflux system. Unlike the $bla_{\text{TEM-1}}$-encoding pUC18, there was no obvious decrease in ertapenem susceptibility in the *Salmonella* TolC mutant. This was possibly due to the lower copy number of pCT and/or lower expression of $bla_{\text{CTX-M-14}}$. However, in the presence of PAβN, an increase in ertapenem MIC values was observed for *S.* Typhimurium 14028s$^{\text{Rif}}$/pCT and 14028s$^{\text{Rif}}$ *acrAB*/pCT.

Efflux systems has been well established for their contribution to antibiotic resistance and TolC forms the outer membrane channel of most efflux pumps found in Gram-negative bacteria. Hence, TolC has been suggested as a potential target for the design of efflux pump component inhibitors (Pagès and Amaral, 2009). From this work, it is suggested that PAβN which causes membrane stress and the loss of function of the TolC and/or its physical protein could increase the resistance of the bacteria against antibiotics which uses outer membrane porins as entry routes to their targets in the cell. Hence, TolC may not be an ideal drug target as the loss of TolC function and/or its protein may cause increase resistance via the down-regulation of outer membrane proteins, especially OmpF.

3.9 Future Work

One main question arising from the data obtained is whether the down-regulation of the porins resulted from the physical loss of the TolC protein or from the loss of function of the protein. This could be addressed by expressing a non-functional TolC protein instead of using a deletion mutant. This could be done by introducing point mutations which affect the function of the TolC protein without affecting its expression or complex with the AcrAB components (Krishnamoorthy et al., 2013). Data arising from this experiment will be important for efflux inhibitor design. If the loss of TolC function was the trigger for the

adaptation in porin repression, a drug designed to inhibit the function of TolC may increase the resistance to certain antibiotics. The main antibiotics tested in this study were carbapenem antibiotics due to the carbapenemases encoded on the plasmids. However, other antibiotics which utilise OmpF as the major route of entry into the cells, such as quinolones, should also be tested (Marshall and Piddock, 1993).

From this study, it was observed that the inactivation of the efflux pump components had different effects in the different species (*K. pneumoniae*, *E. coli* and *Salmonella*). Hence, a TolC inactivated *K. pneumoniae* will be required to investigate whether the susceptibility of the carbapenemase producing strain would show similar susceptibility level as the *E. coli* or *Salmonella* strains. The inactivation of the TolC could be done as previously described by homologous recombination of chimeric PCR amplicons of antibiotic resistance gene (e.g. *aph* or *cat*) with flanking regions homologous to the *tolC* gene (Datsenko and Wanner, 2000).

It is hypothesised that the inactivation of TolC in *Salmonella* and the addition of PAβN (*Salmonella* and *E. coli*) represses porin production. This could be via at least two mechanisms: (1) the *micF* antisense pathway which inhibits OmpF translation and/or destabilisation of *ompF* mRNA transcript (Ramani et al., 1994) or (2) EnvZ-OmpR mechanism where EnvZ phosphorylates OmpR which then represses the transcription of *ompF* (Cai and Inouye, 2002). Hence, qRT-PCR could be used to assess the expression of *micF* and the porin genes in the TolC mutants and, when the wildtype *Salmonella* and *E. coli* strains are treated with PAβN. Another method to confirm that OmpF played a role in the carbapenem susceptibility observed in the *Salmonella* TolC mutant is by expression of OmpF in *trans* on a low copy plasmid in the strain. A low copy plasmid such as pUA66 can be used for the expression of OmpF with an inducible promoter (Zaslaver et al., 2004). If OmpF is

repressed in the TolC mutant, the expression of OmpF in *trans* would restore the carbapenem MIC values to wildtype levels.

3.10 Key Findings

- The AcrAB-TolC efflux system in *K. pneumoniae*, *E. coli* and *S.* Typhimurium is not essential for carbapenem resistance.
- The loss of TolC protein and/or TolC function in *S.* Typhimurium reduces β-lactam antibiotic susceptibility.
- The efflux inhibitor (PAβN) reduces β-lactam antibiotic susceptibility.
- The decrease in carbapenem susceptibility in a *Salmonella* TolC mutant and, when *Salmonella* and *E. coli* are treated with PAβN, is likely due to OmpF repression.

CHAPTER FOUR: COMPARISON OF PLASMIDS pKpQIL-UK WITH pKpQIL-D2

4.1 Background

The *bla*$_{KPC}$-encoding plasmid pKpQIL-UK has been found in bacteria isolated in various countries, including USA, Israel, Italy and Poland. Recently, this plasmid was also discovered in the UK (Woodford, N. Unpublished data). Interestingly, a variant of the plasmid i.e. pKpQIL-D2 was also isolated. A ca. 20 kb backbone region on the pKpQIL-UK has been substituted with a smaller ca. 16 kb in pKpQIL-D2. This variant plasmid was found in different species of Enterobacteriaceae, including *E. cloacae* and *E. coli*. However, it is not known whether the substituted region confers a general advantage to the dissemination and persistence of this variant plasmid in other species and so contributed to the hospital outbreak in the UK.

4.2 Hypothesis and Aims

It was hypothesised that the ca. 16 kb region in the pKpQIL-D2 plasmid confers a fitness advantage in the plasmid-carrying host. Therefore, the aims of the study were:

i) To determine the conjugation frequencies of the pKpQIL-UK and -D2 from their original clinical isolates into representative species of Enterobacteriaceae.

ii) To determine the ability of the plasmid-carrying hosts to form biofilm.

iii) To study the differences in growth rate of the plasmid-carrying hosts.

iv) To investigate the impact of the plasmids on host bacterium chromosomal gene expression.

4.3 Comparison of the Genetic Sequence of pKpQIL-UK with that of pKpQIL-D2

The pKpQIL-UK and pKpQIL-D2 plasmid DNA sequences were identical except for a ca. 20 kb region in pKpQIL-UK which has been substituted with a ca. 16 kb region in pKpQIL-D2.

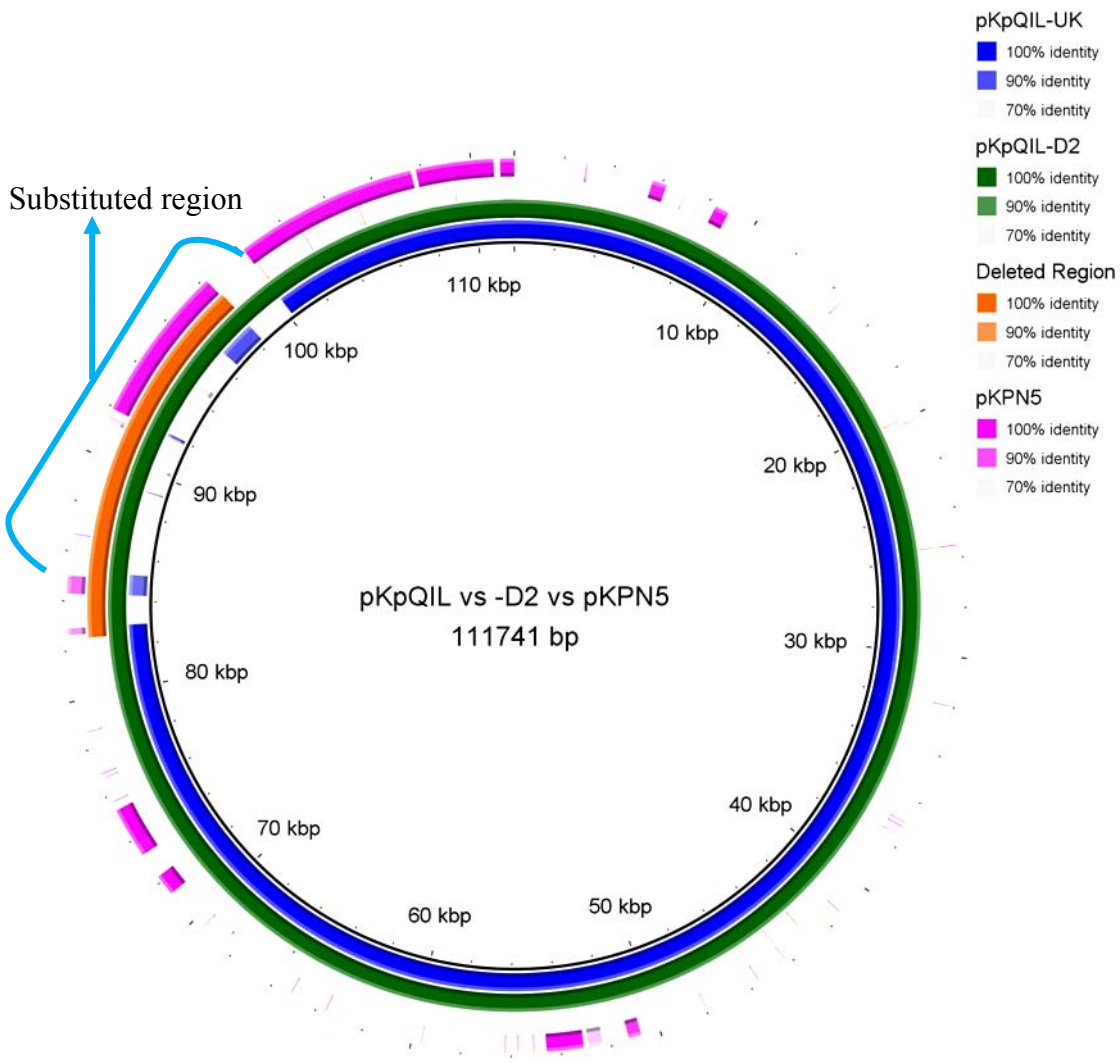
Both of these regions carry genes which are associated with resistance, mobile elements, replicon, plasmid genes (e.g. partition system) (Figure 1.8; On page 42). The majority of the open reading frames within the substituted region in pKpQIL-D2 encode for small hypothetical proteins (100 – 150 amino acids). Protein homology search by Blastp, conserved motif search by PROSITE (http://prosite.expasy.org/) and protein localisation prediction by PSORTb (http://www.psort.org/psortb/) were conducted but no significant hit was obtained for these hypothetical proteins. It is not known whether these were functional proteins. A blastn search (http://blast.ncbi.nlm.nih.gov/) using this region in pKpQIL-D2 showed 46% coverage with high sequence identity (98%) with a region in the plasmid pKPN5 found in the multidrug drug resistant *K. pneumoniae* MGH78578 (Figure 4.1). Among the isolates received by Public Health England, it was found that plasmid pKpQIL-D2 occurred more frequently than pKpQIL-D1. Moreover, pKpQIL-D2 was also found in *E. cloacae*, *E. coli* and non-ST258 *K. pneumoniae*. Therefore, all the fitness experiments conducted in this PhD study focused on the pKpQIL-UK and pKpQIL-D2 plasmids.

4.4 Transfer of Plasmid into Different Species of Enterobacteriaceae

Plasmids pKpQIL-UK and -D2 were large and could not be transferred by electroporation into new bacterial hosts. Hence, rifampicin-resistant mutants were selected from *K. pneumoniae* Ecl8, *E. coli* NCTC10418, *S.* Typhimurium (SL1344 and ATCC14028s), *E. cloacae* NCTC10005 and *S. marcescens* NCTC10211. A single amino acid substitution was found in the *rpoB* gene in representative mutants of all five strains. The amino acid substitution varied between the species. The frequency of mutation to resistance of each strain was typical of that of a mutation in a single gene (Table 4.1) (Miller et al., 2002).

Plasmids pKpQIL-UK and -D2 were transferred by electroporation into highly electrocompetent ElectroMAX^{TM} DH10B^{TM} *E. coli* cells according to manufacturer's

Figure 4.1 Alignment of pKpQIL-UK and -D2 with pKPN5



The diagram was generated using Blast Ring Image Generator. From the inner most ring, (1) Size of the plasmid, (2) pKpQIL-UK (blue), (3) pKpQIL-D2 (green), (4) substituted region (orange) and (5) pKPN5 (pink). The 16 kb region in pKpQIL-D2 which substituted the 20 kb region in pKpQIL-UK is highlighted in orange. It also shows that about 46% of this substituted region (orange) shares about 98% DNA sequence identity with pKPN5 plasmid from *K. pneumoniae* MGH78578. The genes shared between pKPN5 with the substituted region in pKpQIL-D2 include the *repB* replicon, *parAB* partitioning system and *umuCD* DNA damage control (Figure 1.8).

Table 4.1 Rifampicin-resistant mutants of five species of Enterobacteriaceae

Bacteria (Rifampicin-Resistant)	Code	Mutation (Substitution)	Mutation Frequency
K. pneumoniae Ecl8	$Ecl8^{Rif}$	His537Leu	8.6×10^{-9}
E. coli NCTC10418	10418^{Rif}	Ser531Phe	2.2×10^{-8}
S. Typhimurium ATCC14028s	$14028s^{Rif}$	Ser522Tyr	7.3×10^{-10}
S. Typhimurium SL1344	$SL1344^{Rif}$	Ser522Phe	1.1×10^{-9}
E. cloacae NCTC10005	$Ecloacae^{Rif}$	Ser328Ala	1.3×10^{-8}
S. marcescens NCTC10211	$Serratia^{Rif}$	Asp516Gly	6.0×10^{-9}

The table shows the substitution found in each of the rifampicin-resistant strains used and the frequency at which these mutants were selected at. These strains were used as the host for the pKpQIL-UK and -D2 plasmids in the fitness experiments.

instructions. This *E. coli* strain was chosen as it is known to be plasmid-free and possesses a high transformation efficiency with a good ability to maintain large plasmids (Durfee et al., 2008). To verify that the transformants contained the plasmids, PCR was used to detect the bla_{KPC} gene. The PCR amplicon was purified and DNA sequenced; this showed that the DH10B transformants carried the bla_{KPC-2} variant carbapenemase. The DH10B/pKpQIL-UK and DH10B/pKpQIL-D2 strains were used to transfer the plasmids into the respective Enterobacteriaceae species (Figure 4.2).

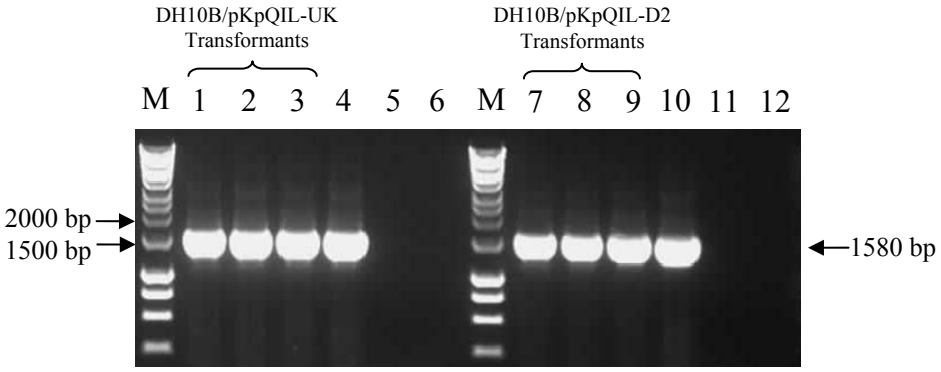
The DH10B/pKpQIL-UK and DH10B/pKpQIL-D2 strains were used as the donor strains of the plasmids into the rifampicin-resistant recipient strains of *E. coli*, *Salmonella*, *E. cloacae* and *S. marcescens* in filter conjugation experiments. The plasmids were also transferred into *K. pneumoniae* ST258 which was naturally kanamycin resistant. All of the transconjugants obtained were checked for the presence of the bla_{KPC} gene and for the plasmid backbone fragment which differentiates the two plasmids (Figure 4.3 – 4.7). For the Serratia^{Rif}/pKpQIL-UK transconjugants, it was shown by PCR to contain the bla_{KPC} gene (Figure 4.5a, Lane 7-9). However, the PCR to check for the presence of a pKpQIL-UK backbone region was unsuccessful (Figure 4.5b, Lane 4-6). Hence, the PCR was repeated (Figure 4.6).

4.5 Growth Rates of Enterobacteriaceae pKpQIL-UK and -D2

Growth kinetics was one of the aspects of fitness which was investigated to determine the impact of the plasmids on its host. It was hypothesised that bacteria carrying the variant plasmid, pKpQIL-D2 grew faster than strains containing pKpQIL-UK. It was further hypothesised that this attribute allowed the pKpQIL-D2 plasmid to spread and persist in the UK. The difference of growth rates was identified by calculating the generation time of each strain/plasmid combination during the logarithmic phase of growth. Under the conditions

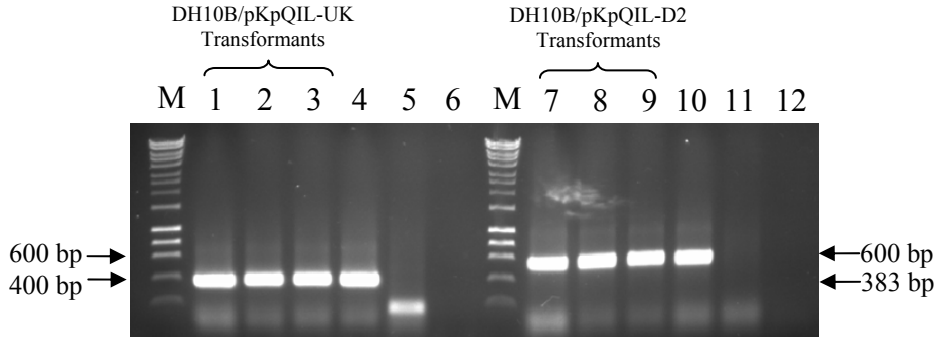
Figure 4.2 PCR verification of the *E. coli* DH10B transformants for the presence of pKpQIL-UK and -D2

(a) PCR check for the presence of bla_{KPC}



The plasmids pKpQIL-UK and -D2 provided by Woodford, N. was electroporated into *E. coli* DH10B to be used as donor strains for conjugation experiments. The transformants were verified by PCR for the presence of the bla_{KPC} gene. M: Hyperladder I (Bioline); Lane 4: pKpQIL-UK plasmid (Positive control); Lane 5: DH10B genomic DNA (Negative control); Lane 6: Water (Contamination control); Lane 10: pKpQIL-D2 plasmid (Positive control); Lane 11: DH10B genomic DNA (Negative control); Lane 12: Water (Contamination control); Primers: KpQIL-KPC-F/R; Expected amplicon: 1580 bp.

(b) PCR check for the presence of pKpQIL-UK



The plasmids pKpQIL-UK and -D2 provided by Woodford, N. was electroporated into *E. coli* DH10B to be used as donor strains for conjugation experiments. The transformants were verified by PCR for the presence of the respective plasmid backbone gene.

Key to lanes:
M: Hyperladder I (Bioline)
Lane 4: pKpQIL-UK plasmid (Positive control)
Lane 5: DH10B genomic DNA (Negative control)
Lane 6: Water (Contamination control)

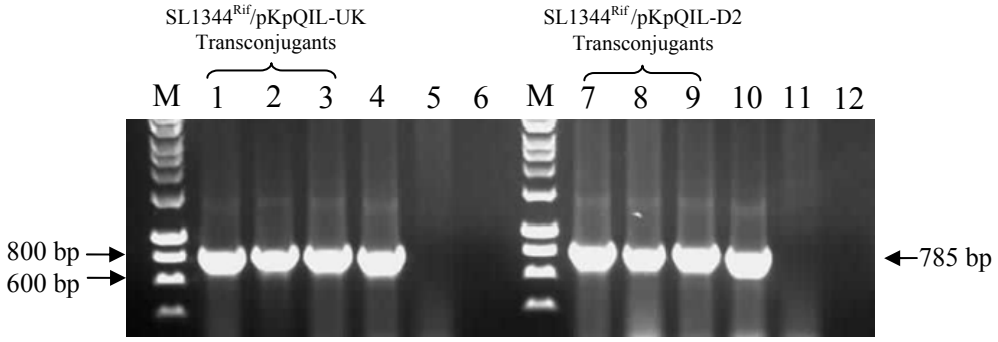
Primers: pQIL-F/R
Expected amplicon: 383 bp

Lane 10: pKpQIL-D2 plasmid (Positive control)
Lane 11: DH10B genomic DNA (Negative control)
Lane 12: Water (Contamination control)

Primers: pMan-F/R
Expected amplicon: 600 bp

Figure 4.3 PCR verification of the *Salmonella* SL1344^{Rif} transconjugants carrying pKpQIL-UK and -D2

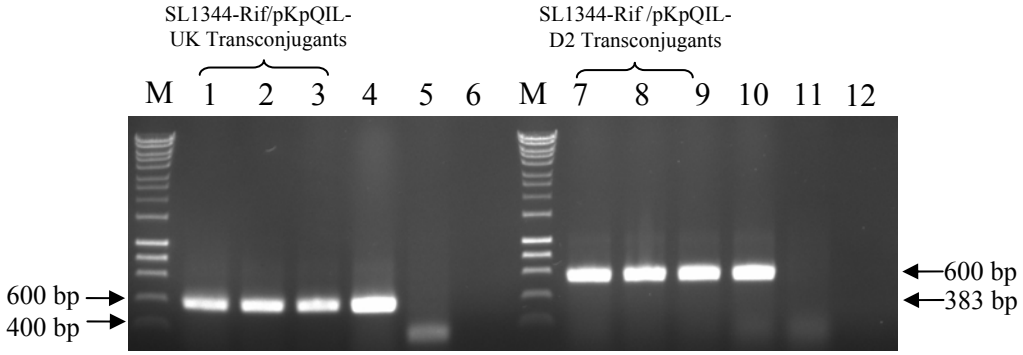
(a) PCR check for the presence of *bla*$_{KPC}$



The transconjugants of *Salmonella* SL1344 carrying pKpQIL-UK & -D2 were verified by PCR for the presence of the *bla*$_{KPC}$ gene. M: Hyperladder I (Bioline); Lane 4: pKpQIL-UK plasmid (Positive control); Lane 5: SL1344^{Rif} genomic DNA (Negative control); Lane 6: Water (Contamination control); Lane 10: pKpQIL-D2 plasmid (Positive control); Lane 11: SL1344^{Rif} genomic DNA (Negative control); Lane 12: Water (Contamination control); Primers: KPCg-colpcr-F/R; Expected amplicon: 785 bp.

*New PCR primers giving a smaller amplicon to detect *bla*$_{KPC}$ gene was designed. These primers are used hereafter.

(b) PCR check for the presence of the specific plasmids



The transconjugants of *Salmonella* SL1344 carrying pKpQIL-UK & -D2 were verified by PCR for the presence of the respective plasmid backbone gene.

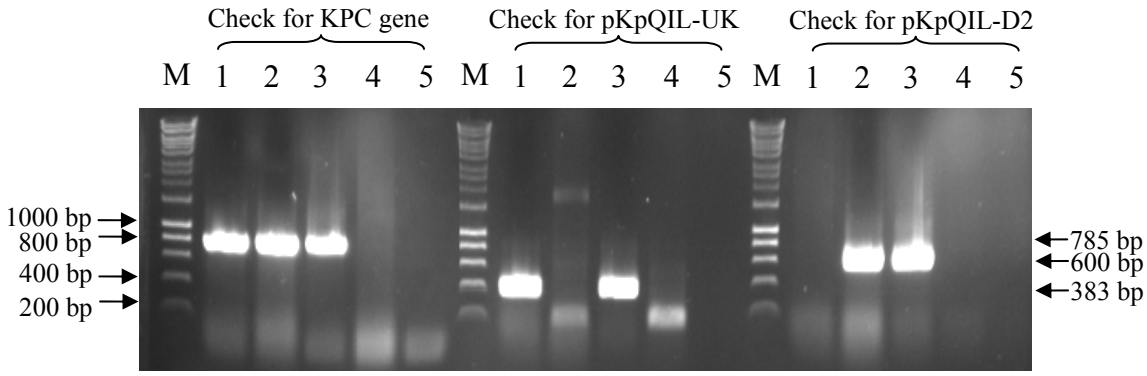
Key to lanes
M: Hyperladder I (Bioline)
Lane 4: pKpQIL-UK plasmid (Positive control)
Lane 5: pKpQIL-D2 plasmid (Negative control)
Lane 6: Water (Contamination control)

Primers: pQIL-F/R
Expected amplicon: 383 bp

Lane 10: pKpQIL-D2 plasmid (Positive control)
Lane 11: pKpQIL-UK plasmid (Negative control)
Lane 12: Water (Contamination control)

Primers: pMan-F/R
Expected amplicon: 600 bp

Figure 4.4 PCR verification of the *E. coli* 10418^{Rif} transconjugants carrying pKpQIL-UK and -D2



The transconjugants of *Salmonella* SL1344 carrying pKpQIL-UK & -D2 were verified by PCR for the presence of the *bla*$_{KPC}$ and respective plasmid backbone gene.

Key to lanes
M: Hyperladder I (Bioline)
Lane 1: 10418^{Rif}/pKpQIL-UK transconjugant
Lane 2: 10418^{Rif}/pKpQIL-D2 transconjugant
Lane 3: Positive control
Lane 4: 10418-Rif genomic DNA (Negative control)
Lane 5: Water (contamination control)

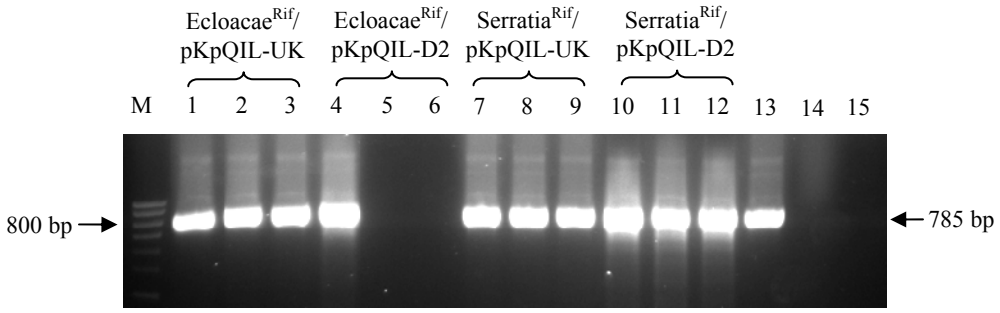
Check for the presence of *bla*$_{KPC}$
Primers: KPCg-colpcr-F/R
Postive control: pKpQIL-UK plasmid
Expected amplicon: 785 bp

Check for the presence of pKpQIL-UK
Primers: pQIL-F/R
Postive control: pKpQIL-UK plasmid
Expected amplicon: 383 bp

Check for the presence of pKpQIL-D2
Primers: pMan-F/R
Postive control: pKpQIL-D2 plasmid
Expected amplicon: 600 bp

Figure 4.5 PCR verification of the Rifampicin-resistant *E. cloacae* and *S. marcescens* transconjugants carrying pKpQIL-UK and -D2

(a) PCR check for the presence of bla_{KPC}



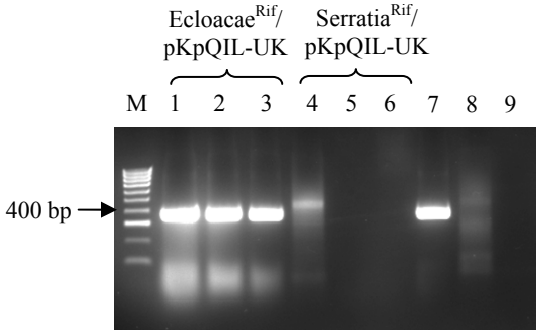
The transconjugants of *E. cloacae* and *S. marcescens* carrying pKpQIL-UK & -D2 were verified by PCR for the presence of the bla_{KPC} gene.

Key to lanes
M: Hyperladder IV (Bioline)
Electrophoresis was done using 2% (w/v) agarose

Lane 13: pKpQIL-UK plasmid (Positive control)
Lane 14: Ecloacae^Rif/Serratia^Rif genomic DNA (Negative control)
Lane 15: Water (Contamination control)

Primers: KPCg-colpcr-F/R
Expected amplicon: 785 bp

(b) PCR check for the presence of pKpQIL-UK

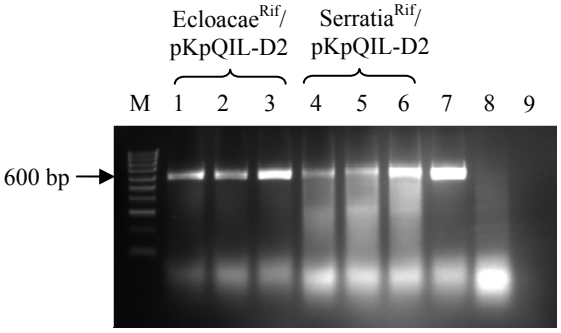


Key to lanes
M: Hyperladder IV (Bioline)
Electrophoresis was done using 2% (w/v) agarose

Lane 7: pKpQIL-UK plasmid (Positive control)
Lane 8: pKpQIL-D2 plasmid (Negative control)
Lane 9: Water (Contamination control)

Primers: pQIL-F/R
Expected amplicon: 383 bp

(c) PCR check for the presence of pKpQIL-D2

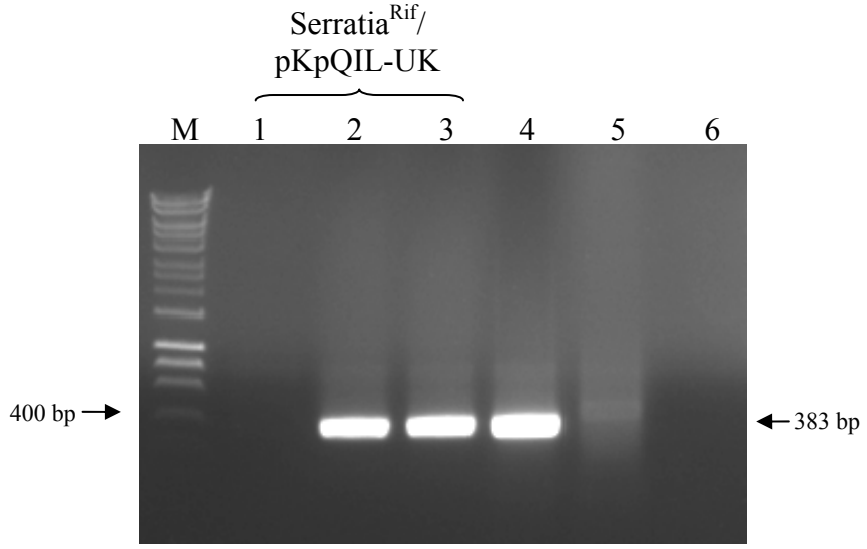


Key to lanes
M: Hyperladder IV (Bioline)
Electrophoresis was done using 2% (w/v) agarose

Lane 7: pKpQIL-D2 plasmid (Positive control)
Lane 8: pKpQIL-UK plasmid (Negative control)
Lane 9: Water (Contamination control)

Primers: pMan-F/R
Expected amplicon: 600 bp

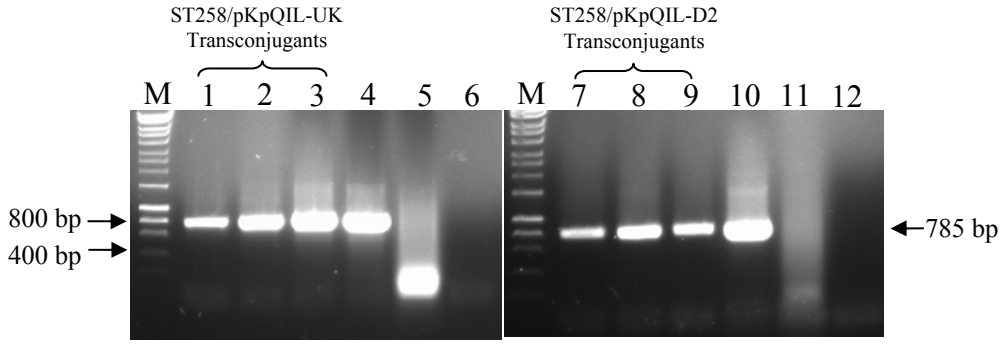
Figure 4.6 Repeat PCR verification of the Rifampicin-resistant *S. marcescens* transconjugants carrying pKpQIL-UK



The transconjugants of *S. marcescens* carrying pKpQIL-UK were verified by PCR for the presence of the plasmid backbone gene. M: Hyperladder I (Bioline); Lane 4: pKpQIL-UK plasmid (Positive control); Lane 5: pKpQIL-D2 plasmid (Negative control); Lane 10 – 12: Water (Contamination control); Primers: pQIL-F/R; Expected amplicon: 383 bp.

Figure 4.7 PCR verification of the *K. pneumoniae* ST258 transconjugants carrying pKpQIL-UK and -D2

(a) PCR check for the presence of bla_{KPC}

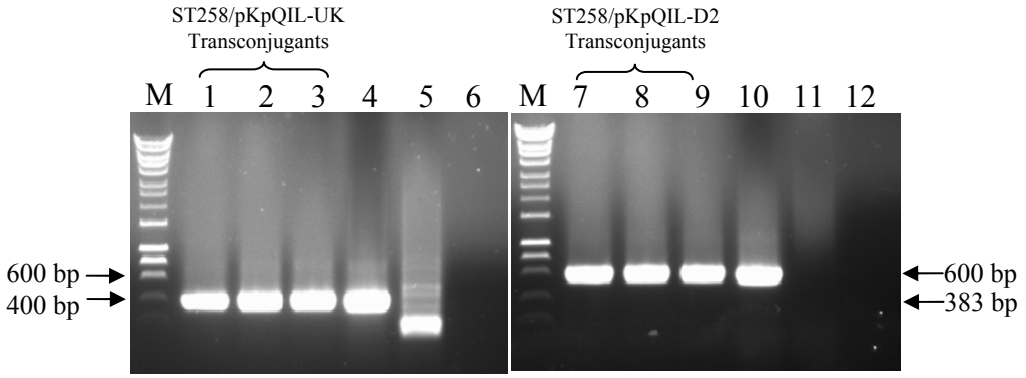


The transconjugants of *K. pneumoniae* ST258 carrying pKpQIL-UK & -D2 were verified by PCR for the presence of the bla_{KPC} gene.

Key to lanes
M: Hyperladder I (Bioline)
Lane 4: pKpQIL-UK plasmid (Positive control)
Lane 5: ST258 genomic DNA (Negative control)
Lane 6: Water (Contamination control)
Lane 10: pKpQIL-D2 plasmid (Positive control)
Lane 11: ST258 genomic DNA (Negative control)
Lane 12: Water (Contamination control)

Primers: KPCg-colpcr-F/R
Expected amplicon: 785 bp

(b) PCR check for the presence of the specific plasmids



The transconjugants of *K. pneumoniae* ST258 carrying pKpQIL-UK & -D2 were verified by PCR for the presence of the respective plasmid backbone gene.

Key to lanes
M: Hyperladder I (Bioline)
Lane 4: pKpQIL-UK plasmid (Positive control)
Lane 5: pKpQIL-D2 plasmid (Negative control)
Lane 6: Water (Contamination control)

Primers: pQIL-F/R
Expected amplicon: 383 bp

Lane 10: pKpQIL-D2 plasmid (Positive control)
Lane 11: pKpQIL-UK plasmid (Negative control)
Lane 12: Water (Contamination control)

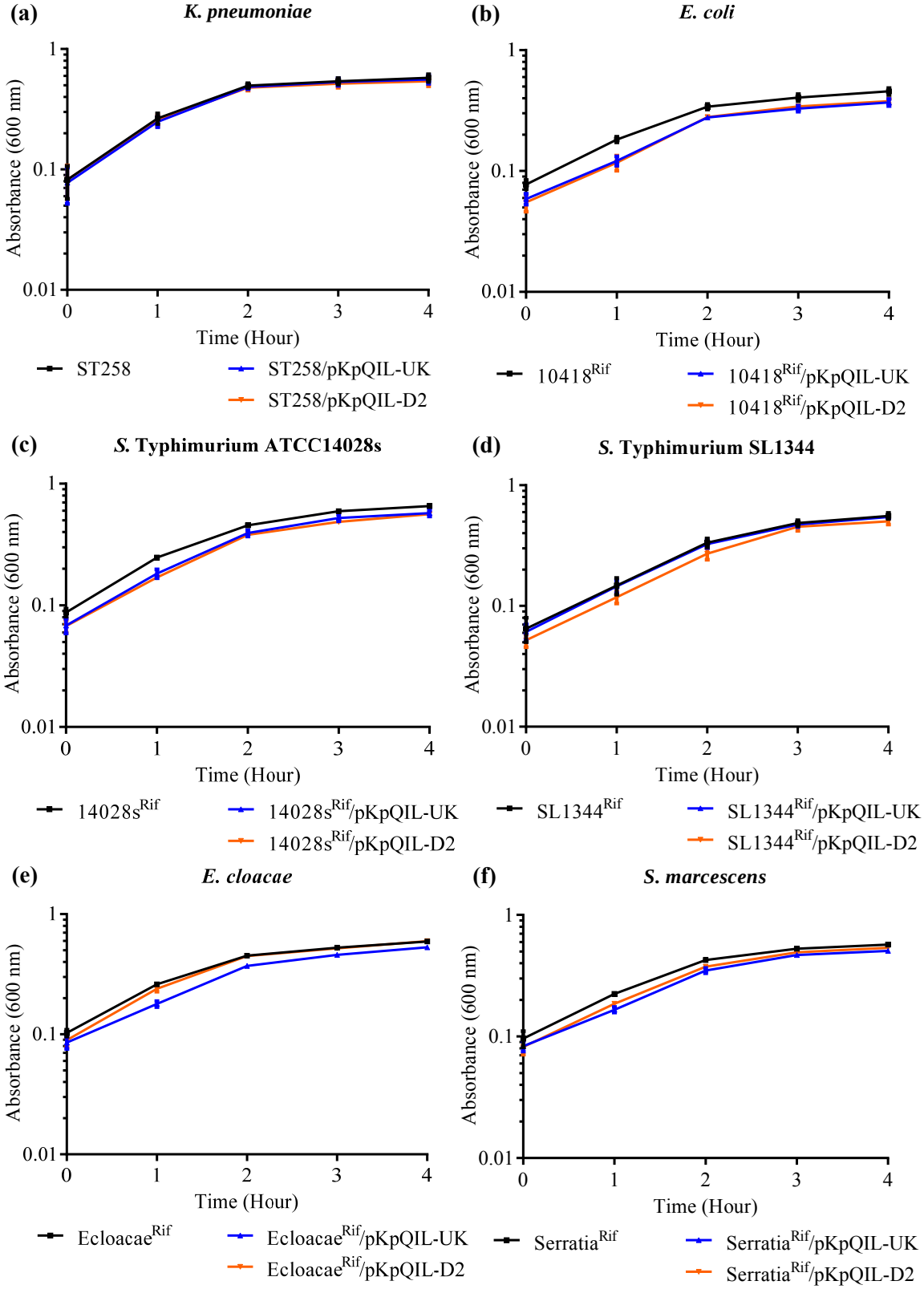
Primers: pMan-F/R
Expected amplicon: 600 bp

tested, *E. cloacae* and *S. marcescens* carrying pKpQIL-UK grew significantly more slowly than when carrying pKpQIL-D2. *S.* Typhimurium 14028s containing pKpQIL-D2 had a significantly slower generation time (Figure 4.8 & Table 4.2). In all other host strains, no difference in the generation time was observed in strains carrying pKpQIL-D2.

4.6 Plasmid Persistence

It was hypothesised that the substituted region in the pKpQIL-D2 allowed the plasmid to persist better than pKpQIL-UK in bacterial host strains, thus allowing it to persist in different bacterial populations. The stability of plasmid carriage among the Enterobacteriaceae species was also assessed by consecutive growth of the plasmid (pKpQIL-UK and -D2) carrying strains over a period of 20 days (ca. 140 generations) without antibiotic selection (Figure 4.9). For the original clinical isolates carrying the plasmids (*K. pneumoniae* ST468/pKpQIL-UK and ST321/pKpQIL-D2) (Figure 4.9a) and the *K. pneumoniae* ST258 transconjugants (Figure 4.9b), both plasmids were able to persist within their hosts over 20 days without detectable loss. However, in both strains of *Salmonella*, it was found that the pKpQIL-D2 plasmid was rapidly lost from the population (Figure 4.9c & d). On day 5, more than half of the population of *Salmonella* has lost pKpQIL-D2 (Figure 4.9c & d). However, pKpQIL-UK was able to persist within the population of *Salmonella* over the duration of time tested (ca. 140 generations). Interestingly, both plasmids were gradually lost from the *E. coli* population (Figure 4.9e). On the 20th day, most of the *E. coli* within the population were plasmid-free. Similar to *K. pneumoniae*, both plasmids were able to persist within the *E. cloacae* population (Figure 4.9f). It was not possible to assess the percentage of the *S. marcescens* population which retained or lost the plasmids as carbapenem-resistant, but plasmid-free, *Serratia* were selected during the course of the experiment.

Figure 4.8 Growth kinetics of various Enterobacteriaceae carrying pKpQIL-UK vs -D2



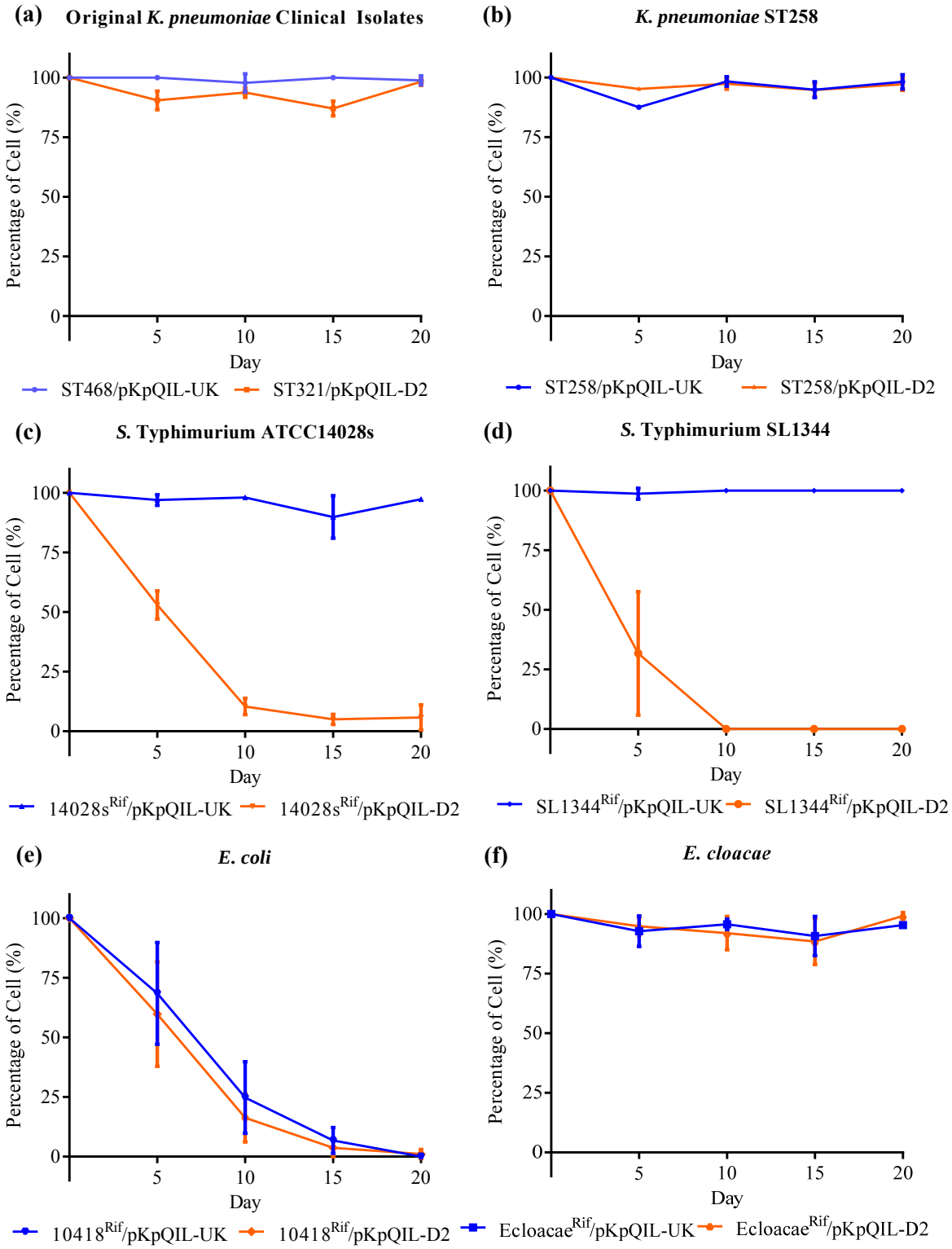
Growth kinetics was one of the aspects of fitness which was investigated to determine the impact of the plasmids on its host. All absorbance values were recorded as mean ± standard deviation of three independent experiments.

Table 4.2 Generation time of various Enterobacteriaceae carrying pKpQIL-UK vs -D2

Species	Strain	Generation Time (min)	Student's *t*-test
K. pneumoniae ST258	ST258	32.7 ± 4.2	-
	ST258/pKpQIL-UK	32.9 ± 4.8	0.91
	ST258/pKpQIL-D2	33.9 ± 5.0	0.44
E. coli NCTC10418	10418^{Rif}	45.1 ± 7.2	-
	10418^{Rif}/pKpQIL-UK	51.3 ± 7.4	0.05
	10418^{Rif}/pKpQIL-D2	50.7 ± 7.3	0.07
S. Typhimurium ATCC14028s	$14028s^{Rif}$	39.3 ± 1.0	-
	$14028s^{Rif}$/pKpQIL-UK	41.6 ± 3.4	0.06
	$14028s^{Rif}$/pKpQIL-D2	44.4 ± 4.9	**0.01***
S. Typhimurium SL1344	$SL1344^{Rif}$	52.3 ± 4.9	-
	$SL1344^{Rif}$/pKpQIL-UK	53.7 ± 5.4	0.57
	$SL1344^{Rif}$/pKpQIL-D2	56.6 ± 3.9	0.10
E. cloacae NCTC10005	$Ecloacae^{Rif}$	40.8 ± 3.2	-
	$Ecloacae^{Rif}$/pKpQIL-UK	51.2 ± 5.0	**<0.001***
	$Ecloacae^{Rif}$/pKpQIL-D2	38.3 ± 2.3	0.05
S. marcescens NCTC10211	$Serratia^{Rif}$	43.1 ± 4.5	-
	$Serratia^{Rif}$/pKpQIL-UK	50.8 ± 4.7	**0.001***
	$Serratia^{Rif}$/pKpQIL-D2	45.5 ± 4.5	0.22

The generation time of each strain was used as a measure for the fitness impact of the plasmids on their hosts. Generation times were recorded as mean ± standard deviation of three independent experiments. Asterisk (*) denotes significant differences observed by Student's *t*-test.

Figure 4.9 The percentage of pKpQIL-UK vs -D2 carrying cells in growth over a period of 20 days without antibiotic selection



The persistence of the plasmids in various Enterobacteriaceae species in LB broth without antibiotic selection over a 20-day period was investigated to determine the stability of the plasmids in their respective hosts. The percentages of cells which retain the plasmids were recorded as mean ± standard deviation of three independent experiments.

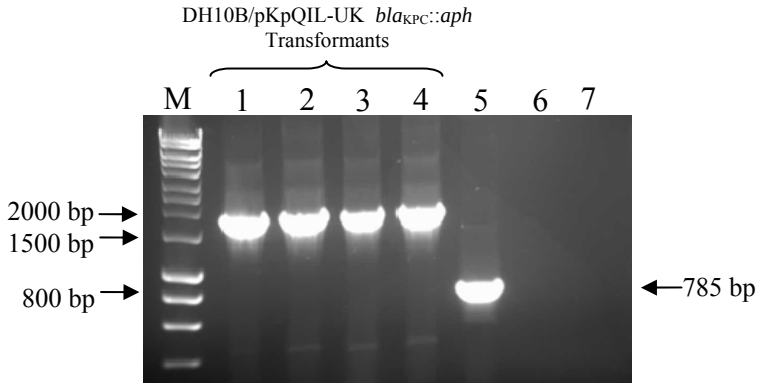
4.7 Pairwise Competition of *K. pneumoniae* Ecl8 carrying pKpQIL-UK and -D2

It was hypothesised that the *K. pneumoniae* carrying pKpQIL-D2 has a competitive advantage against pKpQIL-UK in a bacterial population. Hence, a pairwise competition assay was carried out to study the percentage of bacteria carrying the plasmids within a bacterial population over a period of 20 days. The pKpQIL-UK plasmid has been typically associated with *K. pneumoniae* ST258 (Chen et al., 2014a, Leavitt et al., 2010a). Hence, the competition assay was carried out with *K. pneumoniae*. In order to differentiate the two plasmids pKpQIL-UK and -D2, the bla_{KPC} gene was replaced with the kanamycin resistance gene, *aph*. The inactivated bla_{KPC} was verified by PCR (Figure 4.10). Although ST258 is the ideal host to use for the competition experiment, the ST258 strain obtained from USA was kanamycin resistant. The strain was also resistant to chloramphenicol which the resistant marker is commonly used for gene inactivation. Hence, *K. pneumoniae* Ecl8 was used. The four plasmids (pKpQIL-UK, pKpQIL-UK bla_{KPC}::*aph*, pKpQIL-D2 and pKpQIL-D2 bla_{KPC}::*aph*) were transferred into *K. pneumoniae* $Ecl8^{Rif}$ by filter conjugation. The transconjugants were verified by PCR (Figure 4.11).

In order to exclude the possibility of the kanamycin resistance gene (*aph*) affecting the outcome of the competition experiment, two experiments were carried out, i.e. pKpQIL-UK vs -D2 bla_{KPC}::*aph* and pKpQIL-UK bla_{KPC}::*aph* vs -D2. It was observed that pKpQIL-D2 out-competed pKpQIL-UK over the 20-day period (Figure 4.12a & b). For the pKpQIL-UK bla_{KPC}::*aph* vs -D2 competition, pKpQIL-UK was almost completely loss from the population at the 20^{th} day of the experiment. However, the trend in the loss of the pKpQIL-UK vs -D2 bla_{KPC}::*aph* competition was different as the population of *K. pneumoniae* carrying pKpQIL-UK dropped to ca. 15% on Day-5 and maintained throughout the duration of the experiment. Hence, the percentage of the population carrying the plasmids (pKpQIL-UK vs -D2

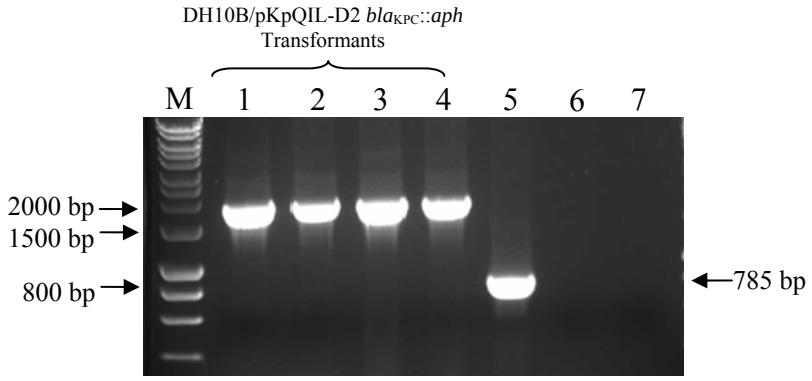
Figure 4.10 PCR verification of the *E. coli* DH10B transformants for the presence of *bla*$_{KPC}$ inactivated pKpQIL-UK and -D2

(a) PCR check for the presence of *bla*$_{KPC}$



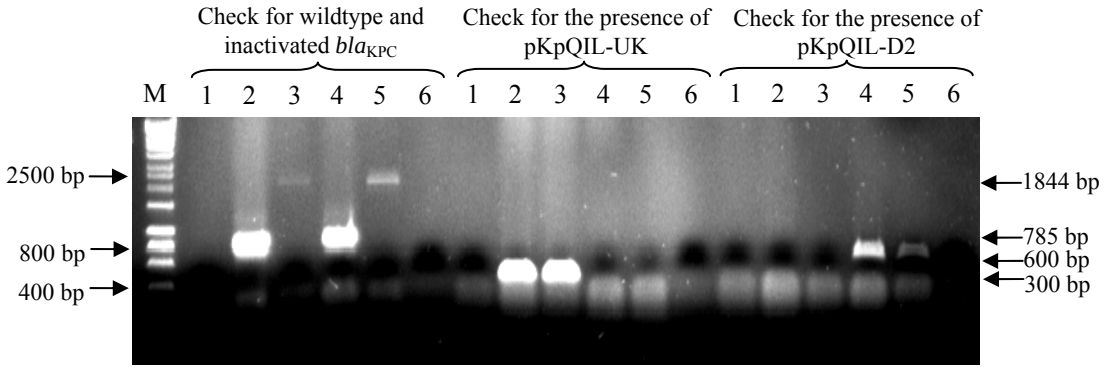
The plasmids pKpQIL-UK which the *bla*$_{KPC}$ gene had been inactivated was electroporated into *E. coli* DH10B. M: Hyperladder I (Bioline); Lane 5: pKpQIL-UK plasmid (Positive control); Lane 6: DH10B genomic DNA (Negative control); Lane 7: Water (Contamination control); Primers: KPCg-colpcrF/R; Expected amplicon: 785 bp (WT); 1844 bp (+*aph*).

(b) PCR check for the presence of pKpQIL-D2



The plasmids pKpQIL-D2 which the *bla*$_{KPC}$ gene had been inactivated was electroporated into *E. coli* DH10B. M: Hyperladder I (Bioline); Lane 5: pKpQIL-D2 plasmid (Positive control); Lane 6: DH10B genomic DNA (Negative control); Lane 7: Water (Contamination control); Primers: KPCg-colpcrF/R; Expected amplicon: 785 bp (WT); 1844 bp (+*aph*).

Figure 4.11 PCR verification of the *K. pneumoniae* Ecl8 transconjugants for the presence of *bla*$_{KPC}$ inactivated pKpQIL-UK and -D2



The rifampicin-resistant *K. pneumoniae* Ecl8 transconjugants carrying the wildtype and *bla*$_{KPC}$-inactivated pKpQIL-UK & -D2 plasmids were verified by PCR for the presence of the inactivated *bla*$_{KPC}$ and respective plasmid backbone genes.

Key to lanes
M: Hyperladder I (Bioline)

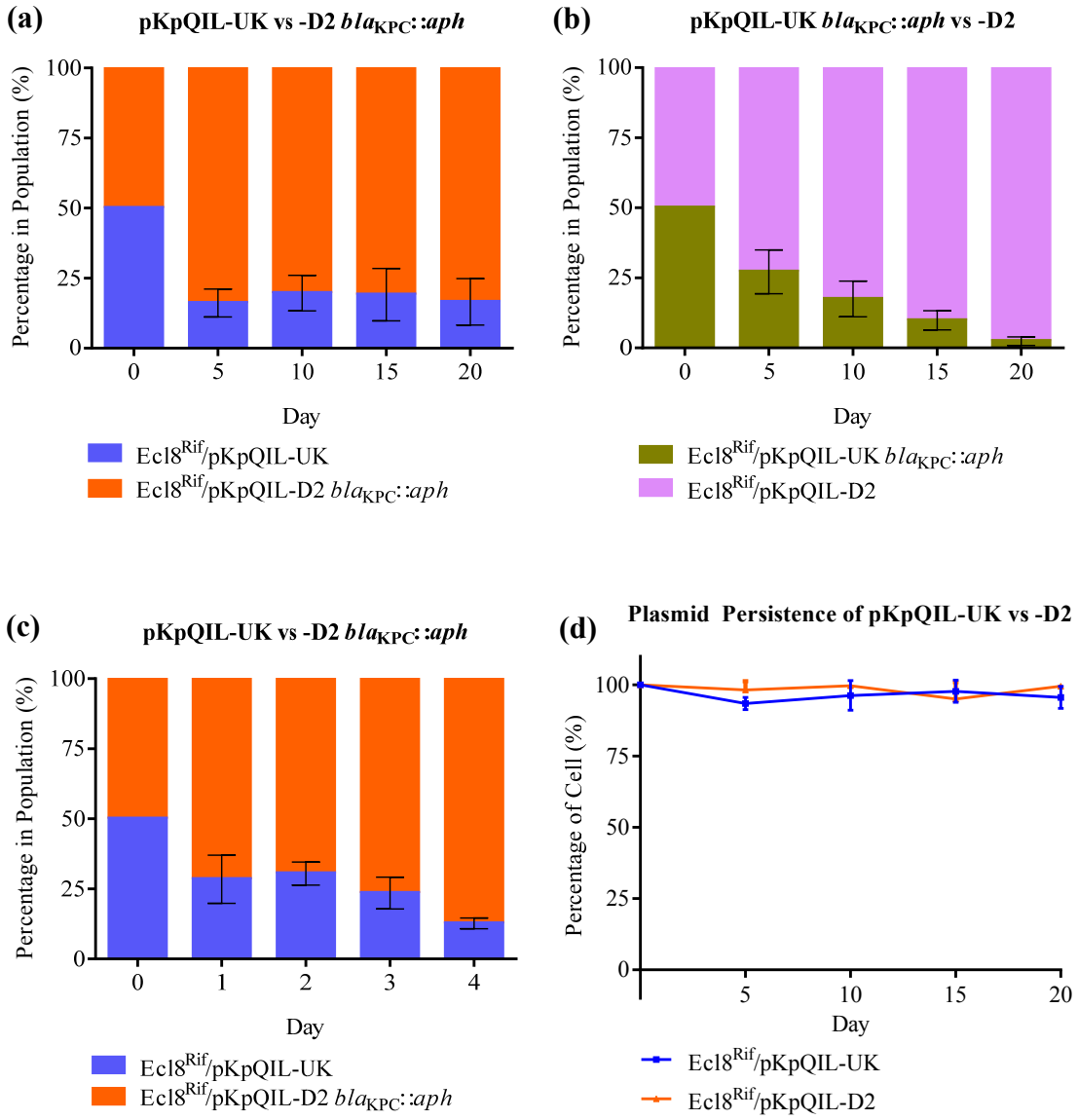
Lane 1: *K. pneumoniae* Ecl8^{Rif}
Lane 2: Ecl8^{Rif}/pKpQIL-UK
Lane 3: Ecl8^{Rif}/pKpQIL-UK *bla*$_{KPC}$::*aph*
Lane 4: Ecl8^{Rif}/pKpQIL-D2
Lane 5: Ecl8^{Rif}/pKpQIL-D2 *bla*$_{KPC}$::*aph*
Lane 6: Water (Contamination control)

Check for wildtype and inactivated *bla*$_{KPC}$
Primers: KPCg-colpcrF/R
Expected amplicon: 785 bp (WT); 1844 bp (+*aph*)

Check for the presence of pKpQIL-UK
Primers: pQIL-F/R
Expected amplicon: 300 bp

Check for the presence of pKpQIL-D2
Primers: pMan-F/R
Expected amplicon: 600 bp

Figure 4.12 Pairwise competition of pKpQIL-UK vs -D2



Pairwise competition was done to study the competition of rifampicin-resistant *K. pneumoniae* Ecl8 carrying (a) pKpQIL-UK vs pKpQIL-D2 marked with an *aph* gene, and (b) pKpQIL-UK marked with an *aph* gene vs pKpQIL-D2. This was done to assess whether the plasmids conferred a competitive advantage to their hosts relative to the other. Percentage of plasmid carrying bacteria was recorded as mean ± standard deviation of three independent experiments. Competition index at the end of the experiment (a) was 0.62, (b) was 0.96. The positive values of the competition index indicated pKpQIL-D2 was fitter than pKpQIL-UK in the experiment. (c) The graph shows that pKpQIL-UK was rapidly displaced from the population by pKpQIL-D2 marked with an *aph* gene within the first five days of the competition. (d) Both plasmids were found to be stable within their host in LB broth without antibiotic selection over a period of 20 days.

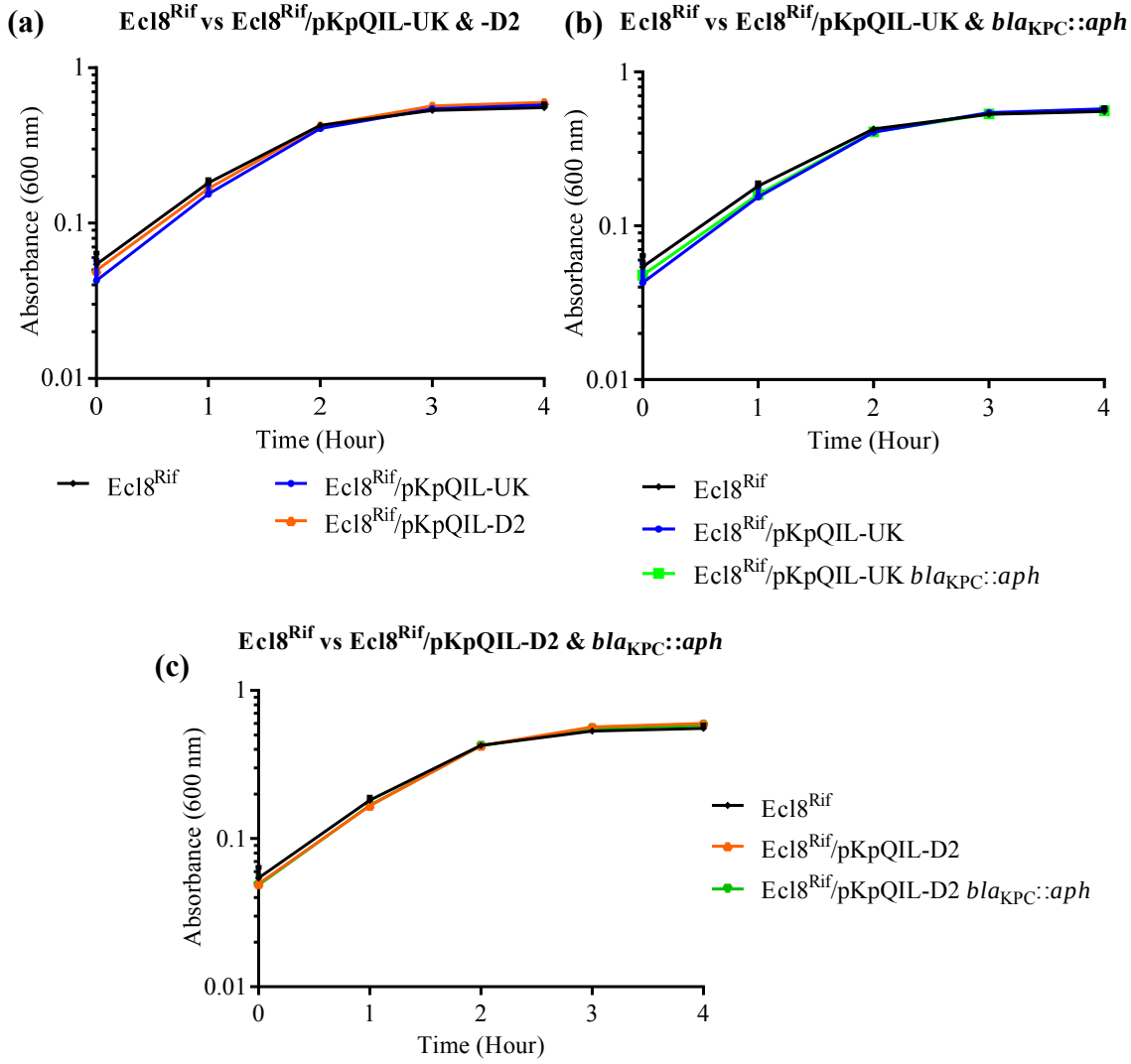
bla$_{KPC}$::*aph*) was determined from the first to the fourth day of the experiment to determine whether the initial input inoculum was equal. It was observed that for the pKpQIL-UK vs -D2 *bla*$_{KPC}$::*aph* competition, there was a rapid loss of pKpQIL-UK from Day 1 (Figure 4.12c) and the percentage of pKpQIL-UK in the population maintained at ca. 15% after Day 4. To assess whether pKpQIL-UK was stable in the *K. pneumoniae* Ecl8 strain, plasmid persistence assay without antibiotic selection was carried out for the Ecl8 strains carrying the plasmids. Unlike *E. coli* and *Salmonella*, both plasmids were able to persist without detectable loss in *K. pneumoniae* Ecl8 over a period of 20 days without antibiotic selection (Figure 4.12d).

It was hypothesised that the pKpQIL-UK plasmid had a slower generation time than its variant plasmid. Hence the generation time for the *K. pneumoniae* Ecl8 carrying the plasmids were determined (Figure 4.13, Table 4.3). There was no difference in generation times of *K. pneumoniae* Ecl8 carrying the various plasmids. The kanamycin resistance gene (*aph*) also had no detectable impact upon the growth rate of the plasmids.

4.8 Conjugation Frequencies of the Plasmids into Various Enterobacteriaceae

In addition to *K. pneumoniae*, the plasmid pKpQIL-D2 was also found in *E. cloacae* and *E. coli* in the UK outbreaks, whereas pKpQIL-UK was generally found in *K. pneumoniae* (Woodford, N. Unpublished data). Hence, it was hypothesised that pKpQIL-D2 possessed a higher transfer rate from its *K. pneumoniae* ST321 host compared to the *K. pneumoniae* ST468 carrying pKpQIL-UK. The transfer potential of the plasmids from their original clinical isolate host strains into four Enterobacteriaceae species was assessed using filter conjugation. pKpQIL-UK showed an 18-fold and 30-fold higher conjugation frequency into *K. pneumoniae* ST258 and *E. coli*, respectively (Figure 4.14a & b, Table 4.4). Both plasmids transferred at similar rates into *S.* Typhimurium (14028s and SL1344) (Figure 4.14c & d) and

Figure 4.13 Growth rates of rifampicin-resistant *K. pneumoniae* Ecl8 carrying pKpQIL-UK & -D2 in the presence & absence of bla_{KPC}



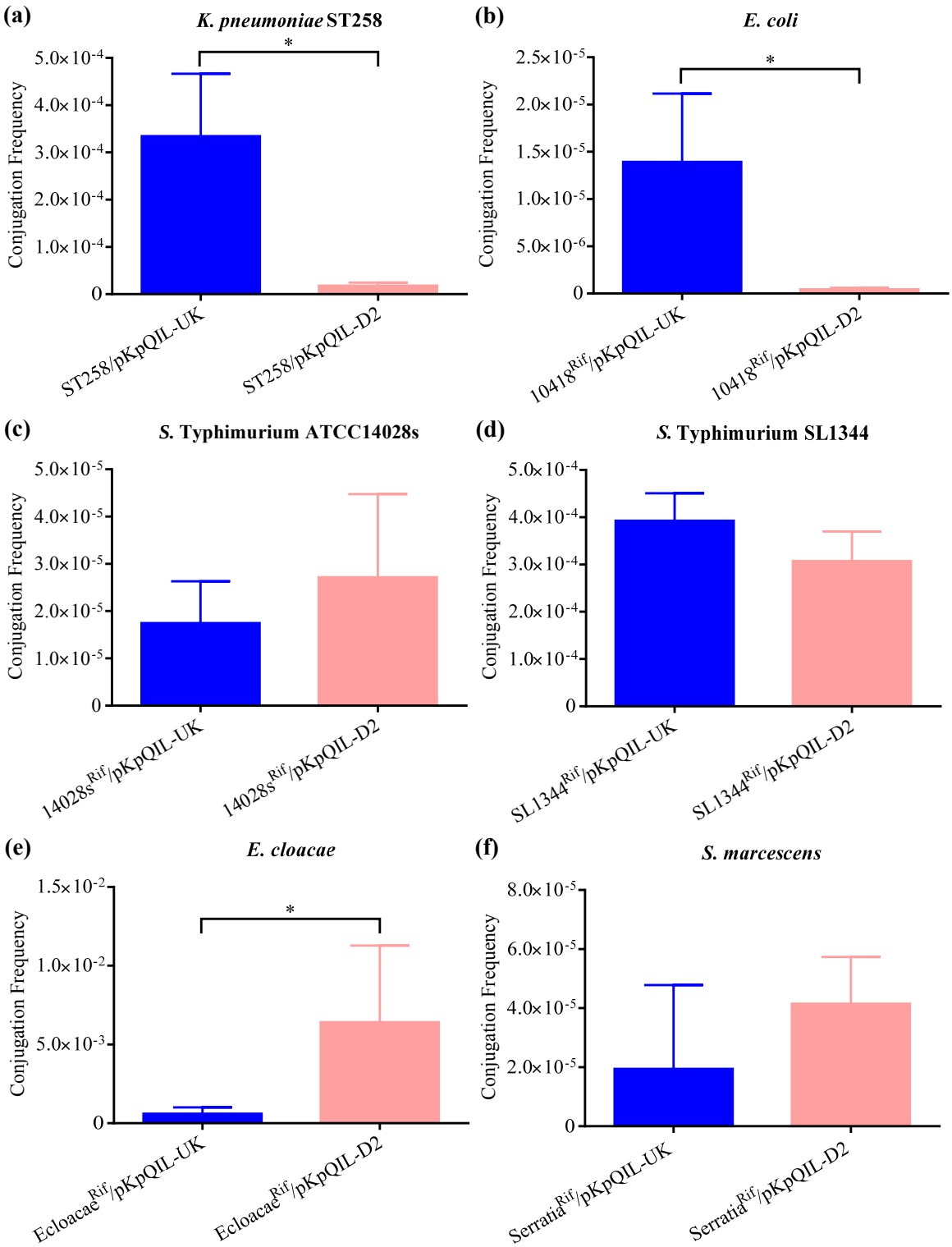
Growth kinetics for the various plasmid-carrying strains were determined to study the fitness impact of the plasmids and its bla_{KPC} genes on their hosts. Generation times were recorded as mean ± standard deviation of three independent experiments. No difference in growth was observed between the *K. pneumoniae* Ecl8 carrying the wildtype plasmids and its corresponding bla_{KPC}-inactivated plasmids.

Table 4.3 Generation times of rifampicin-resistant *K. pneumoniae* Ecl8 carrying pKpQIL-UK & -D2 in the presence & absence of *bla*$_{KPC}$

Strain	Generation Time (min)	Student's *t*-test
K. pneumoniae Ecl8^{Rif}	33.2 ± 3.6	-
Ecl8^{Rif}/pKpQIL-UK	31.8 ± 5.7	0.54
Ecl8^{Rif}/pKpQIL-UK *bla*$_{KPC}$::*aph*	33.2 ± 3.1	0.99
Ecl8^{Rif}/pKpQIL-D2	33.9 ± 5.6	0.76
Ecl8^{Rif}/pKpQIL-D2 *bla*$_{KPC}$::*aph*	33.5 ± 3.2	0.84

The generation time of each strain was used as a measure for the fitness impact of the plasmids in their hosts. Generation times were recorded as mean ± standard deviation of three independent experiments. Compared to the *K. pneumoniae* Ecl8^{Rif}, no difference was observed in the generation time of the various plasmid carrying strains.

Figure 4.14 Conjugation frequencies of pKpQIL-UK and -D2 from their respective clinical isolates into Enterobacteriaceae species



Conjugation frequencies were determined by filter mating to assess the differences in the transfer of the plasmids from their respective clinical isolates into different Enterobacteriaceae species. The values were recorded as mean ± standard deviation of three independent experiments. Significant difference ($p<0.05$) was determined by Student's *t*-test. The values were recorded in Table 4.4.

Table 4.4 Conjugation frequencies of pKpQIL-UK and -D2 into various Enterobacteriaceae

Strain	Conjugation Frequency (per donor cell)		Fold Difference
	pKpQIL-UK	pKpQIL-D2	
K. pneumoniae ST258	3.3×10^{-4}	1.8×10^{-5}	**0.05***
E. coli NCTC10418	1.4×10^{-5}	4.5×10^{-7}	**0.03***
S. Typhimurium ATCC14028s	1.7×10^{-5}	2.7×10^{-5}	1.6
S. Typhimurium SL1344	3.9×10^{-4}	3.1×10^{-4}	0.8
E. cloacae NCTC10005	6.0×10^{-4}	6.4×10^{-3}	**11***
S. marcescens NCTC10211	1.9×10^{-5}	1.4×10^{-4}	7

Filter mating was used to determine whether the substituted region in pKpQIL-D2 conferred the plasmid with higher conjugation frequency from its original host into the various species. Asterisk (*) denotes significant difference ($p<0.05$) by Student's *t*-test. Apart from *K. pneumoniae* ST258 which was kanamycin-resistant, all strains used were rifampicin-resistant. Fold difference was calculated as the ratio of conjugation frequency of pKpQIL-D2 relative to -UK.

S. marcescens (Figure 4.14f). In *E. cloacae*, pKpQIL-D2 transferred at 11 times higher rate from its original host (*K. pneumoniae* ST321) than did pKpQIL-UK (Figure 4.14e).

4.9 Biofilm Formation

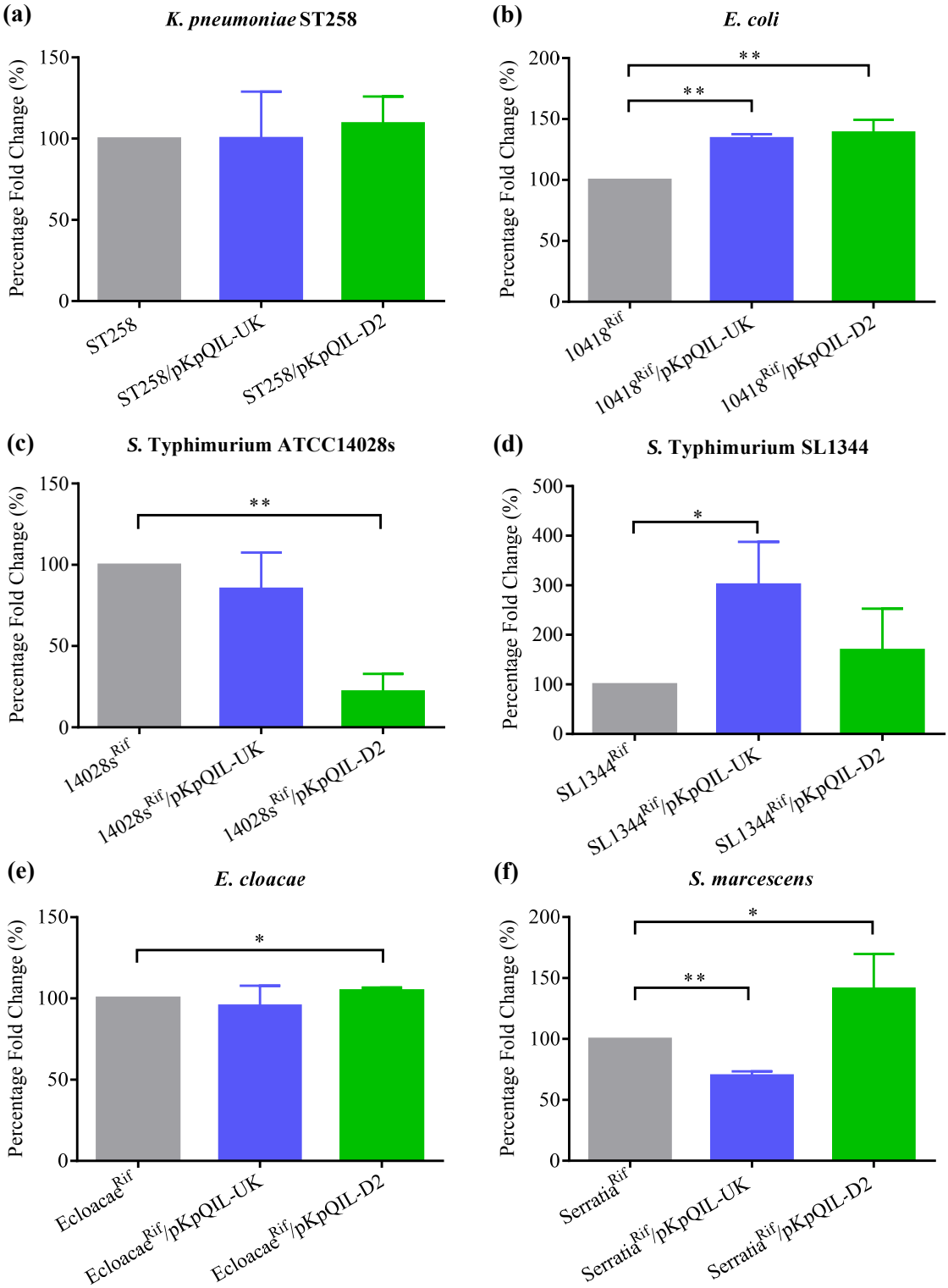
4.9.1 Biofilm Formation on Plastic

The fitness of the plasmid carrying strains was also assessed by measuring the ability of the strains to form a biofilm in comparison to the plasmid-free parental strain. No difference in biofilm formation was observed between *K. pneumoniae* ST258 carrying either plasmid (Figure 4.15a). Both plasmids increased biofilm formation in *E. coli* NCTC10418 ($p<0.001$) (Figure 4.15b). In *S.* Typhimurium ATCC14028s, the carriage of pKpQIL-D2 was associated with significantly ($p<0.001$) lower biofilm formation (Figure 4.15c). However, pKpQIL-UK significantly increase the biofilm formation in *S.* Typhimurium SL1344 (Figure 4.15d) but the variant plasmid had no detectable impact on biofilm formation. In *E. cloacae*, the variant plasmid was found to increase the biofilm formation significantly ($p<0.05$) (Figure 4.15e). However, in *S. marcescens* the opposite was observed: pKpQIL-UK reduced biofilm production whereas pKpQIL-D2 increased production significantly (Figure 4.15f).

4.9.2 Biofilm Formation under Constant Flow of Medium

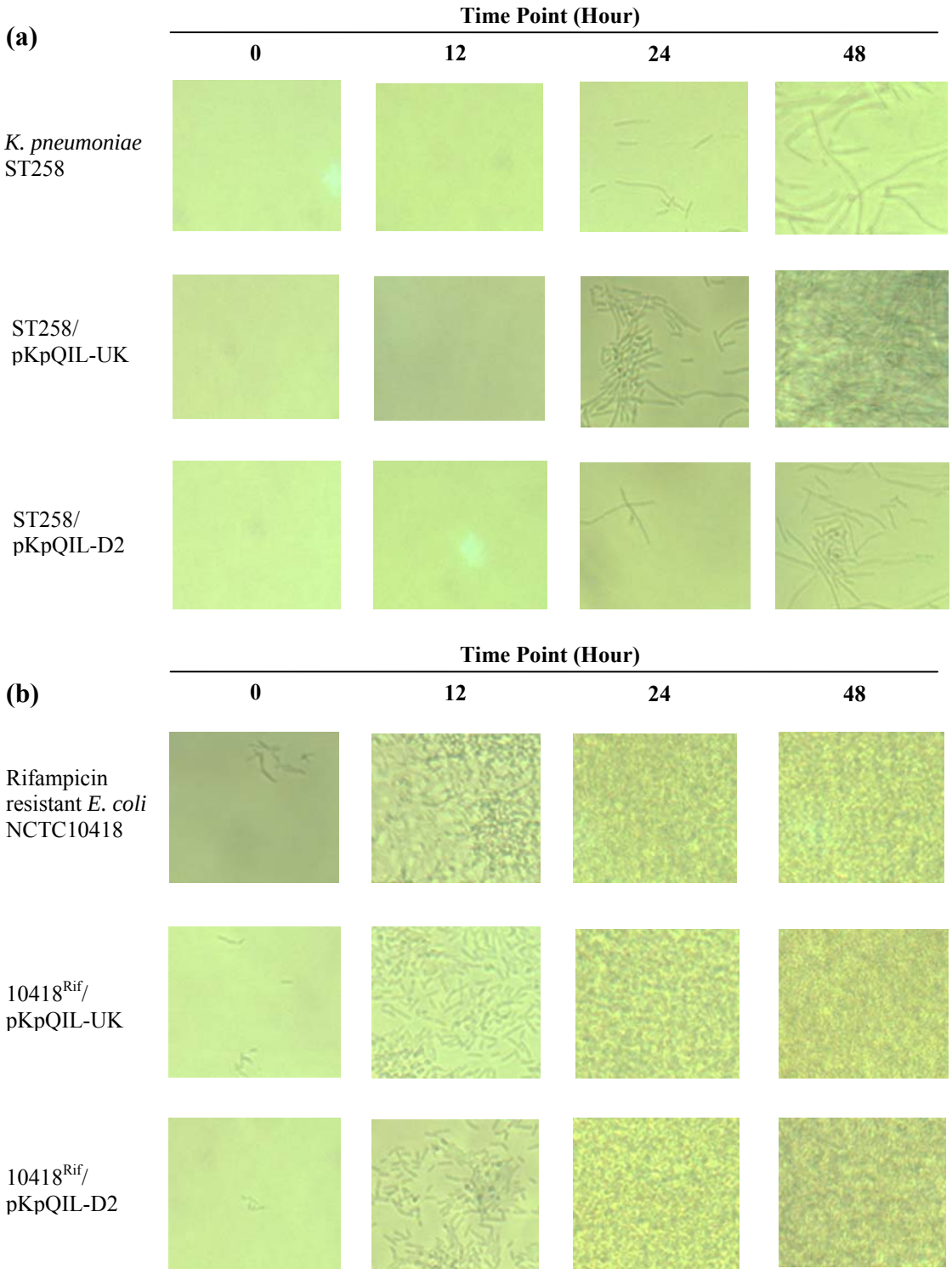
The formation of a biofilm was also monitored under constant flow of LB broth (without salt) at various time points over a period of 48 hours using the BioFlux^{TM} microfluidic channel system. LB broth without salt was used as it has been reported that biofilm formation is inhibited in the presence of salt (Zalewska-Piątek et al., 2013). *S.* Typhimurium SL1344 carrying the plasmids was omitted from this study as SL1344 is weak at forming biofilm (García et al., 2004). Compared to the parental strain and *K. pneumoniae* ST258 carrying pKpQIL-D2, the carriage of pKpQIL-UK increased biofilm formation after 48 hours in this system (Figure 4.16a). The *E. coli*, *E. cloacae* and *S. marcescens* strains formed biofilm

Figure 4.15 Biofilm formation on plastic for the various plasmid carrying Enterobacteriaceae strain



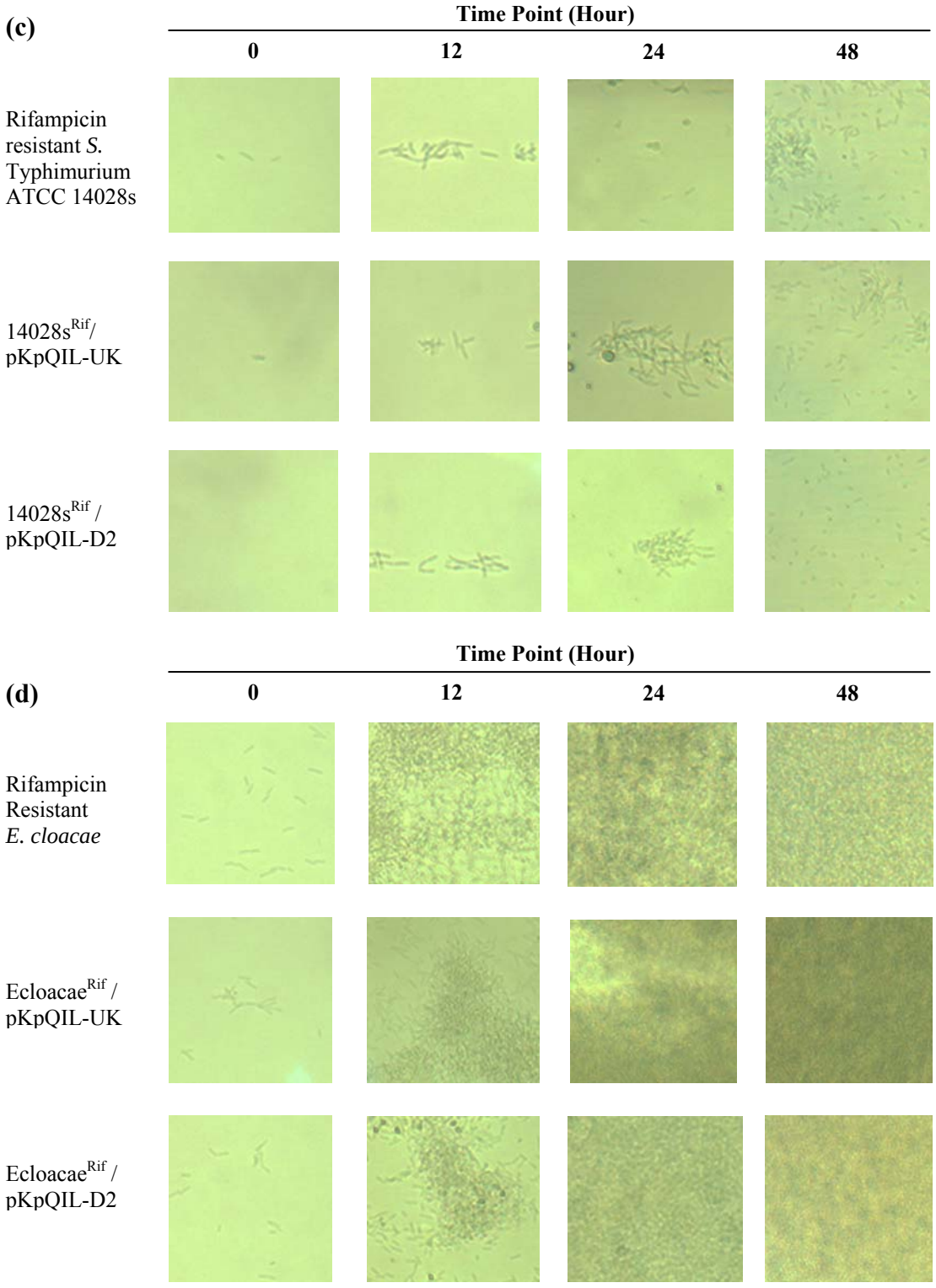
The impact of the plasmids on its hosts' ability to form biofilm was investigated using the microtitre tray and crystal violet dye method. Percentage fold change was recorded as mean ± standard deviation of three independent experiments. Student's *t*-test was used to analyse significant changes and shown with asterisk (*) $p<0.05$ and (**) $p<0.001$. Y-axis was not drawn to similar scale due to the large differences in fold change between species.

Figure 4.16 Images of biofilm formation in microfluidic channel at various time points



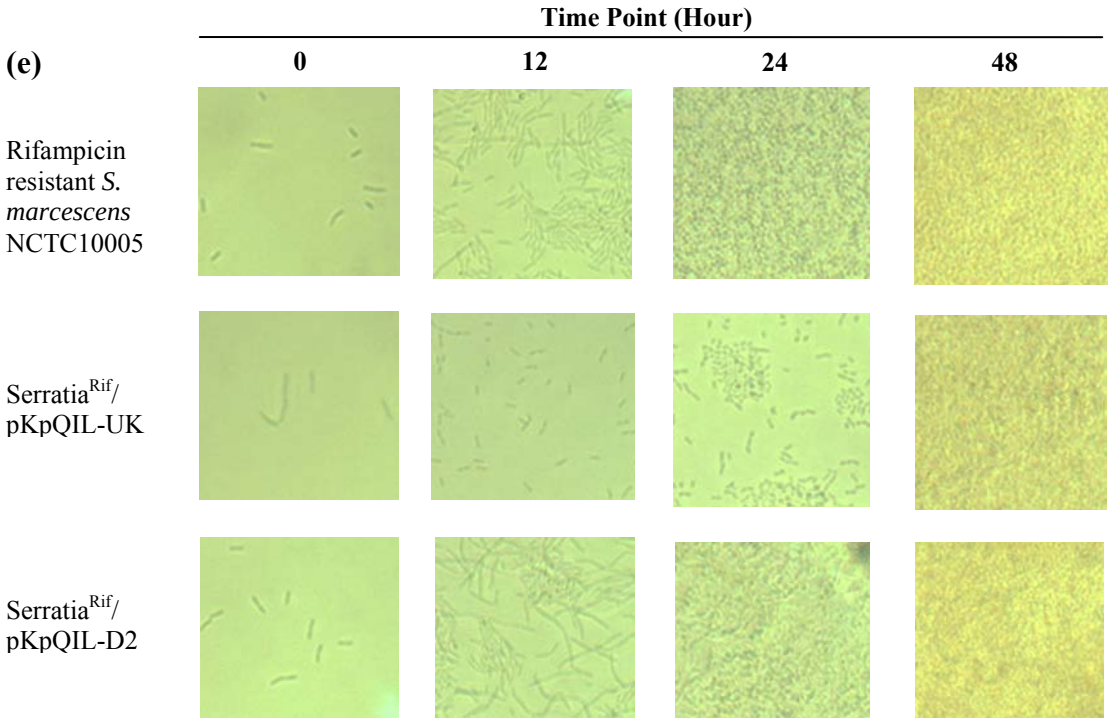
Images were taken at a magnification of 400X.

Figure 4.16 Images of biofilm formation in microfluidic channel at various time points (Continued)



Images were taken at a magnification of 400X.

Figure 4.16 Images of biofilm formation in microfluidic channel at various time points (Continued)



Images were taken at a magnification of 400X.

The ability of the plasmid-carrying hosts to form biofilm was determined under constant flow of LB broth (without salt) using the BioFlux microfluidic system. Images of the biofilm formed were captured at both ends and the middle of the channel. Representative images are shown in this figure.

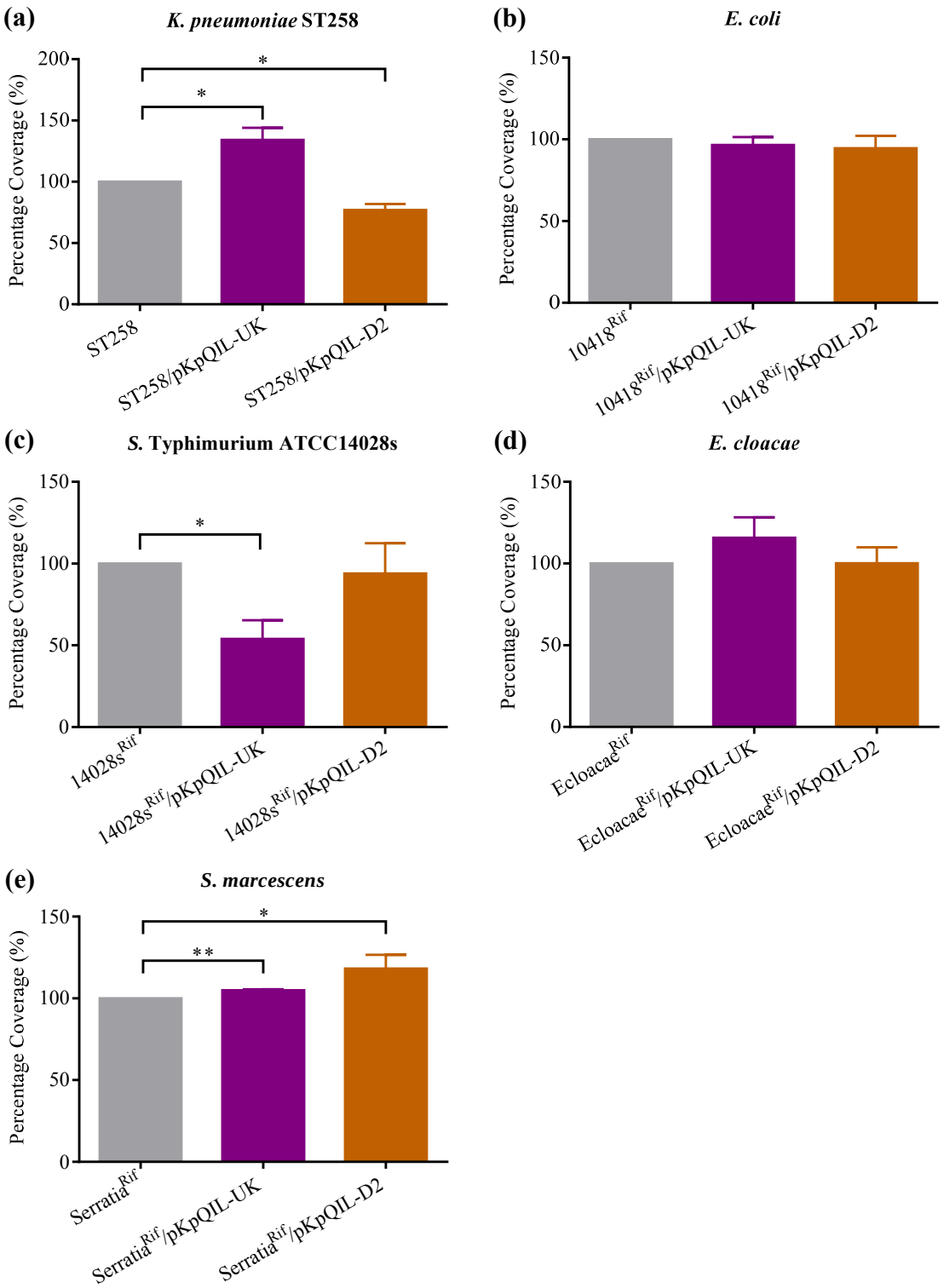
equally well in the microfluidic channel after 48 hours (Figure 4.16b, d & e). Both *E. coli* and *E. cloacae* (with and without plasmids) started forming microcolonies as early as the 12 hours after the attachment of the cells in the microfluidic channel (Figure 4.16b and d). Relative to *E. cloacae* with and without pKpQIL-D2, carriage of pKpQIL-UK conferred the production of a denser biofilm, filling the entire flow channel after 48 hours (4.16d). However, *S. marcescens* carrying pKpQIL-UK formed biofilm slower than the plasmid-free parental strain or when carrying pKpQIL-D2 after 24 hours (Figure 4.16e).

The area in the microfluidic channel which was covered by the plasmid carrying strains relative to the parental strain with no plasmid was also determined (Figure 4.17). The plasmids had different impact on the biofilm forming ability of the *K. pneumoniae* ST258 in the BioFlux system (Figure 4.17a). Plasmid pKpQIL-UK was associated with a higher area of coverage relative to the plasmid-free strain while *K. pneumoniae* with pKpQIL-D2 had lower coverage. Relative to plasmid-free strain, no significant difference was observed for the *E. coli* and *E. cloacae* carrying both pKpQIL-UK and -D2 (Figure 4.17b and d). *S.* Typhimurium ATCC14028s carrying the pKpQIL-UK plasmid had significantly lower coverage compared to the plasmid-free strain and the pKpQIL-D2 counterpart (Figure 4.17c). In *S. marcescens*, both plasmid carrying strains showed significantly higher coverage compared to the plasmid-free strain (Figure 4.17e).

4.10 Infection of *Galleria mellonella* by Plasmid Carrying *K. pneumoniae* ST258

Another aspect of fitness that was investigated in this PhD was the virulence of the plasmid-carrying hosts in an infection model. It was hypothesised that the substituted region in pKpQIL-D2 conferred a difference in virulence of the plasmid carrying *K. pneumoniae* compared to the pKpQIL-UK carrying strain in the *G. mellonella* model. *K. pneumoniae* ST258 was used as the host of the plasmids in the virulence experiment as the plasmids were

Figure 4.17 Percentage area of coverage by biofilm in microfluidic channel after 48 hours under constant flow of LB broth (without salt)



The coverage of biofilm formed on the surface of the microfluidic channel was estimated using ImageJ software. The percentage coverage relative to the parental strain was recorded as mean ± standard deviation of three independent experiments. Student's *t*-test was used to analyse significant changes which are shown with asterisk (*) $p<0.05$ and (**) $p<0.001$.

originally found in this species. No difference was observed between the two populations of *G. mellonella* infected by either of the plasmid carrying *K. pneumoniae* ST258 (Figure 4.18).

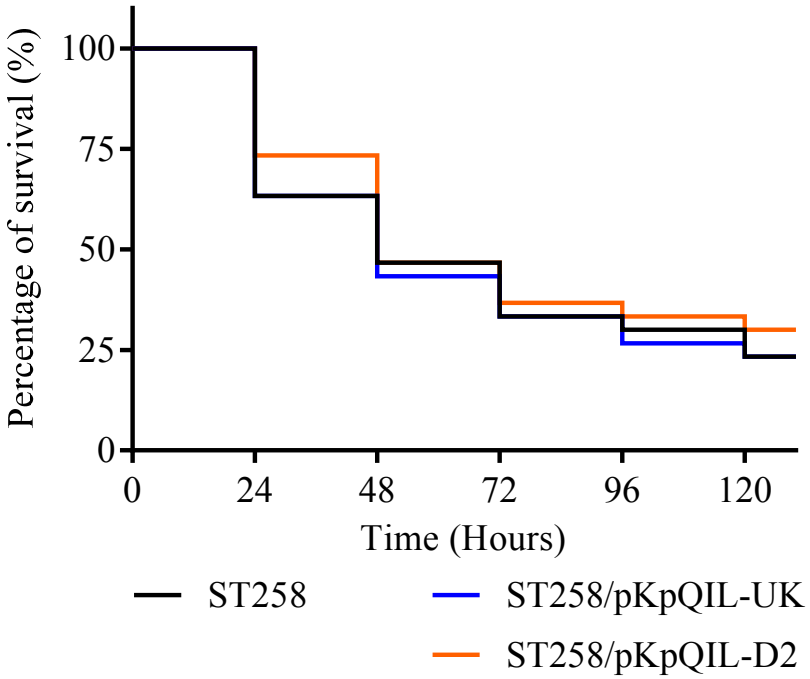
4.11 Minimum Inhibitory Concentration of Antibiotics for Various Plasmid-bearing Hosts

It was hypothesised that the pKpQIL-D2 conferred higher antibiotic resistance in its hosts than pKpQIL-UK and this allow it to persist in other Enterobacteriaceae and frequently occur in the hospital outbreaks in the UK. Hence, susceptibility of the strains to various β-lactam antibiotics, ciprofloxacin and tigecycline was determined. Generally, there was no difference in the MIC values of the different species of Enterobacteriaceae carrying the plasmids (Table 4.5). However, *S. marcescens* carrying pKpQIL-UK was more resistant to carbapenem antibiotics than pKpQIL-D2. Interestingly, there was no difference in the susceptibility of these two strains for the cephalosporins tested. The reverse was observed for *K. pneumoniae* Ecl8 carrying pKpQIL-UK, which was more resistant to cephalosporins than pKpQIL-D2 but not to carbapenem antibiotics.

Based upon this susceptibility data, it was hypothesised that the differences observed in *S. marcescens* and *K. pneumoniae* Ecl8 were due to differences in porin expression. However, no difference was observed in the porin profile of the parental strain and the plasmid-bearing strains (Figure 4.19, Table 4.6).

It was hypothesised that the *K. pneumoniae* ST258 carrying pKpQIL-D2 would grow better than pKpQIL-UK in the presence of a carbapenem. To test this, the strains were grown in LB medium supplemented with 16 μg/ml doripenem (Figure 4.20a). The strains were also challenged with 16 μg/ml doripenem after they have reached OD_{600}~0.6 (Figure 4.20b). In both cases, there was no difference in the growth between the two plasmid carrying strains.

Figure 4.18 Virulence assay of *Galleria mellonella* infected by pKpQIL-UK and -D2 carrying *K. pneumoniae* ST258



Percentage of *G. mellonella* which survived after infection was recorded as mean ± standard deviation of three independent experiments at a 24-hour interval for five days. Each experiment consisted of a group of 10 *Galleria* larvae. Log-rank (Mantel-Cox) test was used to analyse the differences in survival of the two populations infected with plasmid carrying *K. pneumoniae* ST258 compared to the wildtype host. No difference was observed ($p = 0.79$) among the populations.

Table 4.5 Minimum inhibitory concentration of pKpQIL-UK and -D2 carrying hosts

Strains	ETP	IMI	MER	DOR	BIA	FAR	CAZ	CTX	CFX	PIP	CIP	ATM	TIG
***K. pneumoniae* ST258**													
ST258	0.12	0.12	0.03	0.03	0.12	2	256	16	64	512	>16	512	0.5
ST258/UK	32	16	16	8	16	256	512	32	1024	1024	>16	1024	1
ST258/D2	32	8	8	4	8	256	256	32	1024	1024	>16	1024	0.5
***K. pneumoniae* Ecl8**													
Ecl8	0.015	0.12	0.015	0.03	0.03	0.12	0.03	0.008	0.12	0.5	0.008	0.008	0.12
Ecl8^{Rif}	0.008	0.12	0.015	0.03	0.03	0.12	0.03	0.008	0.12	1	0.008	0.008	0.12
Ecl8^{Rif}/UK	4	2	2	2	4	64	**16**	**4**	**256**	256	**0.03**	**64**	0.12
Ecl8^{Rif}/D2	2	4	1	2	4	32	0.25	0.5	64	256	0.008	0.5	0.12
***E.coli* NCTC10418**													
10418	0.015	0.12	0.015	0.03	0.03	1	0.12	0.03	2	2	0.008	0.03	0.12
10418^{Rif}	0.015	0.25	0.03	0.06	0.06	1	0.12	0.03	2	2	0.015	0.03	0.12
10418^{Rif}/UK	0.5	4	0.5	1	2	64	1	1	128	128	0.015	8	0.12
10418^{Rif}/D2	0.5	4	0.5	1	2	64	1	1	64	128	0.015	8	0.12
***E. coli* DH10B**													
DH10B	0.008	0.12	0.015	0.03	0.03	1	0.25	0.03	4	2	0.002	0.06	0.12
DH10B/UK	0.25	1	0.12	0.5	0.5	64	4	1	128	64	0.002	16	0.12
DH10B/D2	0.25	1	0.12	0.5	0.5	64	4	1	128	64	0.002	16	0.12

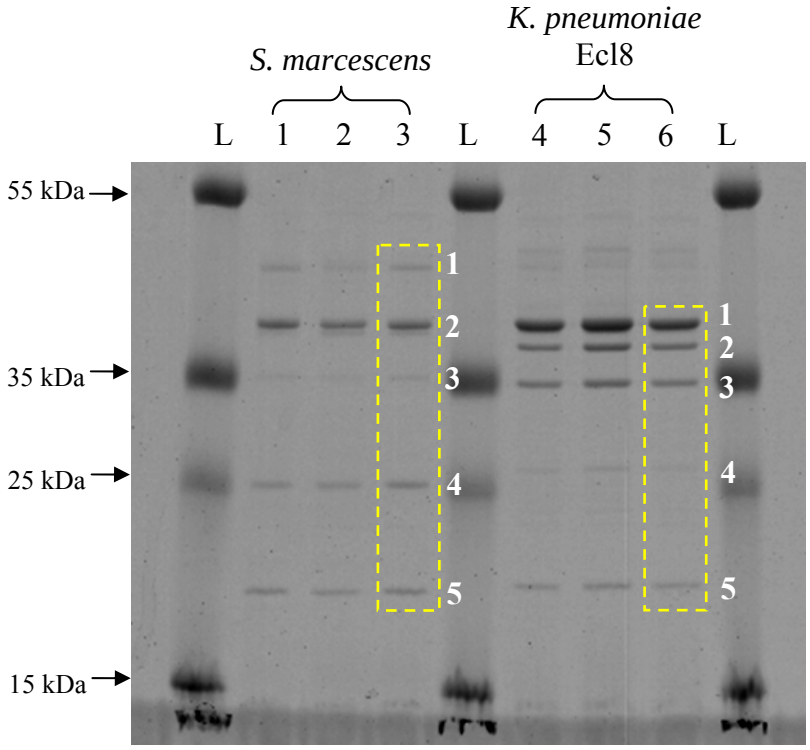
Continued overleaf.

Table 4.5 (Continued) **Minimum inhibitory concentration of pKpQIL-UK and -D2 carrying hosts**

Strains	ETP	IMI	MER	DOR	BIA	FAR	CAZ	CTX	CFX	PIP	CIP	ATM	TIG
***S.* Typhimurium SL1344**													
SL1344	0.015	0.12	0.015	0.03	0.06	0.5	0.25	0.12	8	4	0.015	0.06	0.25
SL1344^{Rif}	0.008	0.12	0.015	0.03	0.03	0.25	0.25	0.25	16	4	0.03	0.03	0.25
SL1344^{Rif}/UK	1	2	0.5	1	4	64	16	16	512	256	0.03	16	0.25
SL1344^{Rif}/D2	0.5	2	0.25	0.5	2	32	16	16	512	256	0.03	16	0.25
***S.* Typhimurium ATCC14028s**													
14028s	0.015	0.12	0.015	0.03	0.06	0.5	0.25	0.12	4	2	0.015	0.06	0.25
14028s^{Rif}	0.008	0.12	0.015	0.03	0.03	0.25	0.25	0.12	8	2	0.015	0.03	0.25
14028s^{Rif}/UK	2	2	0.5	1	2	64	8	8	512	256	0.015	16	0.25
14028s^{Rif}/D2	1	2	0.5	1	2	64	8	8	512	256	0.015	16	0.25
***E. cloacae* NCTC10211**													
Ecloacae	0.5	0.5	0.06	0.12	0.12	4	1	2	256	2	0.008	1	0.12
Ecloacae^{Rif}	0.5	0.25	0.06	0.06	0.06	4	1	1	128	2	0.008	0.12	0.12
Ecloacae^{Rif}/UK	16	2	2	2	2	256	16	8	512	256	0.008	32	0.12
Ecloacae^{Rif}/D2	16	4	4	4	4	256	16	16	1024	512	0.008	64	0.06
***S. marcescens* NCTC10005**													
Serratia	0.03	0.5	0.03	0.12	0.25	8	0.12	0.25	64	2	0.06	0.12	1
Serratia^{Rif}	0.015	0.5	0.06	0.12	0.5	8	0.25	0.12	32	2	0.06	0.06	1
Serratia^{Rif}/UK	**32**	**16**	**32**	**64**	**64**	512	16	32	1024	256	0.06	64	1
Serratia^{Rif}/D2	8	4	4	4	8	512	16	32	1024	128	0.06	64	1

'UK' denotes pKpQIL-UK while 'D2' denotes variant plasmid pKpQIL-D2. Bolded fonts denote significant increase in MIC values. ETP: Ertapenem; IMI: Imipenem; MER: Meropenem; DOR: Doripenem; BIA: Biapenem; FAR: Faropenem; CAZ: Ceftazidime; CTX: Cefotaxime; CFX: Cefuroxime; PIP: Piperacillin; CIP: Ciprofloxacin; ATM: Aztreonam; TIG: Tigecycline.

Figure 4.19 Outer membrane profile of *S. marcescens* and *K. pneumoniae* Ecl8 carrying pKpQIL-UK and -D2



In order to determine whether the down-regulation of porins altered the MIC values of the antibiotics of the pKpQIL-UK carrying strains compared to the -D2 carrying strain. The outer membrane proteins were prepared and separated on a 10% SDS-PAGE gel. The relative abundance of the five outer membrane protein highlighted with the yellow boxes was analysed by densitometry (Table 4.6).

L: PageRuler Plus Prestained Protein Ladder (Thermo Scientific)

Lane 1: *S. marcescens* NCTC10005
Lane 2: Serratia^{Rif}/pKpQIL-UK
Lane 3: Serratia^{Rif}/pKpQIL-D2

Lane 4: *K. pneumoniae* Ecl8^{Rif}
Lane 5: Ecl8^{Rif}/pKpQIL-UK
Lane 6: Ecl8^{Rif}/pKpQIL-D2

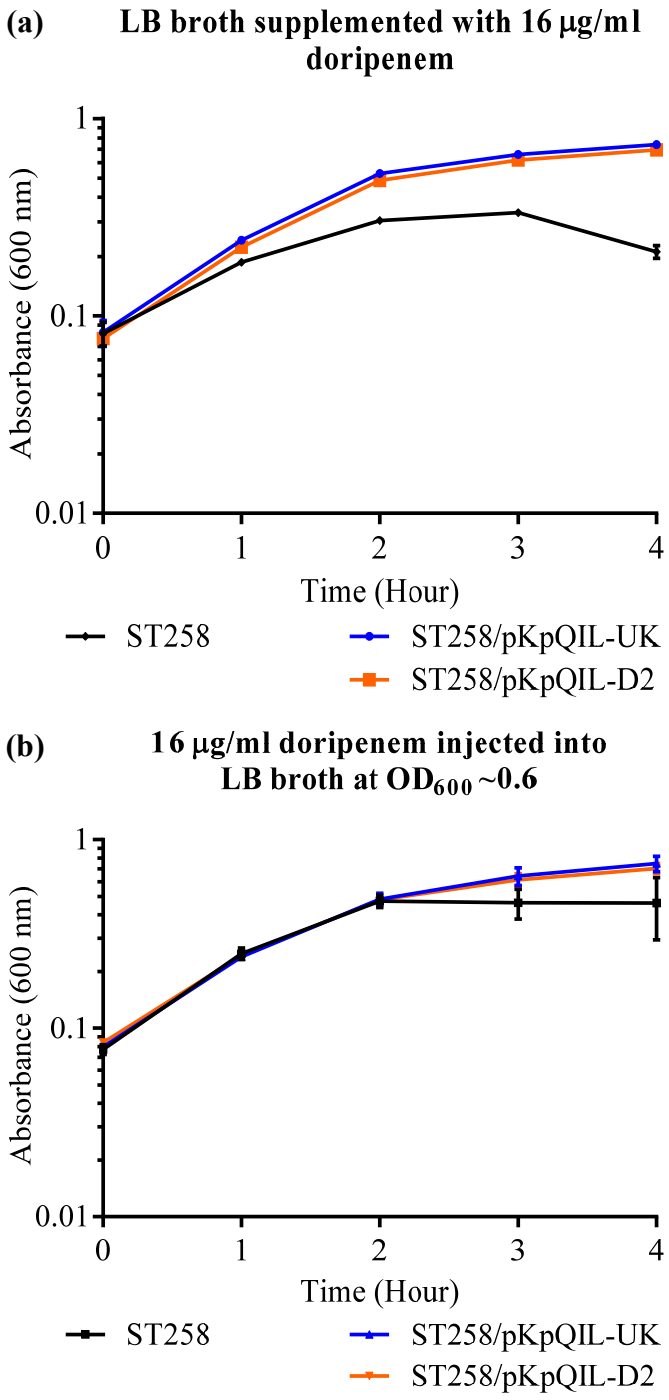
Table 4.6 Relative abundance of the major outer membrane proteins in the plasmid-carrying strains of *S. marcescens* and *K. pneumoniae*

Bands (from top to bottom)	**Lane 1** Serratia^{Rif}	**Lane 2** Serratia^{Rif}/UK	**Lane 3** Serratia^{Rif}/D2	**Lane 4** Ecl8^{Rif}	**Lane 5** Ecl8^{Rif}/UK	**Lane 6** Ecl8^{Rif}/D2
1	0.11	0.08	0.10	0.61	0.57	0.58
2	0.54	0.60	0.50	0.15	0.19	0.19
3	0.05	0.06	0.06	0.15	0.17	0.16
4	0.14	0.14	0.20	0.02	0.02	0.02
5	0.16	0.12	0.14	0.06	0.05	0.05
Total	1.00	1.00	1.00	1.00	1.00	1.00

'UK' denotes pKpQIL-UK, 'D2' denotes pKpQIL-D2

Relative abundance of the major protein bands highlighted in the yellow box was determined by densitometry.

Figure 4.20 Growth kinetics of *K. pneumoniae* ST258/pKpQIL-UK vs -D2 in the presence of doripenem



In order to determine whether the presence of pKpQIL-UK or -D2 affected the growth of its host in the presence of antibiotic, the growth kinetics of the strains were determined when the (a) LB broth was supplemented with 16 µg/ml doripenem and (b) 16 µg/ml doripenem was injected into the LB broth after the strains had achieved mid-logarithmic phase. Absorbance readings were recorded as mean ± standard deviation of three independent experiments.

4.12 Summary of Phenotypes Conferred by Carriage of pKpQIL-UK vs -D2

The spread and persistence of pKpQIL-D2 in different species of Enterobacteriaceae in the UK was hypothesised to be associated to the fitness advantage conferred by the plasmid. However, compared to the pKpQIL-UK, no obvious fitness advantage was observed in the pKpQIL-D2 carrying bacterial hosts in the various experiments (Table 4.7). In general, depending on the fitness assay used, both plasmids affected their hosts in different ways.

4.13 Gene Expression Profiling of Plasmid Carrying *K. pneumoniae* ST258 with RNA Sequencing

4.13.1 Determination of Growth Phase for RNA Extraction

Compared to the *K. pneumoniae* host carrying pKpQIL-UK, it was hypothesised that genes expressed in the pKpQIL-D2 substituted region conferred biological difference in its host as summarised in Table 4.7. In order to identify the genetic determinants conferring the differences, RNA sequencing was used to investigate the gene expression of the *K. pneumoniae* ST258 host in the presence of the pKpQIL-UK and -D2 plasmids. In order to ensure that the results from the study were reproducible, the *K. pneumoniae* ST258 and its isogenic strains carrying the plasmids (pKpQIL-UK and -D2) were grown in defined MOPS minimal medium for RNA preparation. The growth kinetics of the strains showed that the early, mid and late logarithmic phase were at an OD_{600} of 0.2, 0.4 and 0.6, respectively (Figure 4.21a, Table 4.8). Using ST258/pKpQIL-UK as a representative, the total RNA was prepared from the three growth phases to assess the expression level of the bla_{KPC} gene by qRT-PCR. Across the logarithmic phase, it was found that the carbapenemase gene was highly expressed at the early phase (OD_{600} = 0.2) compared to the other phases (Figure 4.21b).

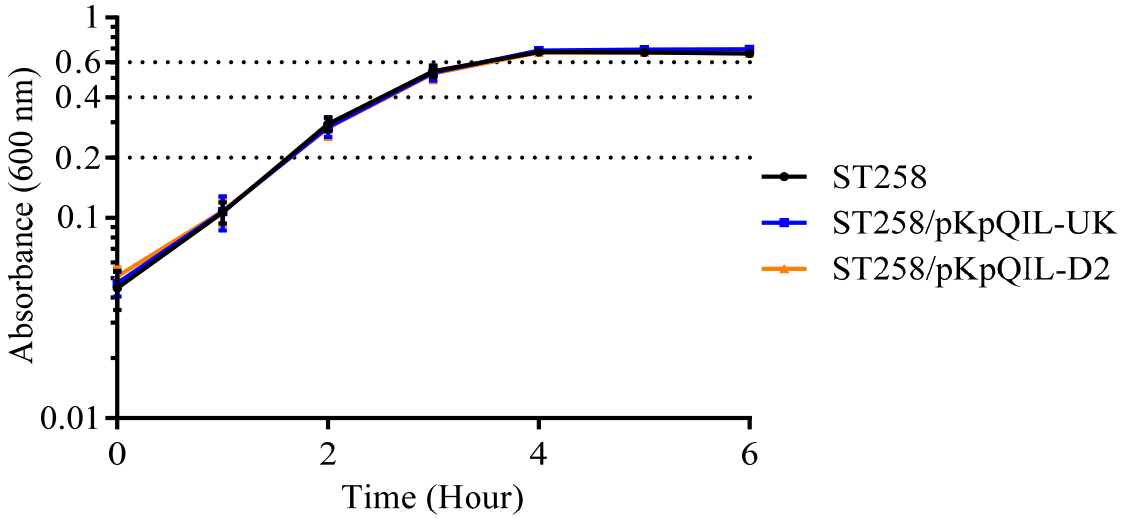
Table 4.7 Summary of the fitness experiments for the various plasmid carrying Enterobacteriaceae

Species	Code	Plasmid	Growth	Persistence	Biofilm (Plastic)	Biofilm (Flow)	Transfer[††]	MIC
K. pneumoniae ST258	ST258	pKpQIL-UK	=	=	=	↑		=
		pKpQIL-D2	=	=	=	↓	↓	=
E. coli NCTC10418	10418^{Rif}	pKpQIL-UK	=	↓	↑	=		=
		pKpQIL-D2	=	↓	↑↑	=	↓	=
S. Typhimurium ATCC14028s	$14028s^{Rif}$	pKpQIL-UK	=	=	=	↓		=
		pKpQIL-D2	↓	↓	↓	=	=	=
S. Typhimurium SL1344	$SL1344^{Rif}$	pKpQIL-UK	=	=	↑	ND		=
		pKpQIL-D2	=	↓	=	ND	=	=
E. cloacae NCTC10005	$Ecloacae^{Rif}$	pKpQIL-UK	↓	=	=	=		=
		pKpQIL-D2	=	=	↑	=	↑	=
S. marcescens NCTC10211	$Serratia^{Rif}$	pKpQIL-UK	↓	ND	↓	↑		↑ *
		pKpQIL-D2	=	ND	↑	↑	=	= *

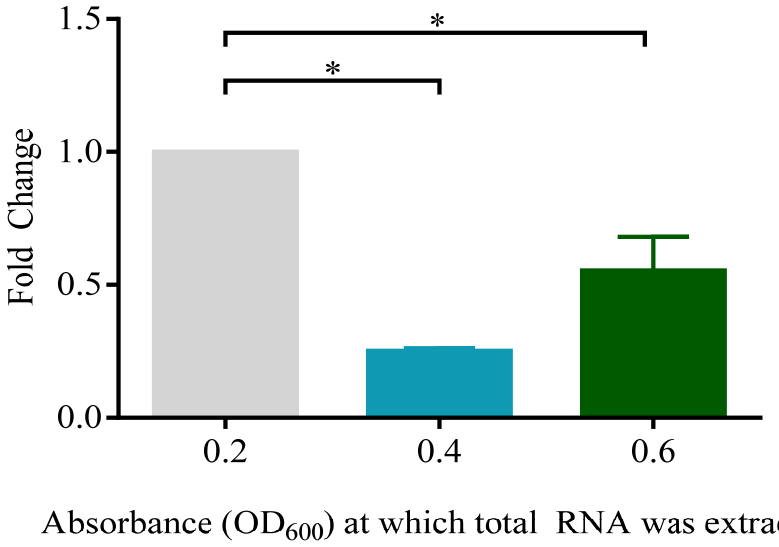
'=', no difference; '↓', significantly lower; '↑', significantly higher; ND, not determined
'††', conjugation frequency was recorded as a comparison of pKpQIL-D2 relative to -UK plasmid
'*', *S. marcescens* carrying pKpQIL-UK was more resistant to carbapenem than -D2, no difference was observed for other β-lactam antibiotics tested

Figure 4.21 Growth kinetics of *K. pneumoniae* ST258 and plasmid carrying isogenic strains in MOPS minimal medium, and bla_{KPC} expression

(a) Growth kinetics of *K. pneumoniae* ST258 and plasmid carrying ST258



(b) Fold Change of bla_{KPC} at Different Growth Phases



(a) The growth kinetics of the plasmid-carrying strains of *K. pneumoniae* ST258 were determined to estimate the approximate early-, mid- and late-logarithmic phase of the strains in MOPS minimal medium. Compared to the ST258, no difference in generation time was observed for the plasmid carrying strains. (b) The bla_{KPC} expression was determined for the various growth phases in MOPS minimal medium and results were recorded as fold change relative to the early growth phase (OD_{600} = 0.2). All values were recorded as mean ± standard deviation of three independent experiments. Student's *t*-test was used to analyse the data for significant changes which is shown with an asterisk (*) $p<0.05$.

Table 4.8 Generation times of plasmid carrying *K. pneumoniae* ST258 in MOPS minimal medium

Strain	Generation Time (min)	Student's *t*-test
ST258	41.4 ± 3.3	-
ST258/pKpQIL-UK	43.2 ± 4.4	0.26
ST258/pKpQIL-D2	44.1 ± 3.8	0.08

Generation time and growth kinetics were determined for the plasmid carrying *K. pneumoniae* ST258 to determine the early-, mid- and -late logarithmic phase of the growth in MOPS minimal media. Generation time was recorded as mean ± standard deviation of three independent experiments. Student's *t*-test was used to analyse the data for significant changes.

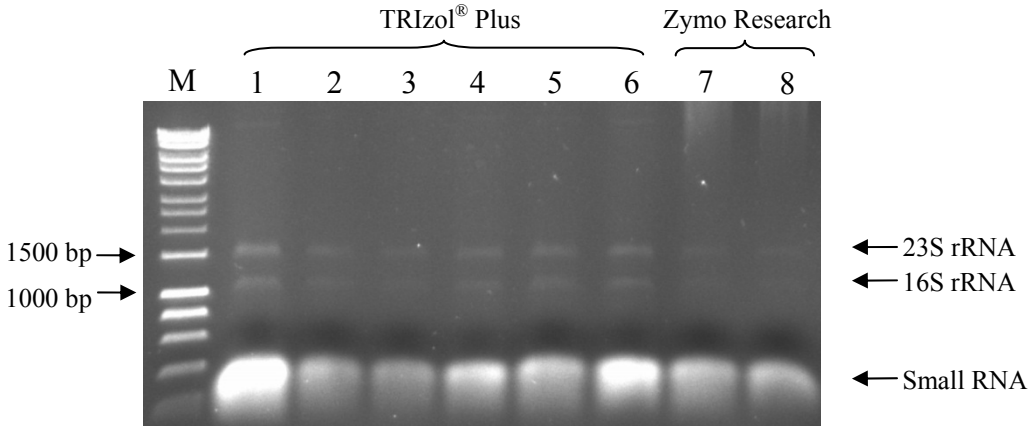
4.13.2 Optimisation of Total RNA Extraction

From the qRT-PCR data, it was found that the carbapenemase gene was highly expressed in the early stage of the logarithmic phase. Hence, the RNA from the early logarithmic phase was used to study the difference in the transcriptome of the *K. pneumoniae* ST258, ST258/pKpQIL-UK and ST258/pKpQIL-D2. Initially, the RNA preparation was carried using the SV Total RNA Isolation System (Promega, Wisconsin, USA, Catalogue No.: Z3105). This kit was used as it is frequently used to prepare RNA from *S.* Typhimurium in the Piddock Group. However, white precipitate (possibly debris from the *Klebsiella* capsule) was observed repeatedly at the final step where the RNA was eluted from the spin column. This white precipitate was also observed in the total RNA extracted for assessing the bla_{KPC} expression in the different growth stages. As RNA sequencing involves many preparation steps and the sequencing reaction is sensitive to contaminants, the RNA preparation conditions were optimised before proceeding to the sequencing stage. The RNA extraction kit used was changed to TRIzol® Plus RNA Purification Kit (Life Technologies, California, USA, Catalogue No.: 12183-555). Using the 'Total RNA Isolation' protocol in the manufacturer's manual, this kit yielded no detectable RNA when quantified by Qubit® RNA HS Assay Kit. Hence, the 'Total Trancriptome Isolation' method (designed to include small RNA during RNA extraction) in the manufacturer's manual was used and yielded about 5 – 7 μg RNA. The eluted RNA was not contaminated with the previously observed white precipitate.

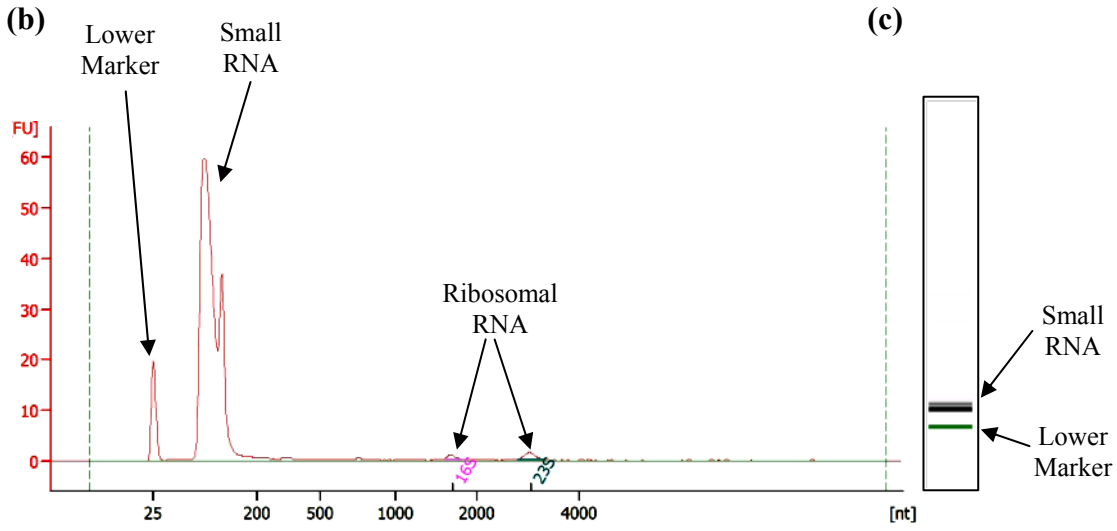
When separated by electrophoresis, the 16S and 23S rRNA could be observed (Figure 4.22a). Although the total amount of RNA extracted was high (5 – 7 μg), but the ribosomal RNA (16S and 23S) bands were relatively faint and possibly suggested a low amount of messenger RNA (mRNA) was present. The RNA samples were analysed using Agilent 2100 Bioanalyzer. However, the RNA samples consisted of mostly of small RNA species (Figure

Figure 4.22 Purification of RNA from *K. pneumoniae* ST258 in the presence and absence of pKpQIL-UK and -D2

(a) Purified total RNA separated on agarose gel



M: Hyperladder I (Bioline)
Lane 1 – 2: ST258
Lane 3 – 4: ST258/pKpQIL-UK
Lane 5 – 6: ST258/pKpQIL-D2
Lane 7 – 8: ST258 (Quick-RNA™, Zymo Research Kit)



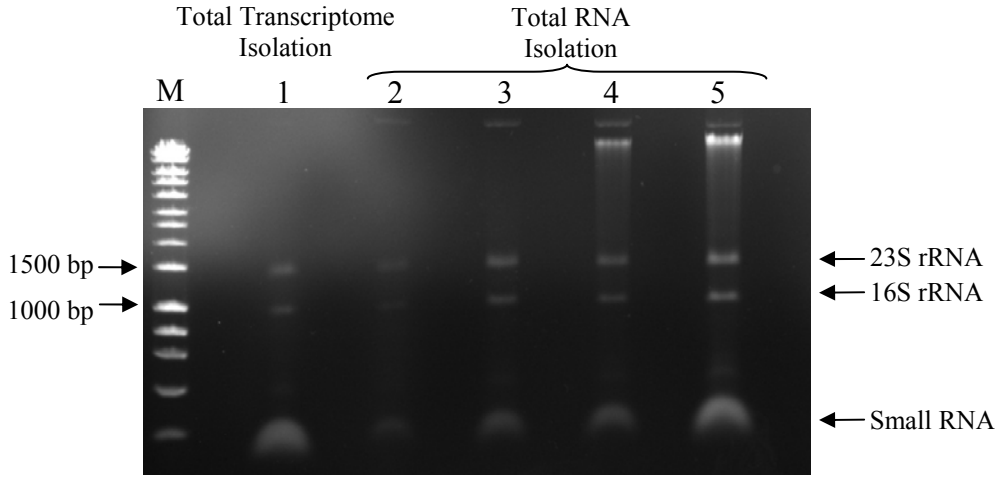
(a) The total RNA samples extracted from the various plasmid-carrying *K. pneumoniae* ST258 strains using different commercial kits were compared in a 1% agarose gel by observing the intensity of the 16 and 23S rRNA bands. A representative image of Agilent 2100 Bioanalyzer (b) electropherogram trace and (c) gel photo, for the RNA samples extracted using TRIzol® Plus Kit and Quick-RNA™ Kit. The RNA samples consisted of mostly small RNA species as the 16 and 23S RNA peaks were very small on the electropherogram.

4.22b & c). A different kit (Quick-RNA^{TM}, Zymo Research, California, USA) was also used to prepare total RNA from ST258. A similar pattern of total RNA composition was observed in the electrophoresis gel (Lane 7 – 8, Figure 4.22a). The composition of RNA obtained from the two different kits suggested that it was not an artefact of the RNA extraction kit but the amount of mRNA at the early logarithmic phase was very low. Hence, a greater volume of culture was use for RNA extraction with the previous 'Total RNA Isolation' protocol in the manufacturer's guide. This protocol was chosen instead of the 'Total Transcriptome Isolation' because the latter omits one of the washing buffers which was needed for on-column DNAse-treatment. The removal of the on-column DNAse-treatment step would result in highly DNA contaminated RNA samples.

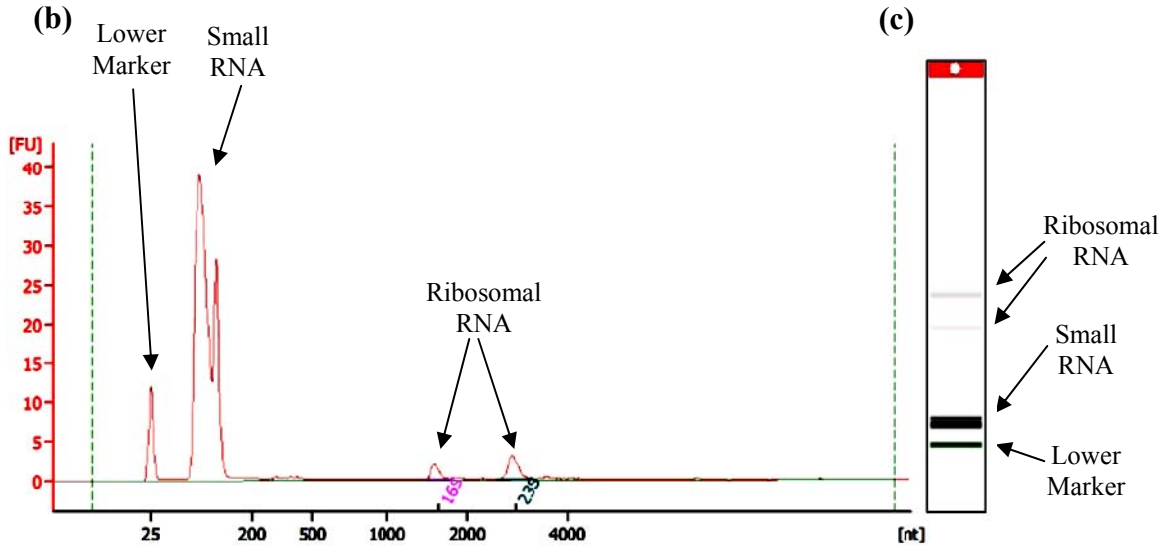
Using an increasing volume of culture (30, 90, 120 and 150 ml) for RNA extraction, it was observed that there was an increase in the intensity of the ribosomal RNA and the smear of mRNA along the electrophoresis lanes (Figure 4.23a). Although the higher volume of culture used for RNA extraction indicated the possibility of increased mRNA transcripts in the samples, the Bioanalyzer analysis showed that the RNA samples still comprised of mostly small RNA species (Figure 4.23b and c). Moreover, the white precipitate was again observed using the larger culture volume. Hence, extraction of RNA using another method (i.e. glass beads) was attempted with the RiboPure^{TM} RNA Purification Kit. The extraction was also carried out using a culture from the late logarithmic phase (OD_{600} = 0.6) to overcome the lower mRNA transcript production at the early growth phase. This kit produced a sufficient amount of total RNA (including mRNA) (ca. 15 – 20 μg) for sequencing. From the Bioanalyzer analyses, the 16S and 23S rRNA peaks were discreet suggesting a good quality of total RNA without obvious degradation (Figure 4.24a and b). The smear on the background

Figure 4.23 Purification of RNA from increasing volume of *K. pneumoniae* ST258 culture

(a) Purified total RNA separated on agarose gel



M: Hyperladder I (Bioline)
Lane 1: ST258 (Total Transcriptome Isolation as positive control)
Lane 2: ST258 (30 ml)
Lane 3: ST258 (90 ml)
Lane 4: ST258 (120 ml)
Lane 5: ST258 (150 ml)
} Total RNA Isolation



(a) The total RNA samples extracted from the various plasmid-carrying *K. pneumoniae* ST258 strains using different culture volumes were compared in a 1% agarose gel by observing the intensity of the 16 and 23S rRNA bands. A representative image of Agilent 2100 Bioanalyzer (b) electropherogram trace and (c) gel photo, for the RNA samples extracted from 150 ml of culture using TRIzol® Plus Kit (Total RNA Isolation Protocol). The RNA samples consisted of mostly small RNA species as the 16 and 23S RNA peaks were very small on the electropherogram.

(Figure 4.24a) and the elevated signal (Figure 4.24b) around the ribosomal RNA peaks showed that mRNA was present in the samples.

4.13.3 Ribosomal RNA Depletion and Library Preparation

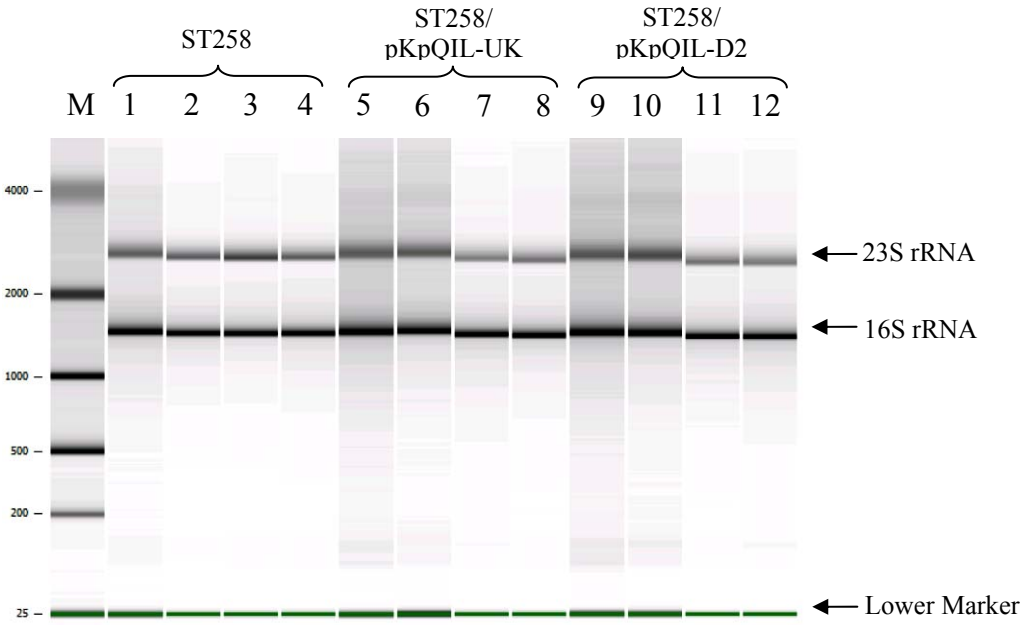
The total RNA in a bacterial cell comprises of mostly ribosomal RNAs with messenger RNA only contributing to about 1 – 5% of the pool of RNA (Giannoukos et al., 2012, He et al., 2010). To increase the sensitivity of detecting an mRNA transcript during sequencing, the depletion of ribosomal RNA was carried out before the sequencing stage. All RNA samples were DNAse-treated to remove DNA contamination as DNA will reduce the ribosomal RNA depletion efficiency. The DNAse-treated RNA samples were then verified by PCR for the *gyrB* gene to be free of DNA contamination (Figure 4.25). The DNA-free RNA samples were then subjected to ribosomal RNA depletion. All samples (Figure 4.26a & b) were successfully depleted of ribosomal RNA as shown by the reduction in the two major ribosomal RNA peaks (16S and 23S) in the Bioanalyzer electropherogram trace (Figure 4.26c & d). The rRNA-depleted RNA samples were used for preparation of the cDNA library for sequencing. Before the library was sequenced, all library preparations were analysed by the Agilent 2200 TapeStation (Figure 4.27a). All the cDNA libraries had an average fragment size of about 260 bp, the recommended fragment size for RNA sequencing (Figure 4.27b).

4.13.4 RNA Sequencing

The sequencing obtained approximately 1.3 to 2.0 million reads per sample. The probability of a base being incorrectly assigned during sequencing was low for most of the length of the reads (Figure 4.28). The sequencing reads were initially mapped on to the published *K. pneumoniae* ST258 chromosome as the reference sequence (Accession No.: CP006923). However, in the subsequent analysis where the gene annotation file for the chromosome sequence was required, the software in GalaxyProject was unable to recognise the gene

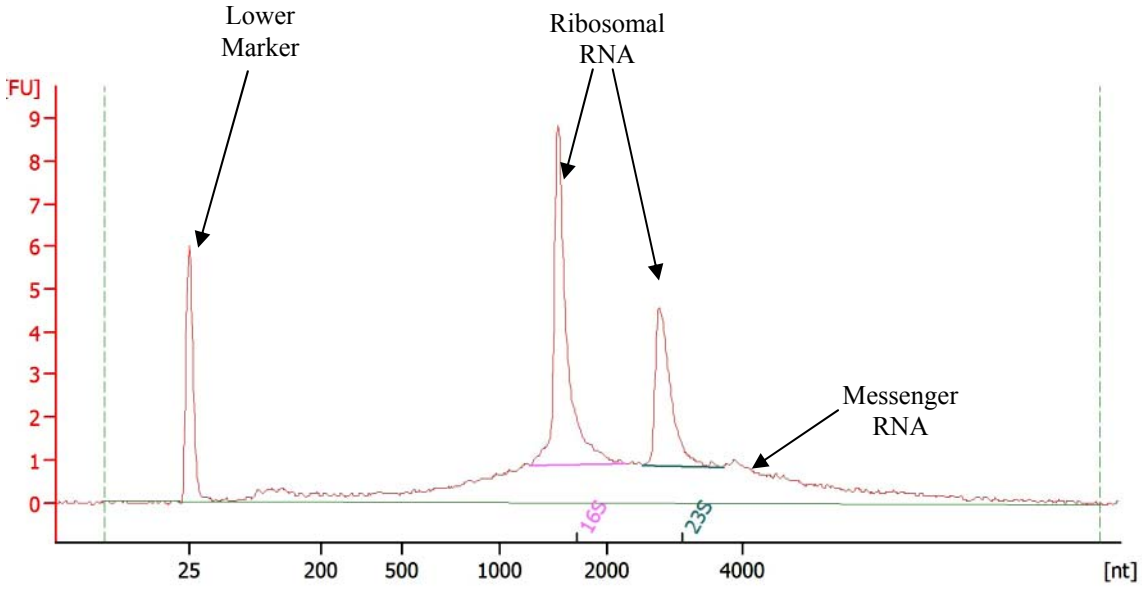
Figure 4.24 Purification of RNA using RiboPure™ RNA Purification Kit

(a) Agilent 2100 BioAnalyzer electrophoresis gel



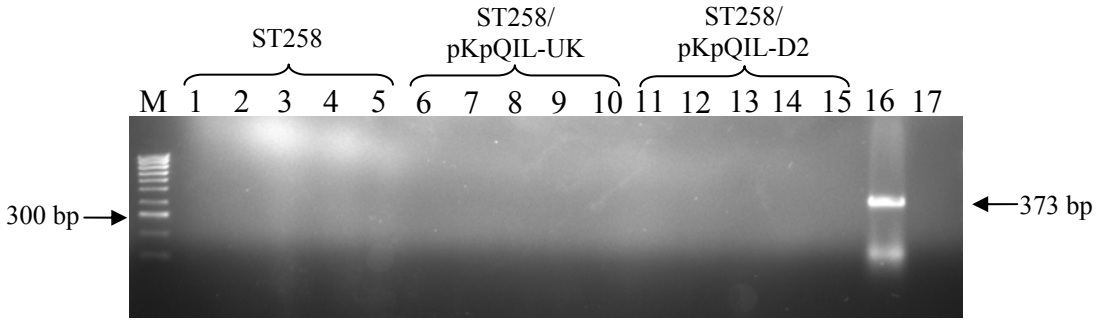
M: Molecular marker (Agilent)

(b) Representative of Agilent 2100 Bioanalyzer electropherogram trace



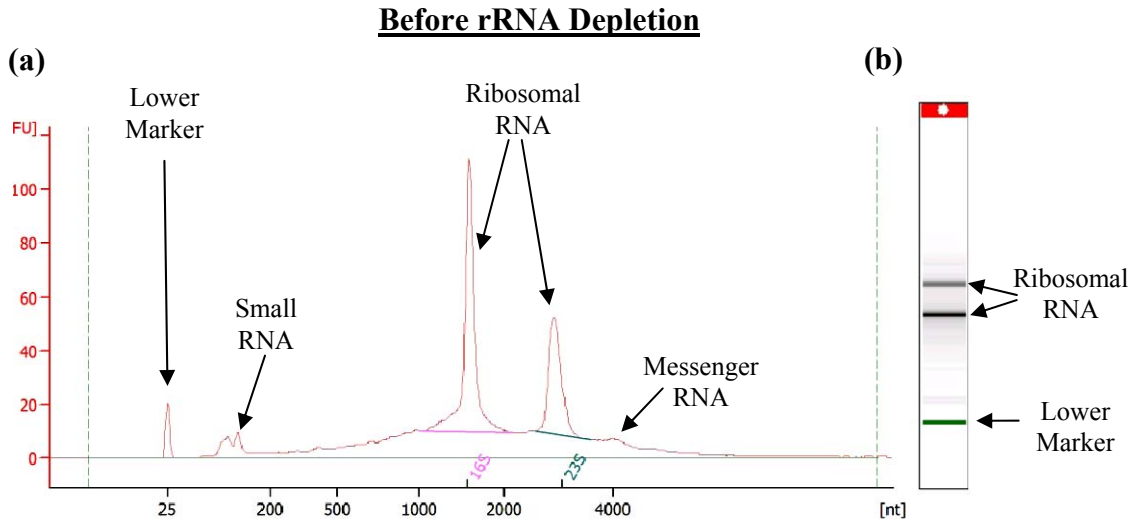
A representative image of Agilent 2100 Bioanalyzer (a) electropherogram trace and (b) gel photo, showing the total RNA samples extracted from the various *K. pneumoniae* ST258 strains using RiboPure RNA Purification Kit. All the samples contained mRNA and the discreet 16 and 23S rRNA peaks suggest good RNA integrity with minimal degradation.

Figure 4.25 Validation of DNA contamination in DNAse-treated RNA samples

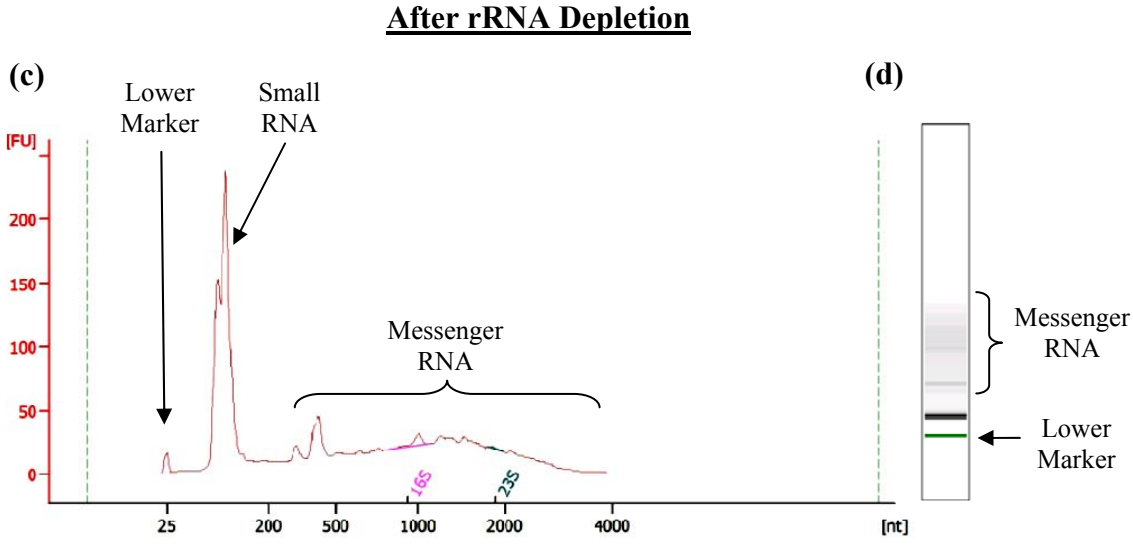


The DNAse-treated RNA samples were checked by PCR (40 cycles) for the presence of the *gyrB* gene. The presence of DNA contamination would allow the gene to be amplified and the amplicons would be visible in the agarose gel. M: HyperLadder IV (Bioline); Lane 16: *K. pneumoniae* ST258 genomic DNA; Lane 17: Water; Primer: gyrB-F/R; Expected amplicon: 373 bp.

Figure 4.26 Depletion of ribosomal RNA



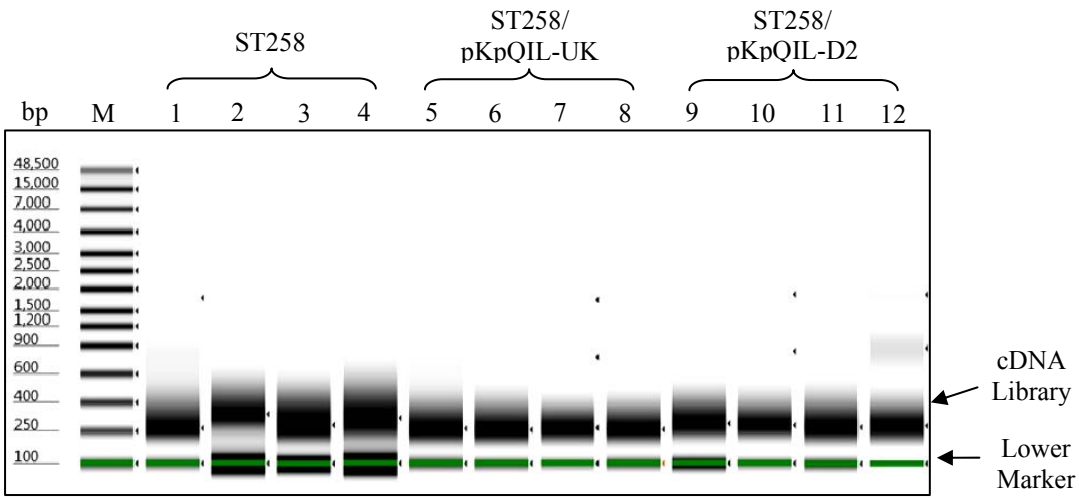
A representative Agilent 2100 Bioanalyzer (a) electropherogram trace and (b) gel photo of DNA-free RNA sample (ST258) before rRNA-depletion.



A representative Agilent 2100 Bioanalyzer (c) electropherogram trace and (d) gel photo of DNA-free RNA sample (ST258) after rRNA-depletion. After the rRNA-depletion step, the 16 and 23S rRNA peaks were clearly reduced.

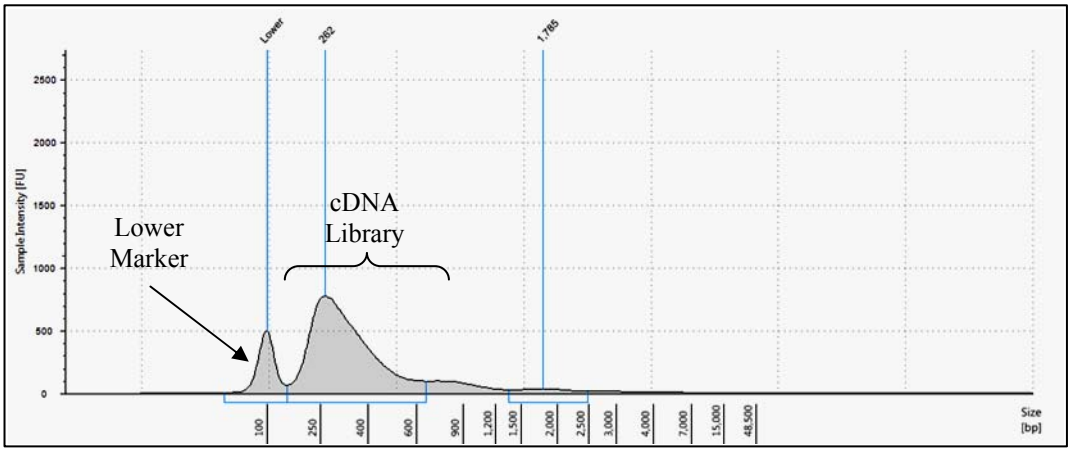
Figure 4.27 Validation of cDNA libraries

(a) Agilent 2200 TapeStation gel photo of all library preparation for sequencing



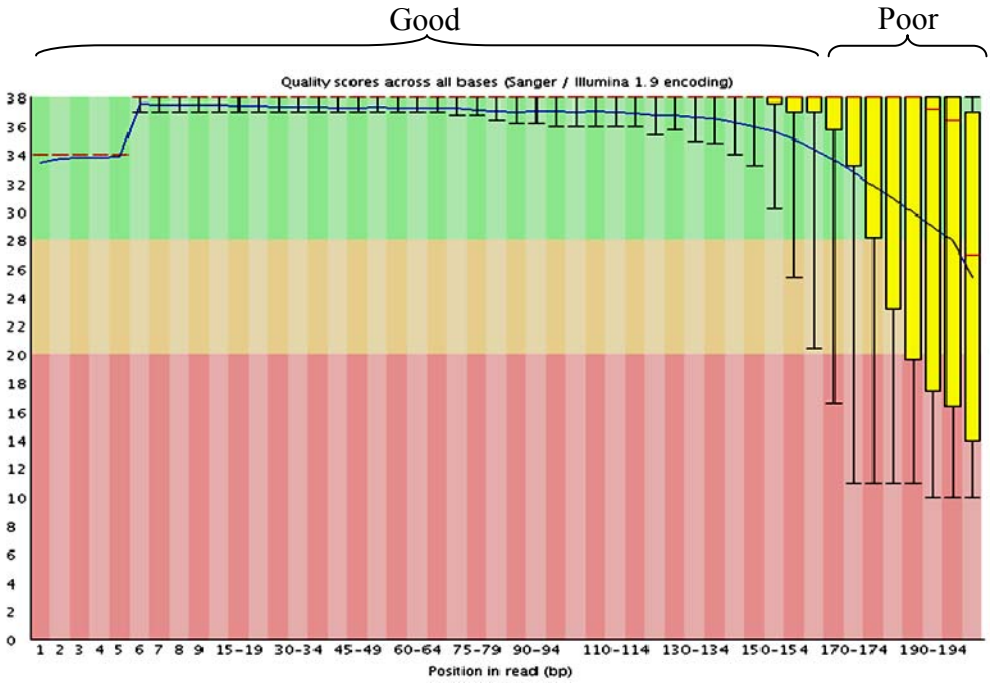
M: Molecular ladder (Agilent)

(b) A representative Agilent 2200 TapeStation electropherogram for a cDNA library sample



The DNase-treated and rRNA-depleted RNA samples were converted into cDNA. The average length of the fragments in these cDNA libraries were determined by the Agilent 2200 TapeStation. (a) The gel photo shows the average length of the cDNA libraries of all samples. (b) A representative electropherogram shows a majority of the cDNA fragment are at the recommended size of about 260 bp for sequencing.

Figure 4.28 Quality check of RNA sequencing reads



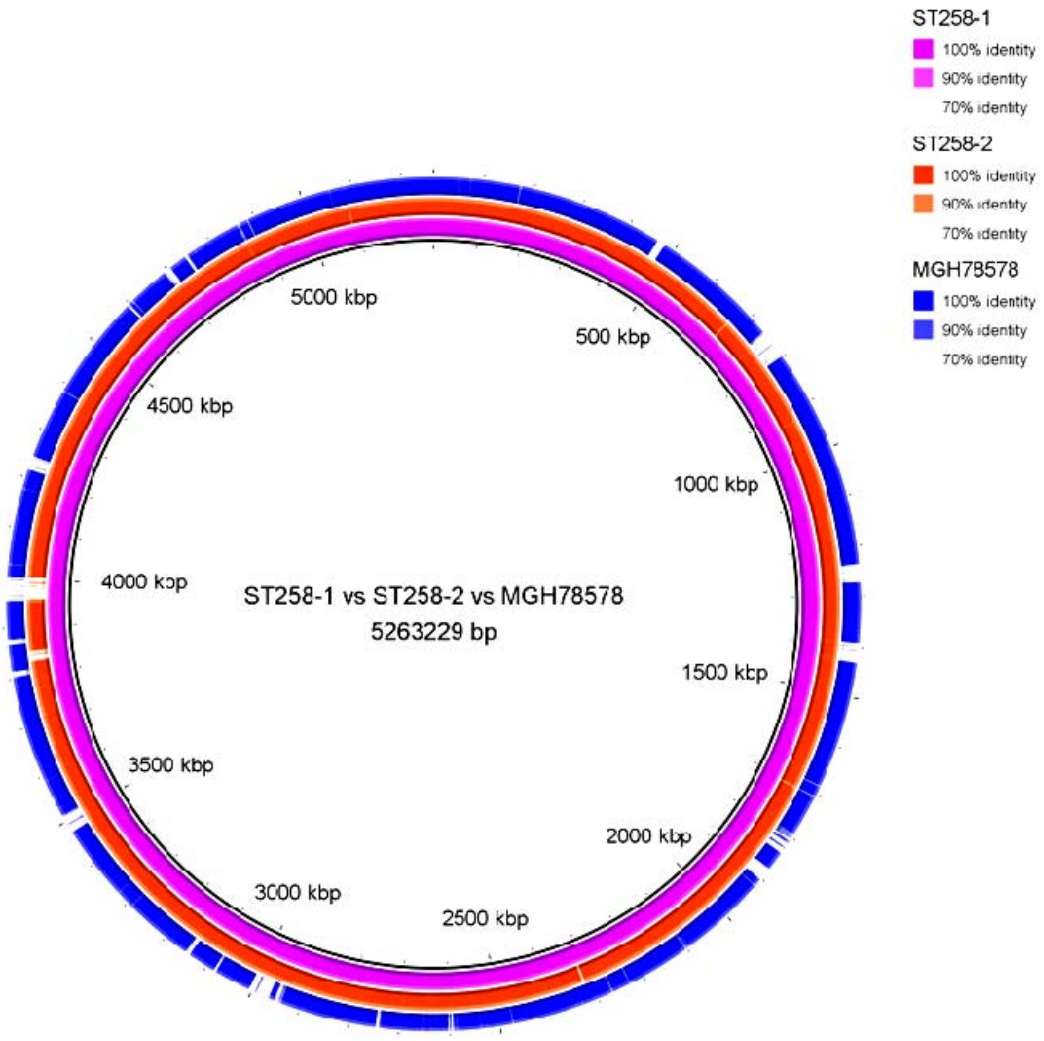
The sequencing reads of each sample were analysed using the FASTQC analysis software. A representative FASTQC analysis shows the majority of the length of the sequencing reads obtained from the MiSeq Sequencer was of good quality i.e. the probability of a base being incorrectly assigned was low.

annotation. This was probably due to incorrect annotation in the original ST258 file. Hence, the chromosome sequence of *K. pneumoniae* MGH78578 (ST38) (Accession No.: CP000647) was used as the reference sequence for the analysis. The MGH78578 shares 91-92% coverage with 99% identity with the other published ST258 chromosome sequence (CP006923 & CP006918) (Figure 4.29).

The difference in *K. pneumoniae* ST258 chromosomal gene expression was studied in the presence of pKpQIL-UK and -D2 (Table 4.9). The genes were categorised according to cluster of orthologous groups (COG). Generally, both plasmids did not significantly alter the gene expression in the *K. pneumoniae* ST258 host. Interestingly, with the exception of four genes, pKpQIL-UK and -D2 affected the expression of two different sets of chromosomal genes. The four genes which were affected by both plasmids were carbon starvation gene (*yjiY*), transcriptional regulator (*ycgE*), short chain dehydrogenase and a gene encoding for a hypothetical protein. Twenty four genes were differentially expressed due to the presence of the plasmid. These were broadly categorised into genes related to cellular processes and signalling (n = 2, Table 4.9a), information storage and processing (n = 4, Table 4.9b), metabolism (n = 9. Table 4.9c) and genes of poorly characterised functions (n = 9, Table 4.9d). Most of the genes which were affected by the plasmids were related to transporters involved in metabolic functions (n = 5, Table 4.9c).

Expression of the pKpQIL-UK and -D2 plasmidic genes were also compared. However, only the genes located in the substituted regions were found to be expressed differently (Table 4.10). By mapping the sequencing reads of *K. pneumoniae* ST258 carrying the plasmids on to the substituted region of the individual plasmids, it was found that pKpQIL-UK's substituted region was transcriptionally more active than the -D2 plasmid (Figure 4.30 – 4.33). Although both *parA* and *parM* genes were found on the substituted

Figure 4.29 Chromosome alignment of *K. pneumoniae* ST258 chromosome sequences with MGH78578



The *K. pneumoniae* chromosome sequence alignment was generated using Blast Ring Image Generator software. From the inner most ring, (1) Size of the chromosome, (2) ST258-1 (Accession No.: CP006923) (pink), (3) ST258-2 (Accession No.: CP006918) (orange), and (4) MGH78578 (blue). The chromosomes of the three *K. pneumoniae* share about 91% coverage which has 99% DNA sequence identity.

Table 4.9 Differentially expressed chromosomal genes in the presence of pKpQIL-UK vs -D2

(a) Cellular Processes and Signaling

Gene ID	Gene Name	Strand	Putative Function	Fold Changes		BlastP Identity (%)	COG ID	COG Group	COG Definition
				pKpQIL-UK	pKpQIL-D2				
KPN_00443	*acrB*	-	Multidrug resistant efflux system	**1.8**	NS	100	COG841	V	Defense mechanisms
KPN_04776	*yjiY*	-	Carbon starvation protein	0.7	0.7	100	COG1966	T	Signal transduction & mechanisms

(b) Information Storage and Processing

Gene ID	Gene Name	Strand	Putative Function	Fold Changes		BlastP Identity (%)	COG ID	COG Group	COG Definition
				pKpQIL-UK	pKpQIL-D2				
KPN_01003	*appY*	+	AraC family transcriptional regulator	NS	0.5	100	COG2207	K	Transcription
KPN_01374	*dbpA*	+	ATP-dependent RNA helicase	NS	0.7	99	COG513	L,K,J	Replication, recombination & repair; Transcription; Translation, ribosomal structure & biogenesis
KPN_01535	*cynR*	+	LysR type transcriptional regulator	**1.9**	NS	100	COG583	K	Transcription
KPN_04733	*ycgE*	-	MerR family transcriptional regulator	0.1	0.1	100	COG789	K	Transcription

Continued overleaf.

(c) Metabolism

Gene ID	Gene Name	Strand	Putative Function	Fold Changes		BlastP Identity (%)	COG ID	COG Group	COG Definition
				pKpQIL-UK	pKpQIL-D2				
KPN_00640	*xylG*	-	ABC sugar transporter	1.8	NS	99	COG1129	G	Carbohydrate transport & metabolism
KPN_00801	*bioF*	+	Biotin metabolism (8-amino-7-oxononanoate synthase)	NS	1.7	100	COG156	H	Coenzyme transport & metabolism
KPN_01007	*oppA*	+	Oligopeptide transport system	NS	0.5	99	COG4166	E	Amino acid transport & metabolism
KPN_02229	*chaA*	+	Calcium/sodium:proton antiporter	0.5	NS	100	COG387	P	Inorganic ion transport & metabolism
KPN_02235	*hemA*	-	Glutamyl-tRNA reductase	0.5	NS	100	COG373	H	Coenzyme transport & metabolism
KPN_02333	*manX*	+	PTS mannose transporter subunit IIAB	0.7	NS	99	COG3444	G	Carbohydrate transport & metabolism
KPN_03184	*cobQ*	-	Cobyric acid synthase	NS	0.8	100	COG1492	H	Coenzyme transport & metabolism
KPN_03259	*yesO*	+	Oligogalacturonate-binding protein OgtD	NS	D2	100	COG1653	G	Carbohydrate transport & metabolism
KPN_03375	*metK*	+	S-adenosylmethionine synthetase	NS	1.4	100	COG192	H	Coenzyme transport & metabolism

(d) Poorly Characterised

Gene ID	Gene Name	Strand	Putative Function	Fold Changes		BlastP Identity (%)	COG ID	COG Group	COG Definition
				pKpQIL-UK	pKpQIL-D2				
KPN_01536	*smrA*	+	DNA endonuclease	**1.9**	NS	100	COG2840	S	Function unknown
KPN_01888	*yddH*	+	Flavin reductase-like protein	**2.6**	NS	100	COG1853	R	General function prediction only
KPN_04023	*ycnE*	+	Hypothetical protein	0.6	0.4	100	COG1359	S	Function unknown
KPN_04732	*sdh*	-	Short chain dehydrogenase/ reductase	0.1	0.1	99	COG300	R	General function prediction only
KPN_00439	*ylaC*	-	Hypothetical protein	**1.8**	NS	99	Unclassified	Unclassified	Unclassified
KPN_01031	-	+	Hypothetical transmembrane protein	**2.0**	NS	100	Unclassified	Unclassified	Unclassified
KPN_01110	*bhsA*	+	Multiple stress resistance protein	NS	**1.4**	100	Unclassified	Unclassified	Unclassified
KPN_01149	*yciG*	+	Hypothetical protein	NS	WT	98	Unclassified	Unclassified	Unclassified
KPN_02999	*alaE*	+	Hypothetical protein	NS	0.6	100	Unclassified	Unclassified	Unclassified

'NS' denotes non-significant changes in gene expression based on q-value. 'WT' denotes the gene was only expressed in the absence of the plasmids. 'D2' denotes the gene was only expressed in the presence of the pKpQIL-D2 plasmid. The genes were categorised according to the cluster of orthologous group (COG). '**Red**' denotes increased in expression whereas 'Blue' denotes the opposite.

Table 4.10 Differentially expressed plasmidic genes

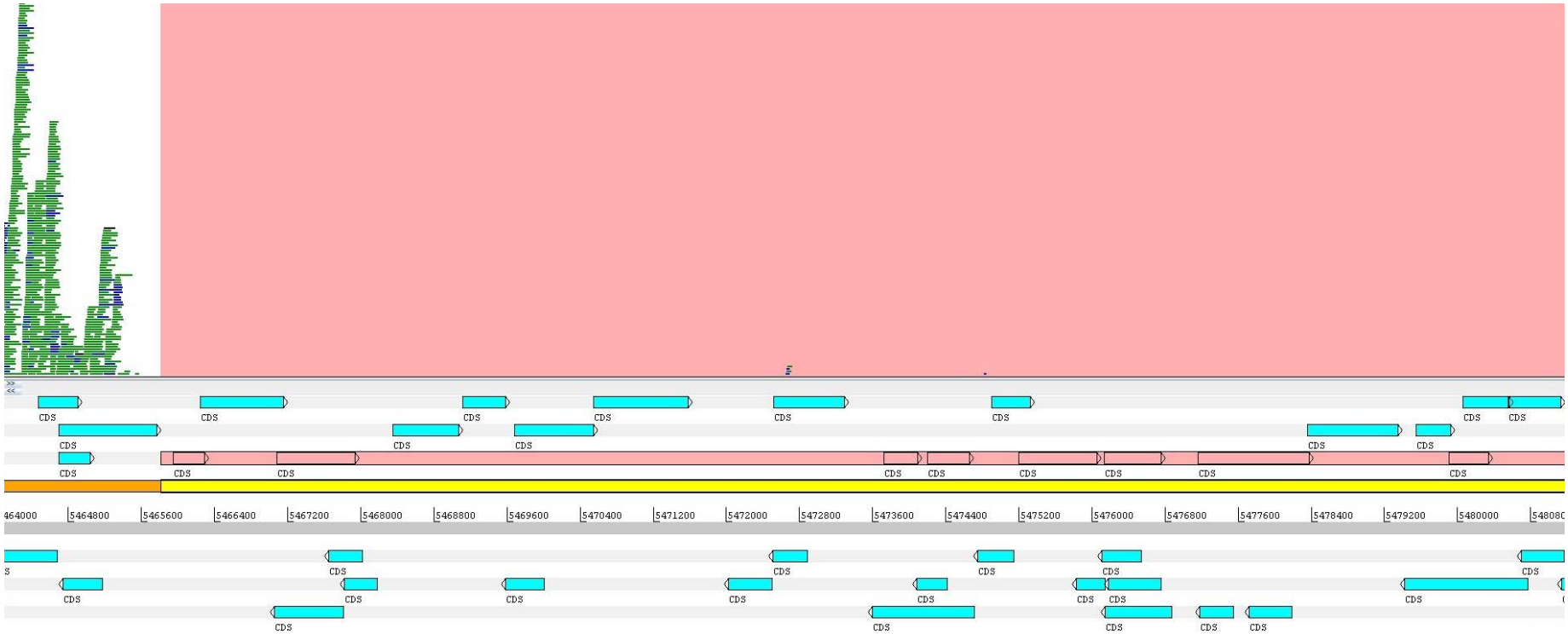
Gene Name	Strand	Putative Function	Plasmid	BlastP Identity (%)
xerC	+	Resolvase	pKpQIL-UK	100
parM	+	Plasmid segregation	pKpQIL-UK	100
parA	-	Plasmid segregation	pKpQIL-UK	99
parA	+	Plasmid segregation	pKpQIL-D2	100
parB	+	Plasmid segregation	pKpQIL-D2	100
parM	+	Plasmid segregation	pKpQIL-D2	99
-	-	Hypothetical protein	pKpQIL-D2	100
-	-	Transposase	pKpQIL-D2	99

The genes were located within the substituted regions of the respective plasmids. Hence, relative expression was not available.

Figure 4.30 Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-UK on to substituted region on pKpQIL-UK DNA sequence

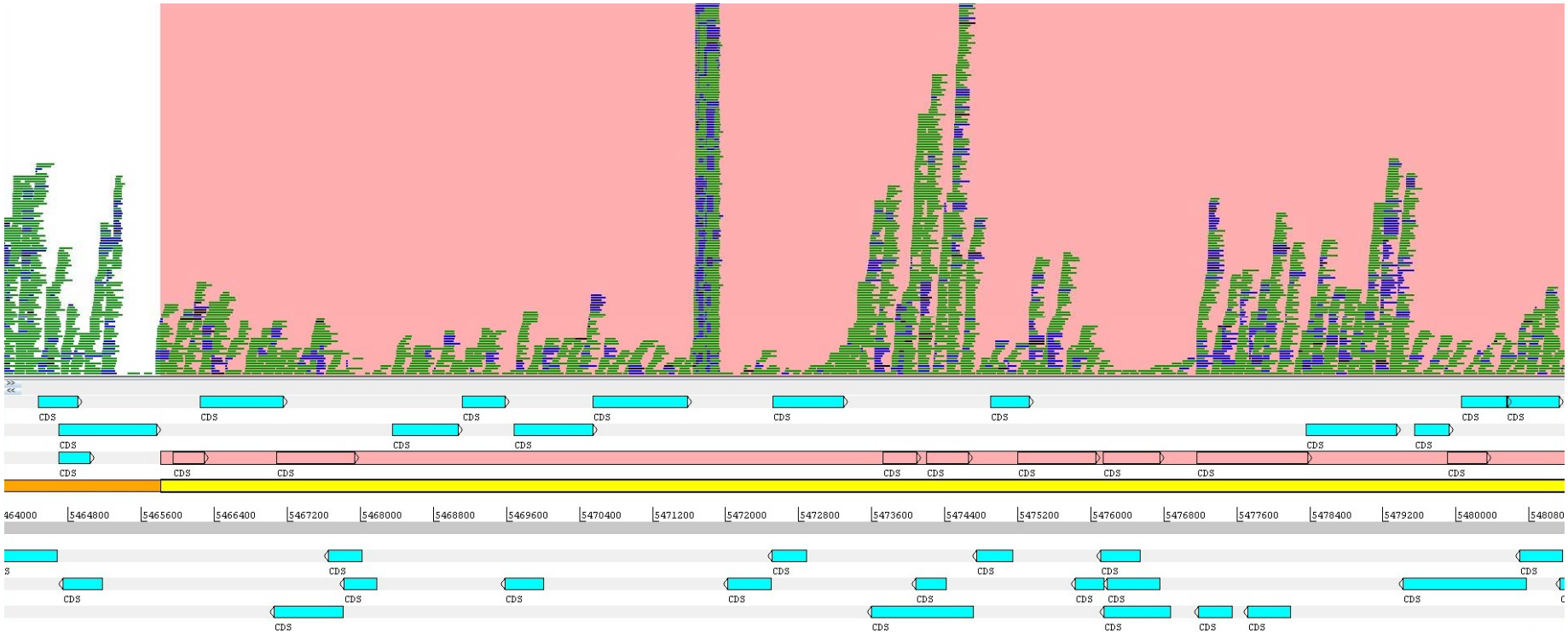
The figure was generated using Artemis (www.sanger.co.uk) by mapping the sequencing reads of ST258/pKpQIL-UK on to the substituted region in pKpQIL-UK plasmid sequence. The region highlighted in pink is the substituted region in pKpQIL-UK. From Figure 4.30-4.33, the mapping results suggest that all samples from the plasmid carrying strains were correct. Relative to pKpQIL-D2's substituted region (Figure 4.32), the corresponding region in pKpQIL-UK (pink) was found to be more transcriptionally active (based on the number of reads mapped on to this region).

Figure 4.31 Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-UK on to substituted region on pKpQIL-D2 DNA sequence



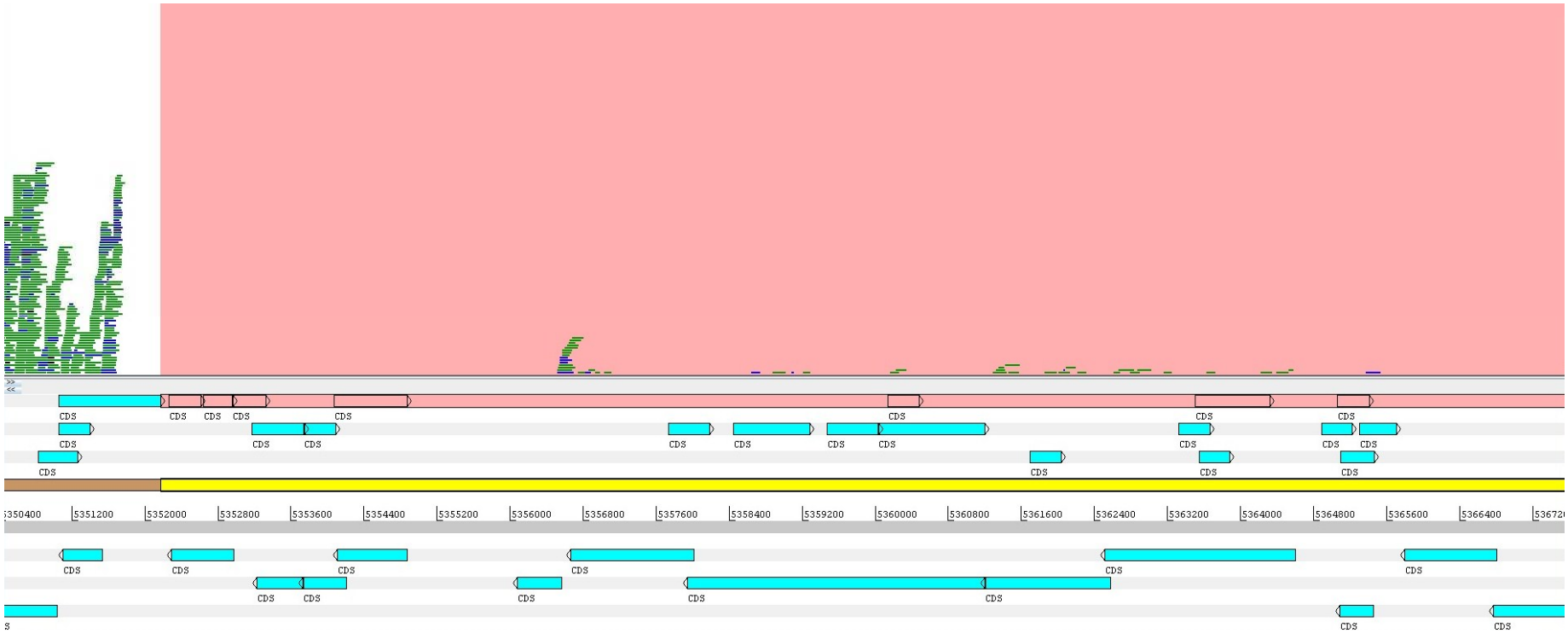
The figure was generated using Artemis (www.sanger.co.uk) by mapping the sequencing reads of ST258/pKpQIL-UK on to the substituted region in pKpQIL-D2 plasmid sequence. The region highlighted in pink is the substituted region in pKpQIL-D2. As the substituted region in pKpQIL-D2 is completely different from the corresponding region in pKpQIL-UK, no sequencing reads were mapped on to the reference sequence. From Figure 4.30-4.33, the mapping results suggest that all samples from the plasmid carrying strains were correct.

Figure 4.32 Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-D2 on to substituted region on pKpQIL-D2 DNA sequence



The figure was generated using Artemis (www.sanger.co.uk) by mapping the sequencing reads of ST258/pKpQIL-D2 on to the substituted region in pKpQIL-D2 plasmid sequence. The region highlighted in pink is the substituted region in pKpQIL-D2. From Figure 4.30-4.33, the mapping results suggest that all samples from the plasmid carrying strains were correct. Relative to pKpQIL-UK's substituted region (Figure 4.30), the corresponding region in pKpQIL-UK (pink) was found to be more transcriptionally active (based on the number of reads mapped on to this region).

Figure 4.33 Alignment of RNA sequencing reads from *K. pneumoniae* ST258 carrying pKpQIL-D2 on to substituted region on pKpQIL-UK DNA sequence



The figure was generated using Artemis (www.sanger.co.uk) by mapping the sequencing reads of ST258/pKpQIL-D2 on to the substituted region in pKpQIL-UK plasmid sequence. The region highlighted in pink is the substituted region in pKpQIL-UK. As the substituted region in pKpQIL-UK is completely different from the corresponding region in pKpQIL-D2, only non-specific sequencing reads were mapped on to the reference sequence. From Figure 4.30-4.33, the mapping results suggest that all samples from the plasmid carrying strains were correct.

region of pKpQIL-UK and -D2, and were highly expressed, these genes shared low amino acid sequence identity to each other (Figure 4.34 & 4.35).

4.14 Discussion

Bacterial fitness is a complex phenomenon which can be broadly defined as the ability of bacteria to alter their metabolism to adapt to the environmental conditions, in order for its genetic trait to compete, survive and reproduce within itself or to transfer across different hosts (Binet and Maurelli, 2005, Pope et al., 2010). For this study, the fitness of the plasmid-bearing strains was investigated in various ways, such as growth rates, biofilm formation, conjugation frequency, antibiotic susceptibility and pairwise competition.

Plasmid carriage has been previously reported to incur a fitness cost, most often translated into slower growth rates (Bouma and Lenski, 1988, Dahlberg and Chao, 2003, Lenski and Bouma, 1987, Ow et al., 2006). In this PhD study, the growth rates of pKpQIL-UK and -D2 were indistinguishable from their parental strains (*K. pneumoniae* ST258, *S.* Typhimurium SL1344 and *E. coli* NCTC10418). Although SL1344 and ATCC14028s are generally regarded as closely related pathogenesis models for *Salmonella* (Brown et al., 2011), pKpQIL-D2 carriage in *S.* Typhimurium ATCC14028s conferred a significantly slower growth rate. These data suggest that minor differences in the host played a role in the differences in fitness impact of the plasmids. pKpQIL-UK, but not -D2, negatively affected the growth rate of *S. marcescens and E. cloacae*. Although both plasmids are very similar with 85% coverage at 99% sequence identity, the differences in the substituted region could have contributed to the differences in observed growth rates. Although a large plasmid size has been implicated with slower growth rates (Smith and Bidochka, 1998, Zünd and Lebek, 1980), such a correlation was not observed in this study (pKpQIL-UK=113 kb; -D2=111 kb).

Figure 4.34 Amino acid alignment of ParA pKpQIL-UK vs -D2

```
pKpQIL-UK-parA      MKRDYGGVGSIARRAGILLESMSRDIEDQRKEFNLTEYFQTFTRNAVAKLPKLSRRIVEQ 60
pKpQIL-D2-parA      -MDNIEQLRKVATRAGKLLTSLSESIRQQKEELKLTEFYQEYSKAALYKLPKLSKGSVEY 59
                       :   : .:* *** ** *:*..*.:*::*::***::* ::: *: ******:  **

pKpQIL-UK-parA      AIKEMEDAGYDFNKKRVGNVEQYALTVQNVIDIYAHRKIPKYREIHQSPYVIFVVNLKGG 120
pKpQIL-D2-parA      AVSEMEAGGYIFKKKPSGNTMKYAMTIQNVIDLYNHRKVPKYRDRFDKAFTIFVCNLKGG 119
                    *:.*** .** *:**  **. :**:*:*****:* ***:****: .:..:.*** *****

pKpQIL-UK-parA      VSKTVSTVTLAHALRVHQDLLRHDLRILVIDLDPQASSTMFLDHTHSIGSILETAAQAML 180
pKpQIL-D2-parA      GSKTVTTASLSHAFRAHPQLLFEDLRILAIDFDPQASLTMFLSHENSVGLVENTAAQAML 179
                     ****:*.:*:**:*.* :** .*****.**:***** ****.* :*:* : :*******

pKpQIL-UK-parA      NDVDAETLRNEVIRPTIIPGVDVIPASIDDGFVASAWKELVSEHLPGVNQYEVLRKIIID 240
pKpQIL-D2-parA      QNVSREELLSDFIVPSIIPGVDVIPASIDDAFLAEGWKGLCEEHLPGKNIHAVLKENIID 239
                    ::*. * * .:.* *:**************.*:*..** * .***** * : **:: ***

pKpQIL-UK-parA      RVADDYDFILIDTGPHLDPFLLNGLAASDLILTPTPPAQVDFHSTLKYLTRLPEMLETLE 300
pKpQIL-D2-parA      KLQHDYDFIFLDSGPHLDAFLKNCIGAADLMLTPLPPATVDFHSSLKFVASLPALIDSIE 299
                    :: .*****::*:*****.** * :.*:**:*** *** *****:**::: ** :::::*

pKpQIL-UK-parA      EEGIEPRLAASIGFMSKMTGKPDHLVSHSLAREVYTSSILDSSLPRLDGFERCGETFDTI 360
pKpQIL-D2-parA      MDGHTCNLIGNVGFMSKILNKSDHKICHSQAKEVFGADMLDMVLPRLDGFERCGETFDTV 359
                     :*   .* ..:*****: .*.** :.** *:**: :.:**  ****************:

pKpQIL-UK-parA      ISANPASYPGSNDALKKARTEAEHFTKAVFDRIEFLRSQSA- 401
pKpQIL-D2-parA      ISANPATYDGSTEALKSAKSAAEDFAKAVFDRIEFIRTNGGM 401
                    ******:* **.:***.*:: **.*:**********:*::..
```

Both substituted region in pKpQIL-UK and -D2 contain genes which have been annotated as the ParA protein involved in plasmid segregation. However from BlastP, both ParA proteins share 97% coverage with only 57% amino acid sequence identity.

Figure 4.35 Amino acid alignment of ParM pKpQIL-UK vs -D2

```
pKpQIL-UK-parM      ------MATISTISQPLRGEFIRTAATAGAVVY-QVDARLPALIPVFFAGQLSAVRLCAV 53
pKpQIL-D2-parM      MNIYCDDGSTNVKLAWFEGDELQTRVSANSFRHGWKVAEFSAATFNYQVGTLKYTWDSVS 60
                           .: ..    :.*: ::* .:*.:. :    *.:.*    : .* *. .  ..

pKpQIL-UK-parM      MALVSGT-----WSSL-----------TGLPDEPENGGAALPVSP---------ETEYQR 88
pKpQIL-D2-parM      RDAIPTTNVEYQYGDLNLLAVHHALLNSGLEPQPVSLTVTLPLSEYYDGDCQRNEENIRR 120
                       :. *     :..*           :**  :* .  .:**:*          * : :*

pKpQIL-UK-parM      RR----YTLTLQNGRSG---------ERVESVLTEASSRLRGDLLRNLIITG----LALH 131
pKpQIL-D2-parM      KRENLMRELVLNKGRAFTVTDVKVMPESLPAAFSRLAELKPGPAETTLIIDLGGTTLDAG 180
                    :*      *.*::**:          * : :.::. :.   *    .***      *

pKpQIL-UK-parM      TTAPELPRLLASMPVPPTSVSELQALVQQMAGTAD------------------------ 166
pKpQIL-D2-parM      VIVGQFDDISAVHGNPSVGVSQVTRAAAGALRAADSETSALIADTIIRNRNDRQYLQRVI 240
                    . . ::  : *    *...**::   .     :**

pKpQIL-UK-parM      -----VNRAAAAVPDTPTASAAPVS--------------GSDTREIKKNMRRAFGD--- 203
pKpQIL-D2-parM      NDAGKIDEVRNKITEAITSLGARVTSELTAFRNVNRVFLVGGGASLIEEAIRQAWPLAPD 300
                         ::..   :.:: *: .* *:               *..:  *:: :*:*:

pKpQIL-UK-parM      ----------------------
pKpQIL-D2-parM      RIEVIGDPQMALAREIALYNKED 323
```

Both substituted region in pKpQIL-UK and -D2 contain genes which have been annotated as the ParM protein involved in plasmid segregation. However, both ParM proteins share only 26% coverage and 45% identity.

Bacterial growth rates have also been implicated to impact upon drug susceptibility (Costerton et al., 1999, Gilbert et al., 1990, Lewis, 2001). However, this was not observed in this study. All strains carrying the two plasmids were inhibited by similar MIC values for the antibiotics tested.

S. marcescens carrying pKpQIL-UK which was found in this PhD study to be more resistant than *S. marcescens* carrying pKpQIL-D2 towards carbapenem antibiotics. A previous study implicated the loss of OmpF and/or OmpC with the overproduction of AmpC β-lactamase as a cause for β-lactam resistance in *S. marcescens* (Weindorf et al., 1998). However, no difference in porin expression was observed in my study. Derepression of AmpC β-lactamase in the presence of antibiotic inducers has been previously reported. Some β-lactam antibiotics such as imipenem, cefoxitin and cefotetan are known to be strong AmpC β-lactamase inducers whilst third generation cephalosporins such as cefotaxime and ceftazidime have poor inducing properties (Sanders et al., 1997, Stapleton et al., 1995). Therefore in *S. marcescens* NCTC10211 carrying the pKpQIL-D2 plasmid, the phenotype maybe caused by incomplete derepression of the chromosomal AmpC β-lactamase.

The high rate of loss of both plasmids from the *E. coli* population was probably due to high fitness cost incurred by the presence of the plasmids. The general fitness cost of plasmid carriage may be attributed to a variety of factors including cytotoxic effect resulting from misfolded proteins, additional usage of cellular resources for physiological processes and alteration of metabolic flux due to introduction of homologues (Baltrus, 2013). The loss of pKpQIL-D2 but not pKpQIL-UK from the *S.* Typhimurium SL1344 and ATCC14028s populations suggests that the fitness cost most probably originated from the substituted region in pKpQIL-D2. The stability of large low copy plasmids within a bacterial population is generally facilitated by post-segregational killing and active partitioning mechanisms encoded

by the plasmids (Sengupta and Austin, 2011). The pKpQIL-UK plasmid is known to encode genes involve in plasmid maintenance, such as the *parA*/*parB* and *stbA*/*stbB* (Leavitt et al., 2010a). The variant plasmid, pKpQIL-D2 also carries the same *stbA*/*stbB* genes but a variant of *parA*/*parB* genes. It is possible that these genes were not able to function efficiently in maintaining the plasmids in some of the genetic backgrounds tested. Although it has been reported that genetic adaptation, which ameliorates the fitness cost of plasmid carriage can reduce the rate of loss of the plasmid (San Millan et al., 2014), this was not observed under the conditions tested. The plasmids exhibited a complete, rather than a delayed loss in the persistence assay. Both plasmids had similar conjugation frequencies into the two *Salmonella* strains, but pKpQIL-D2 was unable to persist. This may suggests that lateral gene transfer play little role in the maintenance of these two plasmids. Previous studies have shown strong correlation between antibiotic usage and the emergence of resistance (Van De Sande-Bruinsma et al., 2008). The reduction in antibiotic consumption has also led to the decrease in resistance (Anonymous, 2008, Seppälä et al., 1997). However, where the genes encoding for antibiotic resistance are found on successful plasmid such as pKpQIL-UK or its variant, the reduction in resistance is unlikely.

Interestingly, it was observed that the percentage of *K. pneumoniae* ST258 and Ecl8 carrying the pKpQIL-UK plasmid dropped at the early stages of the persistence experiment (Figure 4.9b and Figure 4.12d). However, the plasmid carrying population recovered after day 5 (ca. 35 generations). A similar observation was noted with the original clinical isolate (*K. pneumoniae* ST321) carrying the pKpQIL-D2. This may indicate a slight fitness cost of the plasmids in their respective hosts which was rapidly abrogated. A study has shown that chromosomal adaptation could alleviate the fitness cost of the plasmids after 100 generations

of co-existence of the plasmid and its host (San Millan et al., 2014). However, it is unclear whether a similar adaptation had occurred with the persistence experiments in this PhD.

Relative to the host bacterial strain with no plasmid (i.e. before introduction of pKpQIL-UK and -D2), both plasmids affected the ability of various Enterobacteriaceae hosts to form a biofilm on plastic in different ways. Biofilm formation is an important phenomenon as most bacteria (99%) in the natural environment exist in biofilms (Potera, 1996). The establishment of a biofilm by a single or a mixture of bacterial species plays an important role in various infections and resistance to antimicrobial agents (antibiotics and biocides) (Costerton et al., 1999). Conflicting data associating conjugation to biofilm formation have been previously reported (Ghigo, 2001, Reisner et al., 2006, Røder et al., 2013). The carriage of F-plasmids has been previously shown to confer the ability to form biofilm in the *E. coli* host (Ghigo, 2001). When plasmid carrying strains which do not form thick biofilm on their own were co-cultured with the plasmid-free *E. coli* MG1655, the author observed a significant increase in biofilm formation. Moreover, when the *traA* gene involved in pilus formation on the plasmid was inactivated, the ability to form a biofilm was abolished (Ghigo, 2001). This also suggested that the cell-to-cell and/or cell-to-surface contact promoted by the conjugation pilus can influence biofilm formation (Ghigo, 2001). A similar observation was made in another study investigating the effects of co-culturing clinical isolates with plasmid-free *E. coli* on biofilm formation (Reisner et al., 2006). However, a recent study on IncP-1 plasmid carriage in different species showed a reduction in biofilm formation (Røder et al., 2013). In contrast, the inactivation of *traF* gene involved in the conjugation machinery increased the biofilm formation of the host bacterium (Røder et al., 2013). However, as the conjugation machinery involves a large number of genes, it is likely that there is a complex interaction between this machinery with that of biofilm formation. From my study, it was observed that

the bacterial species also contributed to the difference in ability to form a biofilm in the presence of the plasmids.

The pairwise competition assay between the pKpQIL-UK and -D2 in *K. pneumoniae* Ecl8 showed that pKpQIL-D2 conferred a competitive advantage over pKpQIL-UK in this host. Over the 20-day period, the majority of pKpQIL-UK carrying Ecl8 strain was displaced from the population. All the strains used in this competition had comparable growth rates discounting growth rate as the factor for the plasmid loss from the population. Unlike in *S.* Typhimurium, both pKpQIL-UK and -D2 were able to persist within the population of *K. pneumoniae* over a period of 20 days (ca. 140 generation). This suggested that the pKpQIL-D2 plasmid had a competitive advantage over pKpQIL-UK when both plasmid carrying *K. pneumoniae* Ecl8 hosts were grown in the same population. In the competition of pKpQIL-UK vs -D2 bla_{KPC}::*aph*, it was observed that pKpQIL-UK was not completely cleared from the population. It is possible that the kanamycin resistance gene (*aph*) had a fitness impact on the pKpQIL-D2 plasmid reducing its ability to displace the pKpQIL-UK plasmid. However, the *aph* gene was used extensively in our previous work and no apparent fitness cost was observed (Cottell et al., 2014, Cottell et al., 2012). The plasmids pKpQIL-UK, -D2 and their bla_{KPC}-inactivated plasmids were conjugative as the *tra* locus was still present. As both plasmids were of the same incompatibility group, the transfer of pKpQIL-D2 into pKpQIL-UK-carrying Ecl8^{Rif} was unlikely as both plasmids could not coexist in the same host. The possibility of the transfer of pKpQIL-D2 into plasmid-free Ecl8^{Rif} (from the loss of pKpQIL-UK) and displacing the pKpQIL-UK-carrying host from the population was minimal as both plasmids persisted equally well in *K. pneumoniae* Ecl8^{Rif} without any significant loss and all the plasmid carrying strains used in the competition had equal generation times (Figure 4.12d & Figure 4.13 & Table 4.3). Moreover, pKpQIL-UK was found to have a higher conjugation

frequency than pKpQIL-D2. As pKpQIL-UK was out-competed by pKpQIL-D2, it was unlikely that conjugation played a significant role in this experiment.

Another method to assess fitness of the plasmid carrying hosts is by studying the bacteria using infection models. A simple model involving *Galleria mellonella* has been established to study infection by *K. pneumoniae* (Insua et al., 2013, Wand et al., 2013). In this model, there was no detectable difference in virulence between the two plasmid carrying *K. pneumoniae* ST258 under the conditions tested. It is possible that this infection model is not sufficiently sensitive to detect the subtle differences contributed by the plasmids. Hence, additional virulence assays such as murine infection model, serum resistance and macrophage-mediated killing assays are needed to assess the virulence of the pKpQIL-UK and -D2 carrying *K. pneumoniae* ST258 (Diago-Navarro et al., 2014).

The RNA sequencing data revealed a minimal influence of both plasmids on the host chromosomal gene expression. Those chromosomal genes with altered transcription were related to metabolic functions. However, there was no clear association of these pathways with the fitness or virulence of the host. Interestingly, pKpQIL-UK was found to up-regulate the multidrug efflux pump component gene, *acrB*. The up-regulation of this gene did not translate into obvious changes in antibiotic susceptibility of the host for the antibiotics tested. However, *acrB* is also known to affect virulence of the host and biofilm formation (Piddock, 2006b). However, there were no detectable changes in biofilm formation or *Galleria* infection for *K. pneumoniae* ST258 carrying pKpQIL-UK vs -D2. However, the lack of observed difference may also be caused by the poor biofilm forming ability of the ST258 strain used. The lack of many changes in gene expression of the host in the presence of the plasmids may imply that the plasmids are well adapted to *K. pneumoniae*. Hence, they do not significantly change the gene expression of their host resulting in excessive (if any) fitness cost. The

pKpQIL-UK plasmids have been generally found in *K. pneumoniae* since its discovery (Chen et al., 2014a, Leavitt et al., 2010b, Villa et al., 2013). Moreover, the pKpQIL-UK is essentially comprised of pKPN4 with the Tn*4401* harbouring the bla_{KPC-2} gene (Leavitt et al., 2010a). The pKPN4 plasmid was found in the multidrug-resistant *K. pneumoniae* MGH78578 (ATCC700721) isolated from the sputum of a 66-year-old ICU patient with *Klebsiella* caused pneumonia in 1994. The pKpQIL-UK plasmid is relatively stable with less than 10 nucleotide substitution detected in sequenced plasmids (Chen et al., 2014a, Leavitt et al., 2010b). Although deletion or variations of the plasmid have been reported, the significance of these variants is unclear (Chen et al., 2014a, Villa et al., 2013). In the RNA sequencing analysis, it was found that the plasmid segregation genes *parA* and *parM* were highly expressed in both plasmids. However, the genes found on both plasmids share low amino acid sequence identity. It is unclear whether these proteins function differently in the individual plasmids.

The RNA sequencing data suggested no significant difference in the transcriptome profile of the plasmid carrying *K. pneumoniae* ST258. Similarly, the presence of both plasmids in *K. pneumoniae* did not show a significant difference in phenotype under the conditions tested. Taken together, these may suggest that the plasmids are well adapted to the *K. pneumoniae* host. Hence, both plasmids do not confer significant fitness cost on their host, allowing them to thrive in *K. pneumoniae*. However, this is different to the observations with an IncH plasmid commonly found in *S.* Typhimurium (Paytubi et al., 2013). The study found that the IncHI1 plasmid affected different categories of genes to assist its *Salmonella* host to adapt to environmental stresses. A recent study also found that the introduction of a ca. 5 kb plasmid (pNUK73) had tremendous impact on the transcriptional profile of its *P. aeruginosa* PAO1 host (San Millan et al., 2015). This shuttle vector was rapidly loss from its host due to its high fitness cost (San Millan et al., 2014). The fitness cost was a result of the expression of

the Rep plasmid replication protein (San Millan et al., 2015). After 300 generations of co-existence with its host, chromosomal mutations in the helicase or kinase gene successfully reverted the transcriptome profile of the plasmid-carrying strains to that of the plasmid-free *P. aeruginosa* PAO1, reducing the cost of plasmid carriage and stabilising the plasmid in the bacterial population (San Millan et al., 2014, San Millan et al., 2015). These studies suggest that different plasmid incompatibility groups may affect their host differently.

Due to its isolation in many Enterobacteriaceae species in the outbreaks in UK, it was hypothesised that pKpQIL-D2 was more successful than pKpQIL-UK. The data obtained from the competition experiment suggest that the variant plasmid is able to displace pKpQIL-UK from the population under the conditions tested. However, outbreaks in the UK were found to involve both plasmids, suggesting that both plasmids are able to persist equally in the clinical environment.

4.15 Future Work

It has been shown that plasmid copy number can affect the metabolic flux of the plasmid carrying host possibly due to increase gene expression (Adler et al., 2014, Wang et al., 2006). Determination of the plasmid copy number of pKpQIL-UK and -D2 in the various Enterobacteriaceae bacteria could provide more insights to some of the observations made in this study. For example, the loss of pKpQIL-D2 from the *Salmonella* host strains during the 20-day plasmid persistence assay may be due to low plasmid copy number. The information on the plasmid copy number after the inactivation of the bla_{KPC} gene with the kanamycin resistance gene (*aph*) will also exclude the plasmid copy number factor in the interpretation of the competitive advantage observed in pKpQIL-D2. Also, it can be hypothesised that the difference in the plasmid segregation proteins (ParA and ParM) in conjunction with low

plasmid copy number have contributed to the advantage observed in pKpQIL-D2 during the competition experiment or its inability to persist in a *S.* Typhimurium host.

Real time PCR could also be used to determine the expression of the plasmid maintenance genes (*parAB* and *stbAB*) in the *Salmonella* and *E. coli* hosts where the loss of plasmids from the population was significant. The plasmid persistence assay involved daily sub-culturing of the plasmid-carrying bacteria. Hence, bacteria constantly undergo the lag, log and stationary phases of growth. It is hypothesised that both plasmid-carrying bacteria perform differently in the different growth phases leading to the subsequent displacement from the population. Using a chemostat, the growth rate can be kept constant to reduce the variability in the experiment. The same could be done with the pairwise competition assay to determine the competitiveness of the plasmid-carrying strains.

Although pKpQIL-UK has been reported to have crossed the sequence type barrier and is found in other ST strains of *K. pneumoniae* (Chen et al., 2014a), a pairwise competition assay should ideally be done using a carbapenem susceptible ST258 which is the predominant *K. pneumoniae* clone and host of the plasmid. In order to use the current antibiotic resistance gene for inactivation/tagging of the bla_{KPC} gene on the plasmid, a kanamycin or chloramphenicol susceptible ST258 strain will be needed. Otherwise, an alternative resistance marker (e.g. tellurite resistance gene) could be used instead to differentiate the plasmids during competition. However, the alternative marker will need to be tested to confirm that it does not confer any significant cost to the plasmid-carrying host.

The RNA sequencing showed that the presence of either plasmid had little influence on chromosomal gene expression. Nonetheless, the difference in the gene expression in the presence of both plasmids needs to be confirmed by using qRT-PCR. Another study also showed that different culture conditions and growth phases can affect the influence of the

plasmids on their host (Paytubi et al., 2013). It is hypothesised that pKpQIL-UK and -D2 may regulate host gene expression under stress to allow their persistence within the bacterial population. Hence, the RNA sequencing could be repeated using total RNA extracted from stationary phase to investigate the effects of the plasmids on their host under stress (e.g. limited nutrients).

4.16 Key Findings

- Contrary to the hypothesis, pKpQIL-D2 does not confer an obvious fitness advantage to its host over pKpQIL-UK.
- Both pKpQIL-UK and pKpQIL-D2 plasmids influenced their host differently in the various fitness experiments done.
- The pKpQIL-D2 plasmid was found to out-compete pKpQIL-UK in a pairwise competition assay.
- RNA-sequencing suggests that both plasmids have a minimal effect on host chromosomal gene expression.

CHAPTER FIVE: OVERALL DISCUSSION

The continuous spread and persistence of carbapenemase-producing Enterobacteriaceae and deleterious impact on the treatment of infections have attracted increasing attention in the recent years (Otter, 2014). The spread of these carbapenemases and other major β-lactamases is promoted by plasmids (Harris et al., 2015). In addition to antibiotic resistance, plasmid carriage also promotes evolution of the bacterial host via acquisition of new beneficial traits associated with virulence, resistance to heavy metals and additional metabolic functions (Bennett, 2008). It is a widely viewed that the acquisition of plasmids incurs a significant fitness cost upon the bacterial host, which results in plasmid-bearing bacteria to be out-competed by the plasmid-free strains (Bouma and Lenski, 1988, Lenski, 1998, Lenski and Nguyen, 1988).

In this PhD study, the impact of a globally disseminated *bla*$_{KPC}$ carbapenemase encoding plasmid (pKpQIL-UK) and its variant (pKpQIL-D2) on various Enterobacteriaceae host strains was investigated. The pKpQIL-D2 variant has not been described in the published *bla*$_{KPC}$-encoding plasmids to date (31st March 2015). The majority of the pKpQIL-like plasmids (i.e. pKpQIL-UK) investigated (Chen et al., 2014a) do not carry the substituted region observed in the pKpQIL-D2 isolated in the UK. Since the discovery of the pKpQIL plasmid in Israel, the plasmid has since been isolated in various continents including North America, Europe and Asia (Chen et al., 2013, Leavitt et al., 2010a, Richter et al., 2012, Tang et al., 2014, Hrabák et al., 2013, Lee et al., 2014). Variants of the plasmid have also been isolated (Chen et al., 2014a, Villa et al., 2013). The variations in the plasmids obtained can be very drastic. Although the original pKpQIL plasmid was approximately 114 kb, deletion, insertion and fusion events have been observed which resulted in the generation of variant plasmids ranging from 49 kb to 115 kb (Chen et al., 2014a, Villa et al., 2013). The presence

of multiple insertion elements (e.g. IS*26*) on the plasmid is believed to have contributed to the plasticity of this plasmid (Partridge, 2014, Villa et al., 2013).

The historical events leading to the emergence of the pKpQIL-D2 variant plasmid are unclear. The pKpQIL-UK (or referred to as pKpQIL in the literature) plasmid is essentially of pKPN4 plasmid of *K. pneumoniae* MGH 78578 (Accession No.: GCA_000016305) carrying the bla_{KPC} containing Tn*4401* transposon (Leavitt et al., 2010a). As the host *K. pneumoniae* strain MGH 78578 carries five plasmids (pKPN3, 4, 5, 6 and 7), it is possible that the D2 substituted region was a result of recombination of the pKPN4 and pKPN5 plasmids (Figure 1.8 & 4.1). The region between the *repB* and *umuD* (Figure 1.8) in the pKpQIL-D2 16 kb substituted region shares 98% DNA sequence identity with the corresponding region in pKPN5. However, it is unclear whether the bla_{KPC} containing transposon was inserted in pKpQIL-D2 before or after the additional fragment of the substituted region was introduced. The source of the additional fragment is also unclear as Blastn search of the fragment did not provide any significant homology hits apart from pKPN5.

Previous reports have studied the association of bla_{CTX-M} ESBL with specific genotypes of *E. coli* (Branger et al., 2005, Deschamps et al., 2009). Although no conclusive correlation was observed between the type of bla_{CTX-M}-encoding plasmids with the genotype of the *E. coli* host, it was suggested that adaptation between the ESBL gene and the host genotype resulted in increased fitness of the resistant strains which aided in the dissemination of the ESBL gene within the host bacterial genotype (Deschamps et al., 2009). This has been found to be true for $bla_{CTX-M-15}$ which has been reported to be highly associated with the *E. coli* ST131 causing frequent outbreaks around the world (Coque et al., 2008, Mathers et al., 2015, Peirano and Pitout, 2010). Although the bla_{KPC} carbapenemase was initially discovered in a *K. pneumoniae*, it has crossed species boundaries and can be found in various

Enterobacteriaceae including *E. coli*, *S. marcescens*, *E. cloacae*, *A. baumannii* and *P. aeruginosa* (Cai et al., 2008, Chen et al., 2012). In *K. pneumoniae*, this carbapenemase is predominantly associated with *K. pneumoniae* ST258 (Chen et al., 2014b, Chen et al., 2012). However, bla_{KPC} can also be found with sequence type unrelated to ST258 (Giakkoupi et al., 2011, Lavigne et al., 2013). Comparative genomics of published *K. pneumoniae* genome sequence of various sequence types have found high conservation among chromosomal sequences with differences mostly associated to mobile elements (Chen et al., 2014b). Due to the differences in the capsule polysaccharide gene clusters *K. pneumoniae* ST258 is categorised into two clades (DeLeo et al., 2014). The bla_{KPC-2} and bla_{KPC-3} carrying plasmids are associated with clade 1 and 2, respectively (DeLeo et al., 2014). The authors also suggested possible association of plasmid incompatibility groups with the clades (DeLeo et al., 2014). The reason contributing to these associations is unknown. It is possible that factors encoded in the host and/or the plasmids have resulted in this close host-plasmid relationship.

Antibiotic resistance has frequently been associated with a fitness cost incurred on the host bacterium (Andersson and Hughes, 2010). One study compared the fitness impact of a carbapenemase, bla_{SME-1} and a penicillinase, bla_{TEM-1} cloned into a pTrc99A- and pUC18-based plasmids on a *E. coli* host (Marciano et al., 2007). Compared to the bla_{TEM-1} carrying *E. coli*, the authors found that the *E. coli* carrying the bla_{SME-1} gene had a significant slower growth rate, lower cell viability after 24 hours of culture and were unable to persist in the absence of antibiotic selection (Marciano et al., 2007). The lower cell viability was also observed when the bla_{SME-1} was cloned into the *E. coli* chromosome. Hence, the authors concluded that the fitness impact arose from the carriage of the carbapenemase gene (Marciano et al., 2007). Another study, on bla_{VIM-2} carbapenemase in *S.* Typhimurium, also found that the carbapenemase resulted in colony morphology deformity, slower growth,

decreased motility and reduction in invasion of Caco-2 cells (Cordeiro et al., 2014). However, a *bla*$_{\text{OXA-66}}$ carbapenemase had no deleterious effects on the growth and morphology in the same experiment (Cordeiro et al., 2014). Recently, various *bla*$_{\text{KPC-2}}$ containing plasmids of different incompatibility groups (IncN, IncL/M and IncFII) were transferred into a common *K. pneumoniae* ATCC10031 and the fitness impact of these plasmid carrying hosts were assessed using the *Caenorhabditis elegans* infection model (Lavigne et al., 2013). The authors found that the *K. pneumoniae* carrying plasmids isolated from clonal group 258 were more virulent than plasmids from non-ST258 related strains. The *K. pneumoniae* carrying a *bla*$_{\text{KPC-2}}$ cloned into a pBK-CMV expression plasmid was found to be unable to kill the *C. elegans* as efficiently as the plasmid-free host, suggesting the carbapenemase gene was the source of the fitness cost (Lavigne et al., 2013). This also suggested that the observed increase in virulence in the infection model was probably a result of the plasmid backbone.

In this PhD study, the carriage of the pKpQIL-UK or -D2 plasmid showed no detectable adverse impact on the virulence of the *K. pneumoniae* ST258 in the *Galleria* infection model. This correlated to the other fitness experiments done with the *K. pneumoniae* ST258 as both plasmids did not have a significant impact in the generation time, ability to form biofilm and persistence of the plasmids in this strain. Further supporting these, the total transcriptome of both *K. pneumoniae* ST258 carrying pKpQIL-UK and -D2 also showed the plasmids had minimal impact in the host chromosomal gene expression. The carriage of *bla*$_{\text{KPC-2}}$ inactivated pKpQIL-UK and -D2 plasmids in *K. pneumoniae* Ecl8 also showed no changes in generation times of the bacterial host strains. This suggested that the carbapenemase gene does not incur any fitness cost on the growth rate of the host. It was hypothesised that the pKpQIL-D2 plasmid with the new substituted region has a fitness advantage over the original pKpQIL-UK plasmid. However, there was no obvious advantage

across the panel of fitness experiments used to compare the two plasmids in the various Enterobacteriaceae. Of note, the pKpQIL-D2 plasmid was only found to confer a fitness advantage over the pKpQIL-UK plasmid in a pairwise competition experiment in *K. pneumoniae* Ecl8, where the variant plasmid carrying bacterial host strain displaced most of the pKpQIL-UK carrying *K. pneumoniae* Ecl8 after 20 days (ca. 140 generation). No correlation was observed between conjugation frequencies of the plasmids from their original clinical isolates into the various Enterobacteriaceae strains with the persistence of the plasmids in their new bacterial hosts. Virulence experiment using a representative *K. pneumoniae* ST258 isolate and a septicaemia mice model suggested that this sequence type is avirulent (Tzouvelekis et al., 2013). The strain was also prone to human serum killing and phagocytosis. Hence, the authors suggested that the *K. pneumoniae* ST258 generally acts as an opportunistic pathogen and the associated mortality rate with this epidemic strain is mostly by overwhelming of the host immune system with high bacterial load (Tzouvelekis et al., 2013). Antibiotic resistance which leads to therapy failure may also allow the bacteria to achieve the numbers required for infection to occur (Tzouvelekis et al., 2013).

The spread of carbapenemase producing Enterobacteriaceae has threatened one of the most efficacious antibiotics available, i.e. carbapenem for treatment of serious bacterial infections. Correct management of infections should depend on the antibiotic susceptibility of bacteria from patient specimens and epidemiological data. However, as reported, carbapenemase producing Enterobacteriaceae can appear susceptible to carbapenem antibiotics leading to false reports (Nordmann et al., 2012b). Where treatment options is guided by such information, it may lead to counter-productive outcome among patients with bacterial infections (Cortés et al., 2010, Kang et al., 2005, Lueangarun and Leelarasamee, 2012, MacGowan, 2008, Zilberberg et al., 2014). The level of carbapenem resistance is

dependent on various factors including level of gene expression and number of carbapenemase genes, up-regulation of efflux pump and down-regulation of porins (Kitchel et al., 2010). Hence, inappropriate initial antibiotic therapy may select for high level resistant isolates during the course of treatment leading to serious consequences (Adams-Sapper et al., 2015).

Carbapenemase producing Enterobacteriaceae often carry other resistance determinant rendering other antibiotics such as fluoroquinolone and aminoglycosides ineffective (DeLeo et al., 2014, Ho et al., 2011). Hence, the combination therapy of efflux inhibitor and antibiotics may present an alternative option for the treatment of multidrug resistant bacteria (Lomovskaya and Bostian, 2006). The presence of the efflux inhibitor may sensitise the bacteria to a variety of antibiotics which are substrates to the efflux systems. However, data from this PhD study suggest that careful design and judicious use of efflux inhibitors are required to ensure that indirect effects of the inhibitor do not result in reduced susceptibility of bacteria carrying β-lactamase genes. Where the efflux inhibitor exerts an effect (such as down-regulation of porins), the inhibitor should only be used in combination with antibiotics that do not require porins as the main entry route into the bacterial cell.

The recent Review on Antimicrobial Resistance has acknowledged the increasing concerns of mortality and morbidity associated with antibiotic resistant *E. coli* and *K. pneumoniae* (O'Neill, 2014). The widespread prevalence of bla_{KPC} carrying *K. pneumoniae* ST258-related clones poses a serious threat to medical treatment. At present, pKpQIL and its variant have played a significant role in the dissemination of this carbapenemase across various sequence type and species (Chen et al., 2014a). Although the reason for the successful spread and stability of this plasmid within a bacterial population still remains unclear, the findings of this PhD study has provided more evidence that these factors are found on the

plasmid. This work allows us to better understand the interaction of plasmid factor(s) with bacterial host background in determining the success of the plasmid.

REFERENCES

Abraham, E. P. & Chain, E. 1940. An enzyme from bacteria able to destroy penicillin. *Nature,* 146**,** 837-837.

Actis, L. A., Tolmasky, M. E. & Crosa, J. H. 1999. Bacterial plasmids: replication of extrachromosomal genetic elements encoding resistance to antimicrobial compounds. *Frontiers in Bioscience,* 4**,** D43-62.

Adams-Sapper, S., Nolen, S., Donzelli, G. F., Lal, M., Chen, K., Justo Da Silva, L. H., Moreira, B. M. & Riley, L. W. 2015. Rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 59**,** 3281-3289.

Adler, M., Anjum, M., Berg, O. G., Andersson, D. I. & Sandegren, L. 2014. High fitness costs and instability of gene duplications reduce rates of evolution of new genes by duplication-divergence mechanisms. *Molecular Biology and Evolution,* 31**,** 1526-1535.

Albertí, S., Rodríquez-Quiñones, F., Schirmer, T., Rummel, G., Tomás, J. M., Rosenbusch, J. P. & Benedí, V. J. 1995. A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional model, and complement binding. *Infection and Immunity,* 63**,** 903-10.

Allen, K. P., Randolph, M. M. & Fleckenstein, J. M. 2006. Importance of heat-labile enterotoxin in colonization of the adult mouse small intestine by human enterotoxigenic *Escherichia coli* strains. *Infection and Immunity,* 74**,** 869-875.

Alvarez-Martinez, C. E. & Christie, P. J. 2009. Biological diversity of prokaryotic type IV secretion systems. *Microbiology and Molecular Biology Reviews,* 73**,** 775-808.

Ambler, R. P. 1980. The structure of β-lactamases *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 289**,** 321-331.

Anderson, E. S. 1965. Origin of transferable drug-resistance factors in the Enterobacteriaceae. *The British Medical Journal,* 2**,** 1289-1291.

Andersson, D. I. & Hughes, D. 2010. Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nature Reviews Microbiology,* 8**,** 260-271.

Andrews, J. M. 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 48**,** 5-16.

Anonymous 2008. Recent trends in antimicrobial resistance among *Streptococcus pneumoniae* and *Staphylococcus aureus* isolates: the French experience. *Euro*

surveillance : bulletin européen sur les maladies transmissibles = European communicable disease bulletin, 13**,** pii=19035.

Antignac, A., Kriz, P., Tzanakaki, G., Alonso, J. M. & Taha, M. K. 2001. Polymorphism of *Neisseria meningitidis penA* gene associated with reduced susceptibility to penicillin. *Journal of Antimicrobial Chemotherapy,* 47**,** 285-296.

Aoki, H., Sakai, H., Kohsaka, M., Konomi, T. & Hosoda, J. 1976. Nocardicin A, a new monocyclic β-lactam antibiotic. I. Discovery, isolation and characterization. *The Journal of Antibiotics (Tokyo),* 29**,** 492-500.

Arias, C. A. & Murray, B. E. 2009. Antibiotic-resistant bugs in the 21st century — A clinical super-challenge. *New England Journal of Medicine,* 360**,** 439-443.

Arnold, R. S., Thom, K. A., Sharma, S., Phillips, M., Kristie Johnson, J. & Morgan, D. J. 2011. Emergence of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Southern Medical Journal,* 104**,** 40-45.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L. & Mori, H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Molecular Systems Biology,* 2.

Baltrus, D. A. 2013. Exploring the costs of horizontal gene transfer. *Trends in Ecology & Evolution,* 28**,** 489-495.

Baraniak, A., Grabowska, A., Izdebski, R., Fiett, J., Herda, M., Bojarska, K., Żabicka, D., Kania-Pudło, M., Młynarczyk, G., Żak-Puławska, Z., Hryniewicz, W., Gniadkowski, M. & Group, T. K.-P. S. 2011. Molecular characteristics of KPC-producing *Enterobacteriaceae* at the early stage of their dissemination in Poland, 2008–2009. *Antimicrobial Agents and Chemotherapy,* 55**,** 5493-5499.

Barbosa, T. M. & Levy, S. B. 2000. Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. *Journal of Bacteriology,* 182**,** 3467-3474.

Barlow, M. 2009. What antimicrobial resistance has taught us about horizontal gene transfer. *Methods in Molecular Biology,* 532**,** 397-411.

Baroud, M., Dandache, I., Araj, G. F., Wakim, R., Kanj, S., Kanafani, Z., Khairallah, M., Sabra, A., Shehab, M., Dbaibo, G. & Matar, G. M. 2013. Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates at a tertiary care centre in Lebanon: Role of

OXA-48 and NDM-1 carbapenemases. *International Journal of Antimicrobial Agents,* 41**,** 75-79.

Baucheron, S., Tyler, S., Boyd, D., Mulvey, M. R., Chaslus-Dancla, E. & Cloeckaert, A. 2004. AcrAB-TolC directs efflux-mediated multidrug resistance in *Salmonella enterica* serovar typhimurium DT104. *Antimicrobial Agents and Chemotherapy,* 48**,** 3729-3735.

Baugh, S., Ekanayaka, A. S., Piddock, L. J. V. & Webber, M. A. 2012. Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. *Journal of Antimicrobial Chemotherapy,* 67**,** 2409-2417.

Baugh, S., Phillips, C. R., Ekanayaka, A. S., Piddock, L. J. V. & Webber, M. A. 2014. Inhibition of multidrug efflux as a strategy to prevent biofilm formation. *Journal of Antimicrobial Chemotherapy,* 69**,** 673-681.

Bennett, J. W., Herrera, M. L., Lewis, J. S., Ii, Wickes, B. W. & Jorgensen, J. H. 2009. KPC-2-producing *Enterobacter cloacae* and *Pseudomonas putida* coinfection in a liver transplant recipient. *Antimicrobial Agents and Chemotherapy,* 53**,** 292-294.

Bennett, P. M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology,* 153**,** S347-S357.

Bernet, M. F., Brassart, D., Neeser, J. R. & Servin, A. L. 1994. *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut,* 35**,** 483-489.

Bhardwaj, A. K. & Mohanty, P. 2012. Bacterial efflux pumps involved in multidrug resistance and their inhibitors: Rejuvinating the antimicrobial chemotherapy. *Recent Patents on Anti-Infective Drug Discovery,* 7**,** 73-89.

Binet, R. & Maurelli, A. T. 2005. Fitness cost due to mutations in the 16S rRNA associated with spectinomycin resistance in *Chlamydia psittaci* 6BC. *Antimicrobial Agents and Chemotherapy,* 49**,** 4455-4464.

Blahová, J., Králiková, K., Krčméry Sr, V. & Bartoníková, N. 1999. High-frequency transduction of antibiotic resistance in *Pseudomonas aeruginosa* by a wild-type bacteriophage with restricted specificity for recipient strains. *European Journal of Clinical Microbiology & Infectious Diseases,* 18**,** 152-154.

Blumberg, P. M. & Strominger, J. L. 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. *Bacteriological Reviews,* 38**,** 291-335.

Blumer, J. L. 1997. Meropenem: Evaluation of a new generation carbapenem. *International Journal of Antimicrobial Agents,* 8**,** 73-92.

Bogaerts, P., Bouchahrouf, W., De Castro, R. R., Deplano, A., Berhin, C., Piérard, D., Denis, O. & Glupczynski, Y. 2011. Emergence of NDM-1-producing Enterobacteriaceae in Belgium. *Antimicrobial Agents and Chemotherapy,* 55**,** 3036-3038.

Bornet, C., Chollet, R., Malléa, M., Chevalier, J., Davin-Regli, A., Pagès, J. M. & Bollet, C. 2003. Imipenem and expression of multidrug efflux pump in *Enterobacter aerogenes*. *Biochemical and Biophysical Research Communications,* 301**,** 985-990.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards Jr, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. & Bartlett, J. 2009. Bad bugs, no drugs: No ESKAPE! An update from the Infectious Diseases Society of America. *Clinical Infectious Diseases,* 48**,** 1-12.

Bouma, J. E. & Lenski, R. E. 1988. Evolution of a bacteria/plasmid association. *Nature,* 335**,** 351-352.

Bradford, P. A., Bratu, S., Urban, C., Visalli, M., Mariano, N., Landman, D., Rahal, J. J., Brooks, S., Cebular, S. & Quale, J. 2004. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 β-lactamases in New York City. *Clinical Infectious Diseases,* 39**,** 55-60.

Branger, C., Zamfir, O., Geoffroy, S., Laurans, G., Arlet, G., Vu Thien, H., Gouriou, S., Picard, B. & Denamur, E. 2005. Genetic background of *Escherichia coli* and extended-spectrum β-lactamase type. *Emerging Infectious Diseases,* 11**,** 54-61.

Bratu, S., Mooty, M., Nichani, S., Landman, D., Gullans, C., Pettinato, B., Karumudi, U., Tolaney, P. & Quale, J. 2005. Emergence of KPC-possessing *Klebsiella pneumoniae* in Brooklyn, New York: Epidemiology and recommendations for detection. *Antimicrobial Agents and Chemotherapy,* 49**,** 3018-3020.

Brown, A. G., Butterworth, D., Cole, M., Hanscomb, G., Hood, J. D. & Reading, C. 1976. Naturally-occurring β-lactamase inhibitors with antibacterial activity. *Journal of Antibiotics,* 29**,** 668-669.

Brown, M. H., Paulsen, I. T. & Skurray, R. A. 1999. The multidrug efflux protein *NorM* is a prototype of a new family of transporters. *Molecular Microbiology,* 31**,** 394-395.

Brown, N. F., Coombes, B. K., Bishop, J. L., Wickham, M. E., Lowden, M. J., Gal-Mor, O., Goode, D. L., Boyle, E. C., Sanderson, K. L. & Finlay, B. B. 2011. *Salmonella* phage ST64B encodes a member of the SseK/NleB effector family. *PLoS ONE,* 6**,** e17824.

Brown, S. P., Cornforth, D. M. & Mideo, N. 2012. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends in Microbiology,* 20**,** 336-342.

Bryant, K. A., Van Schooneveld, T. C., Thapa, I., Bastola, D., Williams, L. O., Safranek, T. J., Hinrichs, S. H., Rupp, M. E. & Fey, P. D. 2013. KPC-4 is encoded within a truncated Tn*4401* in an IncL/M plasmid, pNE1280, isolated from *Enterobacter cloacae* and *Serratia marcescens*. *Antimicrobial Agents and Chemotherapy,* 57**,** 37-41.

Burton, H. S. & Abraham, E. P. 1951. Isolation of antibiotics from a species of *Cephalosporium*. Cephalosporins P1, P2, P3, P4 and P5. *Biochemical Journal,* 50**,** 168-174.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G. A., Kishony, R., Kreiswirth, B. N., Kutter, E., Lerner, S. A., Levy, S., Lewis, K., Lomovskaya, O., Miller, J. H., Mobashery, S., Piddock, L. J. V., Projan, S., Thomas, C. M., Tomasz, A., Tulkens, P. M., Walsh, T. R., Watson, J. D., Witkowski, J., Witte, W., Wright, G., Yeh, P. & Zgurskaya, H. I. 2011. Tackling antibiotic resistance. *Nature Reviews Microbiology,* 9**,** 894-896.

Bush, K., Jacoby, G. A. & Medeiros, A. A. 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy,* 39**,** 1211-1233.

Cabeen, M. T. & Jacobs-Wagner, C. 2005. Bacterial cell shape. *Nature Reviews Microbiology,* 3**,** 601-610.

Cai, J. C., Zhou, H. W., Zhang, R. & Chen, G. X. 2008. Emergence of *Serratia marcescens, Klebsiella pneumoniae,* and *Escherichia coli* isolates possessing the plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC-2 in intensive care units of a Chinese hospital. *Antimicrobial Agents and Chemotherapy,* 52**,** 2014-2018.

Cai, S. J. & Inouye, M. 2002. EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. *Journal of Biological Chemistry,* 277**,** 24155-24161.

Cascales, E., Bernadac, A., Gavioli, M., Lazzaroni, J. C. & Lloubes, R. 2002. Pal lipoprotein of Escherichia coli plays a major role in outer membrane integrity. *Journal of Bacteriology,* 184**,** 754-759.

CDC 2001. Outbreaks of multidrug-resistant *Salmonella* Typhimurium associated with veterinary facilities--Idaho, Minnesota, and Washington, 1999. *MMWR. Morbidity and mortality weekly report,* 50**,** 701-4.

CDC 2010. Detection of Enterobacteriaceae isolates carrying metallo-β-lactamase - United States, 2010. *MMWR. Morbidity and mortality weekly report,* 59**,** 750.

Chamier, B., Lorenz, M. G. & Wackernagel, W. 1993. Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. *Applied and Environmental Microbiology,* 59**,** 1662-1667.

Chantot, J. F., Klich, M., Teutsch, G., Bryskier, A., Collette, P., Markus, A. & Seibert, G. 1992. Antibacterial activity of RU44790, a new *N*-tetrazolyl monocyclic β-lactam. *Antimicrobial Agents and Chemotherapy,* 36**,** 1756-1763.

Chen, L., Chavda, K. D., Melano, R. G., Jacobs, M. R., Koll, B., Hong, T., Rojtman, A. D., Levi, M. H., Bonomo, R. A. & Kreiswirtha, B. N. 2014a. Comparative genomic analysis of KPC-encoding pKpQIL-like plasmids and their distribution in New Jersey and New York hospitals. *Antimicrobial Agents and Chemotherapy,* 58**,** 2871-2877.

Chen, L., Chavda, K. D., Melano, R. G., Jacobs, M. R., Levi, M. H., Bonomo, R. A. & Kreiswirth, B. N. 2013. Complete sequence of a bla_{KPC-2}-harboring IncFIIK1 plasmid from a *Klebsiella pneumoniae* sequence type 258 strain. *Antimicrobial Agents and Chemotherapy,* 57**,** 1542-1545.

Chen, L., Mathema, B., Chavda, K. D., Deleo, F. R., Bonomo, R. A. & Kreiswirth, B. N. 2014b. Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. *Trends in Microbiology,* 22**,** 686-696.

Chen, L. F., Anderson, D. J. & Paterson, D. L. 2012. Overview of the epidemiology and the threat of *Klebsiella pneumoniae* carbapenemases (KPC) resistance. *Infection and Drug Resistance,* 5**,** 133-141.

Chen, S., Cui, S., Mcdermott, P. F., Zhao, S., White, D. G., Paulsen, I. & Meng, J. 2007. Contribution of target gene mutations and efflux to decreased susceptibility of *Salmonella enterica* serovar typhimurium to fluoroquinolones and other antimicrobials. *Antimicrobial Agents and Chemotherapy,* 51**,** 535-542.

Chen, Y., Zhou, Z., Jiang, Y. & Yu, Y. 2011. Emergence of NDM-1-producing *Acinetobacter baumannii* in China. *Journal of Antimicrobial Chemotherapy,* 66**,** 1255-1259.

Chuanchuen, R., Beinlich, K., Hoang, T. T., Becher, A., Karkhoff-Schweizer, R. R. & Schweizer, H. P. 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: Exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrobial Agents and Chemotherapy,* 45**,** 428-432.

Chuang, Y. C., Chen, Y. C., Chang, S. C., Sun, C. C., Chang, Y. Y., Chen, M. L., Hsu, L. Y. & Wang, J. T. 2010. Secular trends of healthcare-associated infections at a teaching hospital in Taiwan, 1981–2007. *Journal of Hospital Infection,* 76**,** 143-149.

Clancy, C. J., Chen, L., Hong, J. H., Cheng, S., Hao, B., Shields, R. K., Farrell, A. N., Doi, Y., Zhao, Y., Perlin, D. S., Kreiswirth, B. N. & Nguyen, M. H. 2013a. Mutations of the *ompK*36 porin gene and promoter impact responses of sequence Type 258, KPC-2-producing *Klebsiella pneumoniae* strains to doripenem and doripenem-colistin. *Antimicrobial Agents and Chemotherapy,* 57**,** 5258-5265.

Clancy, C. J., Chen, L., Shields, R. K., Zhao, Y., Cheng, S., Chavda, K. D., Hao, B., Hong, J. H., Doi, Y., Kwak, E. J., Silveira, F. P., Abdel-Massih, R., Bogdanovich, T., Humar, A., Perlin, D. S., Kreiswirth, B. N. & Hong Nguyen, M. 2013b. Epidemiology and molecular characterization of bacteremia due to carbapenem-resistant *Klebsiella pneumoniae* in transplant recipients. *American Journal of Transplantation,* 13**,** 2619-2633.

Clewell, D. B., Flannagan, S. E. & Jaworski, D. D. 1995. Unconstrained bacterial promiscuity: the Tn*916*–Tn*1545* family of conjugative transposons. *Trends in Microbiology,* 3**,** 229-236.

Colardyn, F. 2005. Appropriate and timely empirical antimicrobial treatment of ICU infections - A role for carbapenems. *Acta Clinica Belgica,* 60**,** 51-62.

Collu, F., Vargiu, A. V., Dreier, J., Cascella, M. & Ruggerone, P. 2012. Recognition of imipenem and meropenem by the RND-transporter MexB studied by computer simulations. *Journal of the American Chemical Society,* 134**,** 19146-19158.

Colomer-Lluch, M., Jofre, J. & Muniesa, M. 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE,* 6**,** e17549.

Coque, T. M., Novais, Â., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., Baquero, F., Cantón, R. & Nordmann, P. 2008. Dissemination of clonally related *Escherichia coli* strains

expressing extended-spectrum β-lactamase CTX-M-15. *Emerging Infectious Diseases,* 14**,** 195-200.

Cordeiro, N. F., Chabalgoity, J. A., Yim, L. & Vignoli, R. 2014. Synthesis of metallo-β-Lactamase VIM-2 is associated with a fitness reduction in *Salmonella enterica* serovar Typhimurium. *Antimicrobial Agents and Chemotherapy,* 58**,** 6528-6535.

Cortés, J. A., Garzón, D. C., Navarrete, J. A. & Contreras, K. M. 2010. Impact of inappropriate antimicrobial therapy on patients with bacteremia in intensive care units and resistance patterns in Latin America. *Revista Argentina de Microbiologia,* 42**,** 230-234.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. 1999. Bacterial biofilms: A common cause of persistent infections. *Science,* 284**,** 1318-1322.

Cottell, J. L., Saw, H. T. H., Webber, M. A. & Piddock, L. J. V. 2014. Functional genomics to identify the factors contributing to successful persistence and global spread of an antibiotic resistance plasmid. *BMC Microbiology,* 14**,** 168.

Cottell, J. L., Webber, M. A., Coldham, N. G., Taylor, D. L., Cerdeño-Tárraga, A. M., Hauser, H., Thomson, N. R., Woodward, M. J. & Piddock, L. J. V. 2011. Complete sequence and molecular epidemiology of IncK epidemic plasmid encoding *bla*$_{\text{CTX-M-14}}$. *Emerging Infectious Diseases,* 17**,** 645-652.

Cottell, J. L., Webber, M. A. & Piddock, L. J. V. 2012. Persistence of transferable extended-spectrum-β-lactamase resistance in the absence of antibiotic pressure. *Antimicrobial Agents and Chemotherapy,* 56**,** 4703-4706.

Cozens, R. M., Markiewicz, Z. & Tuomanen, E. 1989. Role of autolysins in the activities of imipenem and CGP 31608, a novel penem, against slowly growing bacteria. *Antimicrobial Agents and Chemotherapy,* 33**,** 1819-1821.

D'costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N. & Wright, G. D. 2011. Antibiotic resistance is ancient. *Nature,* 477**,** 457-61.

Dahlberg, C. & Chao, L. 2003. Amelioration of the cost of conjugative plasmid carriage in *Eschericha coli* K12. *Genetics,* 165**,** 1641-1649.

Datsenko, K. A. & Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 6640-6645.

Datta, N. & Hughes, V. M. 1983. Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics. *Nature,* 306**,** 616-7.

Davison, J. 1999. Genetic exchange between bacteria in the environment. *Plasmid,* 42**,** 73-91.

De Cristóbal, R. E., Vincent, P. A. & Salomón, R. A. 2006. Multidrug resistance pump AcrAB-TolC is required for high-level, Tet(A)-mediated tetracycline resistance in *Escherichia coli*. *Journal of Antimicrobial Chemotherapy,* 58**,** 31-36.

De Kraker, M. E. A., Davey, P. G., Grundmann, H. & On Behalf of The, B. S. G. 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: Estimating the burden of antibiotic resistance in Europe. *PLoS Medicine,* 8**,** e1001104.

De La Cruz, M. A. & Calva, E. 2010. The complexities of porin genetic regulation. *Journal of Molecular Microbiology and Biotechnology,* 18**,** 24-36.

De Majumdar, S., Veleba, M., Finn, S., Fanning, S. & Schneiders, T. 2013. Elucidating the regulon of multidrug resistance regulator *rarA* in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 57**,** 1603-1609.

Deleo, F. R., Chen, L., Porcella, S. F., Martens, C. A., Kobayashi, S. D., Porter, A. R., Chavda, K. D., Jacobs, M. R., Mathema, B., Olsen, R. J., Bonomo, R. A., Musser, J. M. & Kreiswirth, B. N. 2014. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 *Klebsiella pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 4988-4993.

Demain, A. L. & Elander, R. P. 1999. The β-lactam antibiotics: past, present, and future. *Antonie van Leeuwenhoek,* 75**,** 5-19.

Denap, J. C. B., Thomas, J. R., Musk, D. J. & Hergenrother, P. J. 2004. Combating drug-resistant bacteria: Small molecule mimics of plasmid incompatibility as antiplasmid compounds. *Journal of the American Chemical Society,* 126**,** 15402-15404.

Deschamps, C., Clermont, O., Hipeaux, M. C., Arlet, G., Denamur, E. & Branger, C. 2009. Multiple acquisitions of CTX-M plasmids in the rare D2 genotype of *Escherichia coli* provide evidence for convergent evolution. *Microbiology,* 155**,** 1656-1668.

Dhamdhere, G. & Zgurskaya, H. I. 2010. Metabolic shutdown in *Escherichia coli* cells lacking the outer membrane channel TolC. *Molecular Microbiology,* 77**,** 743-754.

Dhanji, H., Khan, P., Cottell, J. L., Piddock, L. J., Zhang, J., Livermore, D. M. & Woodford, N. 2012. Dissemination of pCT-like IncK plasmids harboring CTX-M-14 extended-

spectrum β-lactamase among clinical *Escherichia coli* isolates in the United Kingdom. *Antimicrobial Agents and Chemotherapy,* 56**,** 3376-7.

Diago-Navarro, E., Chen, L., Passet, V., Burack, S., Ulacia-Hernando, A., Kodiyanplakkal, R. P., Levi, M. H., Brisse, S., Kreiswirth, B. N. & Fries, B. C. 2014. Carbapenem-resistant *Klebsiella pneumoniae* exhibit variability in capsular polysaccharide and capsule associated virulence traits. *Journal of Infectious Diseases,* 210**,** 803-813.

Doern, C. D., Michael Dunne Jr, W. & Burnham, C. a. D. 2011. Detection of *Klebsiella pneumoniae* Carbapenemase (KPC) production in non-*Klebsiella pneumoniae Enterobacteriaceae* isolates by use of the Phoenix, Vitek 2, and disk diffusion methods. *Journal of Clinical Microbiology,* 49**,** 1143-1147.

Domenech-Sanchez, A., Martinez-Martinez, L., Hernandez-Alles, S., Canejo, M. D., Pascual, A., Tomas, J. M., Alberti, S. & Benedi, V. J. 2003. Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrobial Agents and Chemotherapy,* 47**,** 3332-3335.

Donetz, A. P., Harvey, R. A. & Greco, R. S. 1984. Stability of antibiotics bound to polytetrafluoroethylene with cationic surfactants. *Journal of Clinical Microbiology,* 19**,** 1-3.

Doumith, M., Ellington, M. J., Livermore, D. M. & Woodford, N. 2009. Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. *Journal of Antimicrobial Chemotherapy,* 63**,** 659-667.

Drancourt, M., Bollet, C., Carta, A. & Rousselier, P. 2001. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *International Journal of Systematic and Evolutionary Microbiology,* 51**,** 925-932.

Drusano, G. 1997. Meropenem: laboratory and clinical data. *Clinical Microbiology and Infection,* 3, Supplement 4**,** 4S51-4S59.

Durfee, T., Nelson, R., Baldwin, S., Plunkett Iii, G., Burland, V., Mau, B., Petrosino, J. F., Qin, X., Muzny, D. M., Ayele, M., Gibbs, R. A., Csörgo, B., Pósfai, G., Weinstock, G. M. & Blattner, F. R. 2008. The complete genome sequence of Escherichia coli DH10B: Insights into the biology of a laboratory workhorse. *Journal of Bacteriology,* 190**,** 2597-2606.

ECDC. 2013a. *Annual Epidemiological Report 2013* [Online]. Stockholm. Available: http://ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf [Accessed 14th June 2015].

ECDC. 2013b. *Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals 2011-2012* [Online]. Stockholm. Available: http://ecdc.europa.eu/en/publications/Publications/healthcare-associated-infections-antimicrobial-use-PPS.pdf [Accessed 14th June 2015].

ECDC. 2015. *Surveillance of seven priority food- and waterborne diseases in the EU/EEA* [Online]. Stockholm. Available: http://ecdc.europa.eu/en/publications/Publications/food-and-waterborne-diseases-surveillance-report-2015.pdf [Accessed 14th June 2015].

ECDC & EMEA. 2009. *Technical Report: The bacterial challenge - time to react* [Online]. Stockholm: European Centre for Disease Prevention and Control. Available: http://ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf [Accessed 14th June 2015].

Eicher, T., Cha, H. J., Seeger, M. A., Brandstätter, L., El-Delik, J., Bohnert, J. A., Kern, W. V., Verrey, F., Grütter, M. G., Diederichs, K. & Pos, K. M. 2012. Transport of drugs by the multidrug transporter AcrB involves an access and a deep binding pocket that are separated by a switch-loop. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 5687-5692.

El Amin, N., Giske, C. G., Jalal, S., Keijser, B., Kronvall, G. & Wretlind, B. 2005. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa*: alterations of porin OprD and efflux proteins do not fully explain resistance patterns observed in clinical isolates. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica,* 113**,** 187-196.

Eng, R. H. K., Padberg, F. T., Smith, S. M., Tan, E. N. & Cherubin, C. E. 1991. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrobial Agents and Chemotherapy,* 35**,** 1824-1828.

Engelberg-Kulka, H. & Glaser, G. 1999. Addiction modules and programmed cell death and antideath in bacterial cultures. *Annual Review of Microbiology,* 53**,** 43-70.

Erdei, J., Forsgren, A. & Naidu, A. S. 1994. Lactoferrin binds to porins OmpF and OmpC in *Escherichia coli*. *Infection and Immunity,* 62**,** 1236-1240.

Esterly, J. S., Wagner, J., Mclaughlin, M. M., Postelnick, M. J., Qi, C. & Scheetz, M. H. 2012. Evaluation of clinical outcomes in patients with bloodstream infections due to Gram-negative bacteria according to carbapenem MIC stratification. *Antimicrobial Agents and Chemotherapy,* 56**,** 4885-4890.

Fàbrega, A. & Vila, J. 2013. *Salmonella enterica* serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clinical Microbiology Reviews,* 26**,** 308-341.

Falagas, M. E., Tansarli, G. S., Karageorgopoulos, D. E. & Vardakas, K. Z. 2014. Deaths attributable to carbapenem-resistant Enterobacteriaceae infections. *Emerging Infectious Diseases,* 20**,** 1170-1175.

Fernebro, J. 2011. Fighting bacterial infections - Future treatment options. *Drug Resistance Updates,* 14**,** 125-139.

Fey, P. D., Safranek, T. J., Rupp, M. E., Dunne, E. F., Ribot, E., Iwen, P. C., Bradford, P. A., Angulo, F. J. & Hinrichs, S. H. 2000. Ceftriaxone-resistant salmonella infection acquired by a child from cattle. *New England Journal of Medicine,* 342**,** 1242-1249.

Fisher, J. F., Meroueh, S. O. & Mobashery, S. 2005. Bacterial resistance to β-lactam antibiotics: Compelling opportunism, compelling opportunity. *Chemical Reviews,* 105**,** 395-424.

Fleming, A. 1929. On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. *British Journal of Experimental Pathology,* 10**,** 226-236.

Fong, J. J., Rosé, L. & Radigan, E. A. 2012. Clinical outcomes with ertapenem as a first-line treatment option of infections caused by extended-spectrum β-lactamase producing Gram-negative bacteria. *Annals of Pharmacotherapy,* 46**,** 347-352.

Foster, T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiological Reviews,* 47**,** 361-409.

Franceschini, N., Segatore, B., Perilli, M., Vessillier, S., Franchino, L. & Amicosante, G. 2002. Meropenem stability to β-lactamase hydrolysis and comparative in vitro activity against several β-lactamase-producing Gram-negative strains. *Journal of Antimicrobial Chemotherapy,* 49**,** 395-398.

Frost, L. S. & Koraimann, G. 2010. Regulation of bacterial conjugation: balancing opportunity with adversity. *Future Microbiology,* 5**,** 1057-1071.

Gagliotti, C., Balode, A., Baquero, F., Degener, J., Grundmann, H., Gur, D., Jarlier, V., Kahlmeter, G., Monen, J., Monnet, D. L., Rossolini, G. M., Suetens, C., Weist, K. &

Heuer, O. 2011. *Escherichia coli* and *Staphylococcus aureus*: bad news and good news from the European Antimicrobial Resistance Surveillance Network (EARS-Net, formerly EARSS), 2002 to 2009. *Euro surveillance,* 16.

Garai, P., Gnanadhas, D. P. & Chakravortty, D. 2012. *Salmonella enterica* serovars Typhimurium and Typhi as model organisms: Revealing paradigm of host-pathogen interactions. *Virulence,* 3**,** 377-388.

García-Fernández, A., Villa, L., Carta, C., Venditti, C., Giordano, A., Venditti, M., Mancini, C. & Carattoli, A. 2012. *Klebsiella pneumoniae* ST258 producing KPC-3 identified in Italy carries novel plasmids and OmpK36/OmpK35 porin variants. *Antimicrobial Agents and Chemotherapy,* 56**,** 2143-2145.

García, B., Latasa, C., Solano, C., García-Del Portillo, F., Gamazo, C. & Lasa, I. 2004. Role of the GGDEF protein family in Salmonella cellulose biosynthesis and biofilm formation. *Molecular Microbiology,* 54**,** 264-277.

Garzon, A., Cano, D. A. & Casadesus, J. 1995. Role of Erf recombinase in P22-mediated plasmid transduction. *Genetics,* 140**,** 427-434.

Gaynes, R., Edwards, J. R. & Natl Nosocomial, I. 2005. Overview of nosocomial infections caused by Gram-negative bacilli. *Clinical Infectious Diseases,* 41**,** 848-854.

Georgopapadakou, N. H. 1993. Penicillin-binding proteins and bacterial resistance to β-lactams. *Antimicrobial Agents and Chemotherapy,* 37**,** 2045-2053.

Ghigo, J. M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature,* 412**,** 442-445.

Ghosh, S. K., Hajra, S., Paek, A. & Jayaram, M. 2006. Mechanisms for chromosome and plasmid segregation. *Annual Review of Biochemistry,* 75**,** 211-241.

Ghuysen, J. M. 1997. Penicillin-binding proteins. Wall peptidoglycan assembly and resistance to penicillin: Facts, doubts and hopes. *International Journal of Antimicrobial Agents,* 8**,** 45-60.

Giakkoupi, P., Papagiannitsis, C. C., Miriagou, V., Pappa, O., Polemis, M., Tryfinopoulou, K., Tzouvelekis, L. S. & Vatopoulos, A. C. 2011. An update of the evolving epidemic of bla_{KPC-2}-carrying *Klebsiella pneumoniae* in Greece (2009-10). *Journal of Antimicrobial Chemotherapy,* 66**,** 1510-1513.

Giannoukos, G., Ciulla, D. M., Huang, K., Haas, B. J., Izard, J., Levin, J. Z., Livny, J., Earl, A. M., Gevers, D., Ward, D. V., Nusbaum, C., Birren, B. W. & Gnirke, A. 2012.

Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. *Genome Biology,* 13**,** R23.

Gilbert, P., Collier, P. J. & Brown, M. R. W. 1990. Influence of growth rate on susceptibility to antimicrobial agents: Biofilms, cell cycle, dormancy, and strigent response. *Antimicrobial Agents and Chemotherapy,* 34**,** 1865-1868.

Gillis, R. J., White, K. G., Choi, K. H., Wagner, V. E., Schweizer, H. P. & Iglewski, B. H. 2005. Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy,* 49**,** 3858-3867.

Giraud, E., Cloeckaert, A., Kerboeuf, D. & Chaslus-Dancla, E. 2000. Evidence for active efflux as the primary mechanism of resistance to ciproffoxacin in *Salmonella enterica* serovar typhimurium. *Antimicrobial Agents and Chemotherapy,* 44**,** 1223-1228.

Goffin, C., Fraipont, C., Ayala, J., Terrak, M., Nguyendisteche, M. & Ghuysen, J. M. 1996. The non-penicillin-binding module of the tripartite penicillin-binding protein 3 of *Escherichia coli* is required for folding and/or stability of the penicillin-binding module and the membrane-anchoring module confers cell septation activity on the folded structure. *Journal of Bacteriology,* 178**,** 5402-5409.

Gootz, T. D., Lescoe, M. K., Dib-Hajj, F., Dougherty, B. A., He, W., Della-Latta, P. & Huard, R. C. 2009. Genetic organization of transposase regions surrounding bla_{KPC} carbapenemase genes on plasmids from *Klebsiella* strains isolated in a New York city hospital. *Antimicrobial Agents and Chemotherapy,* 53**,** 1998-2004.

Gordon, J. I., Hooper, L. V., Mcnevin, M. S., Wong, M. & Bry, L. 1997. Epithelial cell growth and differentiation. III. Promoting diversity in the intestine: conversations between the microflora, epithelium, and diffuse GALT. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 273**,** G565-G570.

Gould, I. M. 2008. The epidemiology of antibiotic resistance. *International Journal of Antimicrobial Agents,* 32**,** S2-S9.

Graham, J. P., Boland, J. J. & Silbergeld, E. 2007. Growth promoting antibiotics in food animal production: An economic analysis. *Public Health Reports,* 122**,** 79-87.

Greenfield, T. J., Ehli, E., Kirshenmann, T., Franch, T., Gerdes, K. & Weaver, K. E. 2000. The antisense RNA of the *par* locus of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. *Molecular Microbiology,* 37**,** 652-660.

Gupta, N., Limbago, B. M., Patel, J. B. & Kallen, A. J. 2011. Carbapenem-resistant *Enterobacteriaceae*: Epidemiology and prevention. *Clinical Infectious Diseases,* 53**,** 60-67.

Hall, M. A., Cole, C. B., Smith, S. L., Fuller, R. & Rolles, C. J. 1990. Factors influencing the presence of fecal lactobacilli in early infancy. *Archives of Disease in Childhood,* 65**,** 185-188.

Hamad, B. 2010. The antibiotics market. *Nature Reviews Drug Discovery,* 9**,** 675-675.

Hammond, M. L. 2004. Ertapenem: A group 1 carbapenem with distinct antibacterial and pharmacological properties. *Journal of Antimicrobial Chemotherapy,* 53**,** ii7-ii9.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology,* 166**,** 557-580.

Harris, P., Paterson, D. & Rogers, B. 2015. Facing the challenge of multidrug-resistant Gram-negative bacilli in Australia. *Medical Journal of Australia,* 202**,** 243-246.

Hawkey, P. M. 2015. Multidrug-resistant Gram-negative bacteria: A product of globalization. *Journal of Hospital Infection,* 89**,** 241-247.

Hawkey, P. M. & Livermore, D. M. 2012. Carbapenem antibiotics for serious infections. *BMJ (Clinical research ed.),* 344**,** e3236.

He, S., Wurtzel, O., Singh, K., Froula, J. L., Yilmaz, S., Tringe, S. G., Wang, Z., Chen, F., Lindquist, E. A., Sorek, R. & Hugenholtz, P. 2010. Validation of two ribosomal RNA removal methods for microbial metatranscriptomics. *Nature Methods,* 7**,** 807-812.

Hedegaard, J., De a Steffensen, S. A., Nørskov-Lauritsen, N., Mortensen, K. K. & Sperling-Petersen, H. U. 1999. Identification of Enterobacteriaceae by partial sequencing of the gene encoding translation initiation factor 2. *International Journal of Systematic Bacteriology,* 49**,** 1531-1538.

Helling, R. B., Kinney, T. & Adams, J. 1981. The maintenance of plasmid-containing organisms in populations of *Escherichia coli*. *Journal of General Microbiology,* 123**,** 129-141.

Hernandez-Alles, S., Alberti, S., Alvarez, D., Domenech-Sanchez, A., Martinez-Martinez, L., Gil, J., Tomas, J. M. & Benedi, V. J. 1999. Porin expression in clinical isolates of *Klebsiella pneumoniae*. *Microbiology,* 145**,** 673-679.

Hernandez-Alles, S., Alberti, S., Rubires, X., Merino, S., Tomas, J. M. & Benedi, V. J. 1995. Isolation of FC3–11, a bacteriophage-specific for the *Klebsiella pneumoniae* porin

OmpK36, and its use for the isolation of porin-deficient mutants. *Canadian Journal of Microbiology,* 41**,** 399-406.

Hidron, Alicia i., Edwards, Jonathan r., Patel, J., Horan, Teresa c., Sievert, Dawn m., Pollock, Daniel a. & Fridkin, Scott k. 2008. NHSN annual update: Antimicrobial-resistant pathogens associated with healthcare-associated infections: Annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control and Hospital Epidemiology,* 29**,** 996-1011.

Hilas, O., Ezzo, D. C. & Jodlowski, T. Z. 2008. Doripenem (doribax), a new carbapenem antibacterial agent. *P & T,* 33**,** 134-80.

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. *European Journal of Cancer Prevention,* 6**,** S43-S45.

Ho, P. L., Lo, W. U., Yeung, M. K., Lin, C. H., Chow, K. H., Ang, I., Tong, A. H. Y., Baojessie, J. Y. J., Lok, S. & Lo, J. Y. C. 2011. Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in Hong Kong. *PLoS ONE,* 6**,** e17989.

Holden, C. 2002. Cell biology: Alliance launched to model *E. coli*. *Science,* 297**,** 1459-1460.

Holtje, J. V. 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiology and Molecular Biology Reviews,* 62**,** 181-203.

Hooper, L. V., Midtvedt, T. & Gordon, J. I. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual Review of Nutrition,* 22**,** 283-307.

Hopwood, D., Levy, S., Wenzel, R. P., Georgo-Papadakou, N., Baltz, R. H., Bhavnani, S. & Cox, E. 2007. News Feature: A call to arms. *Nature Reviews Drug Discovery,* 6**,** 8-12.

Hornish, R. E. & Kotarski, S. F. 2002. Cephalosporins in veterinary medicine - ceftiofur use in food animals. *Current Topics in Medicinal Chemistry,* 2**,** 717-31.

HPA 2011. Health Protection Report - Carbapenemase-producing *Enterobacteriaceae* in the UK, 2003-2007.

Hrabák, J., Papagiannitsis, C. C., Študentová, V., Jakubu, V., Fridrichová, M., Zemlickova, H., Adamkova, V., Bartonikova, N., Bartova, M., Bendova, E., Bergerova, T., Bohunova, Z., Capova, E., Chmelarova, E., Dovalova, M., Glasnak, M., Hanslianova, M., Haskova, V., Heinigeova, B., Hornikova, M., Horova, B., Janeckova, J., Jezek, P., Jindrak, V., Kolar, M., Kolarova, L., Kůrková, V., Linhart, P., Nedvedova, H.,

Niemczykova, J., Nyc, O., Petkov, V., Pokorna, Z., Pomykal, J., Puchalkova, B., Rumlerova, M., Ryskova, L., Scharfen, J., Sekacova, A., Skacaniova, H., Simeckova, E., Sosikova, M., Stastna, E., Steinerova, A., Stolbova, M., Tejkalova, R., Trojan, L., Typovska, H., Uhlirova, E., Vesela, E., Zalabska, E., Zamazalova, D. & Zaruba, R. 2013. Carbapenemase-producing *Klebsiella pneumoniae* in the Czech Republic in 2011. *Eurosurveillance,* 18.

Hughes, V. M. & Datta, N. 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature,* 302**,** 725-726.

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., Hilton, A., Armstrong, R., Smith, C., Grant, J., Shoo, S. & Forsythe, S. 2009. Neonatal enteral feeding tubes as loci for colonisation by members of the Enterobacteriaceae. *BMC Infectious Diseases,* 9**,** 146.

Iaconis, J. P., Pitkin, D. H., Sheikh, W. & Nadler, H. L. 1997. Comparison of antibacterial activities of meropenem and six other antimicrobials against *Pseudomonas aeruginosa* isolates from North American studies and clinical trials. *Clinical Infectious Diseases,* 24**,** S191-S196.

IDSA 2011. Combating antimicrobial resistance: Policy recommendations to save lives. *Clinical Infectious Diseases,* 52**,** S397-S428.

Insua, J. L., Llobet, E., Moranta, D., Pérez-Gutiérrez, C., Tomás, E., Garmendia, E. & Bengoechea, J. A. 2013. Modeling *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella*. *Infection and Immunity,* 81**,** 3552-3565.

Ishino, F., Mitsui, K., Tamaki, S. & Matsuhashi, M. 1980. Dual enzyme-activities of cell wall peptidoglycan synthesis, peptidoglycan transglycosylase and penicillin-sensitive transpeptidase, in purified preparations of *Escherichia coli* penicillin-binding protein-1a. *Biochemical and Biophysical Research Communications,* 97**,** 287-293.

Jacoby, G. A., Mills, D. M. & Chow, N. 2004. Role of β-lactamases and porins in resistance to ertapenem and other β-lactams in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 48**,** 3203-3206.

Jain, A., Hopkins, K. L., Turton, J., Doumith, M., Hill, R., Loy, R., Meunier, D., Pike, R., Livermore, D. M. & Woodford, N. 2014. NDM carbapenemases in the United Kingdom: An analysis of the first 250 cases. *Journal of Antimicrobial Chemotherapy,* 69**,** 1777-1784.

Jain, S. & Chen, J. 2007. Attachment and biofilm formation by various serotypes of *Salmonella* as influenced by cellulose production and thin aggregative fimbriae biosynthesis. *Journal of Food Protection,* 70**,** 2473-2479.

Jakobsen, Ø. M., Benichou, A., Flickinger, M. C., Valla, S., Ellingsen, T. E. & Brautaset, T. 2006. Upregulated transcription of plasmid and chromosomal ribulose monophosphate pathway genes is critical for methanol assimilation rate and methanol tolerance in the methylotrophic bacterium *Bacillus methanolicus*. *Journal of Bacteriology,* 188**,** 3063-3072.

Jean, S. S., Lee, W. S., Lam, C., Hsu, C. W., Chen, R. J. & Hsueh, P. R. 2015. Carbapenemase-producing Gram-negative bacteria: Current epidemics, antimicrobial susceptibility and treatment options. *Future Microbiology,* 10**,** 407-425.

Jovetic, S., Zhu, Y., Marcone, G. L., Marinelli, F. & Tramper, J. 2010. β-lactam and glycopeptide antibiotics: first and last line of defense? *Trends in Biotechnology,* 28**,** 596-604.

Kaczmarek, F. S., Gootz, T. D., Dib-Hajj, F., Shang, W. C., Hallowell, S. & Cronan, M. 2004. Genetic and molecular characterization of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* with unusually high resistance to ampicillin. *Antimicrobial Agents and Chemotherapy,* 48**,** 1630-1639.

Kahan, J. S., Kahan, F. M., Goegelman, R., Currie, S. A., Jackson, M., Stapley, E. O., Miller, T. W., Miller, A. K., Hendlin, D., Mochales, S., Hernandez, S., Woodruff, H. B. & Birnbaum, J. 1979. Thienamycin, a new β-lactam antibiotic.1. Discovery, taxonomy, isolation and physical properties. *Journal of Antibiotics,* 32**,** 1-12.

Kallen, A. J., Beekmann, S. E., Limbago, B., Lentnek, A. L., Polgreen, P. M., Patel, J. & Srinivasan, A. 2011. Prevalence of β-lactam nonsusceptible Gram-negative bacilli and use and interpretation of current susceptibility breakpoints: a survey of infectious disease physicians. *Diagnostic Microbiology and Infectious Disease,* 71**,** 316-319.

Kalpoe, J. S., Sonnenberg, E., Factor, S. H., Del Rio Martin, J., Schiano, T., Patel, G. & Huprikar, S. 2012. Mortality associated with carbapenem-resistant *Klebsiella pneumoniae* infections in liver transplant recipients. *Liver Transplantation,* 18**,** 468-474.

Kang, C. I., Kim, S. H., Wan, B. P., Lee, K. D., Kim, H. B., Kim, E. C., Oh, M. D. & Choe, K. W. 2005. Bloodstream infections caused by antibiotic-resistant Gram-negative bacilli:

Risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. *Antimicrobial Agents and Chemotherapy,* 49**,** 760-766.

Katayama, Y., Zhang, H. Z. & Chambers, H. F. 2004. PBP 2a mutations producing very-high-level resistance to β-lactams. *Antimicrobial Agents and Chemotherapy,* 48**,** 453-459.

Kenyon, T. A., Valway, S. E., Ihle, W. W., Onorato, I. M. & Castro, K. G. 1996. Transmission of multidrug-resistant *Mycobacterium tuberculosis* during a long airplane flight. *New England Journal of Medicine,* 334**,** 933-938.

Keseler, I. M., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I. T., Peralta-Gil, M. & Karp, P. D. 2005. EcoCyc: A comprehensive database resource for *Escherichia coli*. *Nucleic Acids Research,* 33**,** D334-D337.

Kinana, A. D., Vargiu, A. V. & Nikaido, H. 2013. Some ligands enhance the efflux of other ligands by the *Escherichia coli* multidrug pump AcrB. *Biochemistry,* 52**,** 8342-8351.

Kitchel, B., Rasheed, J. K., Endimiani, A., Hujer, A. M., Anderson, K. F., Bonomo, R. A. & Patel, J. B. 2010. Genetic factors associated with elevated carbapenem resistance in KPC-producing *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 54**,** 4201-4207.

Kitchel, B., Rasheed, J. K., Patel, J. B., Srinivasan, A., Navon-Venezia, S., Carmeli, Y., Brolund, A. & Giske, C. G. 2009. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: Clonal expansion of multilocus sequence Type 258. *Antimicrobial Agents and Chemotherapy,* 53**,** 3365-3370.

Klevens, R. M., Edwards, J. R., Richards, C. L., Jr., Horan, T. C., Gaynes, R. P., Pollock, D. A. & Cardo, D. M. 2007. Estimating health care-associated infections and deaths in U.S. hospitals, 2002. *Public Health Reports* 122**,** 160-166.

Kresse, H., Belsey, M. J. & Rovini, H. 2007. The antibacterial drugs market. *Nature Reviews Drug Discovery,* 6**,** 19-20.

Krishnamoorthy, G., Tikhonova, E. B., Dhamdhere, G. & Zgurskaya, H. I. 2013. On the role of TolC in multidrug efflux: The function and assembly of AcrAB-TolC tolerate significant depletion of intracellular TolC protein. *Molecular Microbiology,* 87**,** 982-997.

Kumar, A. & Schweizer, H. P. 2005. Bacterial resistance to antibiotics: Active efflux and reduced uptake. *Advanced Drug Delivery Reviews,* 57**,** 1486-1513.

Kwon, D. H., Dore, M. P., Kim, J. J., Kato, M., Lee, M., Wu, J. Y. & Graham, D. Y. 2003. High-level β-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy,* 47**,** 2169-2178.

Lambert, P. A. 2005. Bacterial resistance to antibiotics: Modified target sites. *Advanced Drug Delivery Reviews,* 57**,** 1471-1485.

Lamers, R. P., Cavallari, J. F. & Burrows, L. L. 2013. The efflux inhibitor phenylalanine-arginine β-naphthylamide (PAβN) permeabilizes the outer membrane of Gram-negative bacteria. *PLoS ONE,* 8**,** e60666.

Landman, D., Bratu, S. & Quale, J. 2009. Contribution of OmpK36 to carbapenem susceptibility in KPC-producing *Klebsiella pneumoniae*. *Journal of Medical Microbiology,* 58**,** 1303-1308.

Landman, D., Salamera, J., Singh, M. & Quale, J. 2011. Accuracy of carbapenem nonsusceptibility for identification of KPC-possessing Enterobacteriaceae by use of the revised CLSI breakpoints. *Journal of Clinical Microbiology,* 49**,** 3931-3933.

Landman, D., Urban, C., Bäcker, M., Kelly, P., Shah, N., Babu, E., Bratu, S. & Quale, J. 2010. Susceptibility profiles, molecular epidemiology, and detection of KPC-producing *Escherichia coli* isolates from the New York City vicinity. *Journal of Clinical Microbiology,* 48**,** 4604-4607.

Lavigne, J. P., Cuzon, G., Combescure, C., Bourg, G., Sotto, A. & Nordmann, P. 2013. Virulence of *Klebsiella pneumoniae* isolates harboring bla_{KPC-2} carbapenemase gene in a *Caenorhabditis elegans* model. *PLoS ONE,* 8**,** e67847.

Lavigne, J. P., Sotto, A., Nicolas-Chanoine, M. H., Bouziges, N., Bourg, G., Davin-Regli, A. & Pagès, J. M. 2012. Membrane permeability, a pivotal function involved in antibiotic resistance and virulence in *Enterobacter aerogenes* clinical isolates. *Clinical Microbiology and Infection,* 18**,** 539-545.

Leavitt, A., Chmelnitsky, I., Carmeli, Y. & Navon-Venezia, S. 2010a. Complete nucleotide sequence of KPC-3-encoding plasmid pKpQIL in the epidemic *Klebsiella pneumoniae* sequence type 258. *Antimicrobial Agents and Chemotherapy,* 54**,** 4493-4496.

Leavitt, A., Chmelnitsky, I., Ofek, I., Carmeli, Y. & Navon-Venezia, S. 2010b. Plasmid pKpQIL encoding KPC-3 and TEM-1 confers carbapenem resistance in an extremely drug-resistant epidemic *Klebsiella pneumoniae* strain. *Journal of Antimicrobial Chemotherapy,* 65**,** 243-248.

Leavitt, A., Navon-Venezia, S., Chmelnitsky, I., Schwaber, M. J. & Carmeli, Y. 2007. Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* strains in an Israeli hospital. *Antimicrobial Agents and Chemotherapy,* 51**,** 3026-3029.

Lee, Y., Kim, B. S., Chun, J., Yong, J. H., Lee, Y. S., Yoo, J. S., Yong, D., Hong, S. G., D'souza, R., Thomson, K. S., Lee, K. & Chong, Y. 2014. Clonality and resistome analysis of KPC-producing *Klebsiella pneumoniae* strain isolated in Korea using whole genome sequencing. *BioMed Research International,* 2014**,** 1-6.

Lenski, R. E. 1998. Bacterial evolution and the cost of antibiotic resistance. *International Microbiology,* 1**,** 265-270.

Lenski, R. E. & Bouma, J. E. 1987. Effects of segregation and selection on instability of plasmid pACYC184 in *Escherichia coli* B. *Journal of Bacteriology,* 169**,** 5314-5316.

Lenski, R. E. & Nguyen, T. T. 1988. Stability of recombinant DNA and its effects on fitness. *Trends in Biotechnology,* 6**,** S18-S20.

Lenski, R. E., Simpson, S. C. & Nguyen, T. T. 1994. Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *Journal of Bacteriology,* 176**,** 3140-3147.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy,* 45**,** 999-1007.

Li, Y., Powell, D. A., Shaffer, S. A., Rasko, D. A., Pelletier, M. R., Leszyk, J. D., Scott, A. J., Masoudi, A., Goodlett, D. R., Wang, X., Raetze, C. R. H. & Ernst, R. K. 2012. LPS remodeling is an evolved survival strategy for bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 8716-8721.

Liebana, E., Batchelor, M., Hopkins, K. L., Clifton-Hadley, F. A., Teale, C. J., Foster, A., Barker, L., Threlfall, E. J. & Davies, R. H. 2006. Longitudinal farm study of extended-spectrum β-lactamase-mediated resistance. *Journal of Clinical Microbiology,* 44**,** 1630-1634.

Liew, Y.-X., Krishnan, P., Yeo, C. L., Tan, T. Y., Lee, S. Y., Lim, W. P., Lee, W., Hsu, L. Y. & Network for Antimicrobial Resistance, S. 2011. Surveillance of broad-spectrum antibiotic prescription in Singaporean hospitals: A 5-Year longitudinal study. *PLoS ONE,* 6**,** e28751.

Lim, S. P. & Nikaido, H. 2010. Kinetic parameters of efflux of penicillins by the multidrug efflux transporter AcrAB-TolC of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy,* 54**,** 1800-1806.

Lioy, V. S., Rey, O., Balsa, D., Pellicer, T. & Alonso, J. C. 2010. A toxin-antitoxin module as a target for antimicrobial development. *Plasmid,* 63**,** 31-39.

Lleo, M. D., Canepari, P., Cornaglia, G., Fontana, R. & Satta, G. 1987. Bacteriostatic and bactericidal activities of β-lactams against *Streptococcus (enterococcus)-faecium* are associated with saturation of different penicillin-binding proteins. *Antimicrobial Agents and Chemotherapy,* 31**,** 1618-1626.

Loftie-Eaton, W. & Rawlings, D. E. 2010. Evolutionary competitiveness of two natural variants of the IncQ-like plasmids, pRAS3.1 and pRAS3.2. *Journal of Bacteriology,* 192**,** 6182-6190.

Lomovskaya, O. & Bostian, K. A. 2006. Practical applications and feasibility of efflux pump inhibitors in the clinic—A vision for applied use. *Biochemical Pharmacology,* 71**,** 910-918.

Lomovskaya, O., Lee, A., Hoshino, K., Ishida, H., Mistry, A., Warren, M. S., Boyer, E., Chamberland, S. & Lee, V. J. 1999. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy,* 43**,** 1340-1346.

Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S., Renau, T., Leger, R., Hecker, S., Watkins, W., Hoshino, K., Ishida, H. & Lee, V. J. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: Novel agents for combination therapy. *Antimicrobial Agents and Chemotherapy,* 45**,** 105-116.

Lorenz, M. G., Gerjets, D. & Wackernagel, W. 1991. Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. *Archives of Microbiology,* 156**,** 319-326.

Lorenz, M. G. & Wackernagel, W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews,* 58**,** 563-602.

Lueangarun, S. & Leelarasamee, A. 2012. Impact of inappropriate empiric antimicrobial therapy on mortality of septic patients with bacteremia: A retrospective study. *Interdisciplinary Perspectives on Infectious Diseases,* 2012**,** 1-13.

Lugtenberg, B. & Van Alphen, L. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes,* 737**,** 51-115.

Lujan, S. A., Guogas, L. M., Ragonese, H., Matson, S. W. & Redinbo, M. R. 2007. Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 12282-12287.

Luo, N., Pereira, S., Sahin, O., Lin, J., Huanq, S., Michel, L. & Zhanq, Q. 2005. Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 541-546.

Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *Journal of Bacteriology,* 175**,** 6299-6313.

Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Molecular Microbiology,* 16**,** 45-55.

Ma, L., Siu, L. K., Lin, J. C., Wu, T. L., Fung, C. P., Wang, J. T., Lu, P. L. & Chuang, Y. C. 2013. Updated molecular epidemiology of carbapenem-non-susceptible *Escherichia coli* in Taiwan: First identification of KPC-2 or NDM-1-producing *E. coli* in Taiwan. *BMC Infectious Diseases,* 13**,** 599.

Macgowan, A. P. 2008. Clinical implications of antimicrobial resistance for therapy. *Journal of Antimicrobial Chemotherapy,* 62**,** ii105-ii114.

Maclean, R. C., Hall, A. R., Perron, G. G. & Buckling, A. 2010. The evolution of antibiotic resistance: insight into the roles of molecular mechanisms of resistance and treatment context. *Discovery Medicine,* 10**,** 112-8.

Marciano, D. C., Karkouti, O. Y. & Palzkill, T. 2007. A fitness cost associated with the antibiotic resistance enzyme SME-1 β-lactamase. *Genetics,* 176**,** 2381-2392.

Marger, M. D. & Saier Jr, M. H. 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends in Biochemical Sciences,* 18**,** 13-20.

Marshall, A. J. H. & Piddock, L. J. V. 1993. Effect of cations and EDTA upon the activity of 18 quinolones, gentamicin, ceftazidime and polymixin against Gram-negative and Gram-positive bacteria. *Drugs,* 46**,** 150-151.

Martínez-Flores, I., Cano, R., Bustamante, V. H., Calva, E. & Puente, J. L. 1999. The *ompB* operon partially determines differential expression of OmpC in *Salmonella typhi* and *Escherichia coli*. *Journal of Bacteriology,* 181**,** 556-562.

Martínez-Martínez, L., Pascual, A., Conejo, M. D. C., García, I., Joyanes, P., Doménech-Sánchez, A. & Benedí, V. J. 2002. Energy-dependent accumulation of norfloxacin and porin expression in clinical isolates of *Klebsiella pneumoniae* and relationship to extended-spectrum β-lactamase production. *Antimicrobial Agents and Chemotherapy,* 46**,** 3926-3932.

Mathers, A. J., Peirano, G. & Pitout, J. D. D. 2015. The role of epidemic resistance plasmids and international high- risk clones in the spread of multidrug-resistant Enterobacteriaceae. *Clinical Microbiology Reviews,* 28**,** 565-591.

Matic, V., Bozdogan, B., Jacobs, M. R., Ubukata, K. & Appelbaum, P. C. 2003. Contribution of β-lactamase and PBP amino acid substitutions to amoxicillin/clavulanate resistance in β-lactamase-positive, amoxicillin/clavulanate-resistant *Haemophilus influenzae*. *Journal of Antimicrobial Chemotherapy,* 52**,** 1018-1021.

Matsumoto, Y., Hayama, K., Sakakihara, S., Nishino, K., Noji, H., Iino, R. & Yamaguchi, A. 2011. Evaluation of multidrug efflux pump inhibitors by a new method using microfluidic channels. *PLoS ONE,* 6**,** e18547.

Mcclelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R. & Wilson, R. K. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature,* 413**,** 852-856.

Mcmurry, L. M., Oethinger, M. & Levy, S. B. 1998. Overexpression of *marA*, *soxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiology Letters,* 166**,** 305-309.

Miller, K., O'neill, A. J. & Chopra, I. 2002. Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes. *Journal of Antimicrobial Chemotherapy,* 49**,** 925-934.

Miriagou, V., Tzouvelekis, L. S., Rossiter, S., Tzelepi, E., Angulo, F. J. & Whichard, J. A. 2003. Imipenem resistance in a Salmonella clinical strain due to plasmid-mediated class a carbapenemase KPC-2. *Antimicrobial Agents and Chemotherapy,* 47**,** 1297-1300.

Mitsuhashi, S., Hashimoto, H., Egawa, R., Tanaka, T. & Nagai, Y. 1967. Drug resistance of enteric bacteria. IX. Distribution of R factors in gram-negative bacteria from clinical sources. *Journal of Bacteriology,* 93**,** 1242-1245.

Moellering, R. C., Eliopoulos, G. M. & Sentochnik, D. E. 1989. The carbapenems: New broad spectrum β-lactam antibiotics. *Journal of Antimicrobial Chemotherapy,* 24**,** 1-7.

Mohr, J. F. 2008. Update on the efficacy and tolerability of meropenem in the treatment of serious bacterial infections. *Clinical Infectious Diseases,* 47**,** S41-S51.

Moland, E. S., Hanson, N. D., Herrera, V. L., Black, J. A., Lockhart, T. J., Hossain, A., Johnson, J. A., Goering, R. V. & Thomson, K. S. 2003. Plasmid-mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. *Journal of Antimicrobial Chemotherapy,* 51**,** 711-714.

Moran, N. A., Russell, J. A., Koga, R. & Fukatsu, T. 2005. Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Applied and Environmental Microbiology,* 71**,** 3302-3310.

Morris, D., Boyle, F., Ludden, C., Condon, I., Hale, J., O'connell, N., Power, L., Boo, T. W., Dhanji, H., Lavallee, C., Woodford, N. & Cormican, M. 2011. Production of KPC-2 carbapenemase by an *Escherichia coli* clinical isolate belonging to the international ST131 clone. *Antimicrobial Agents and Chemotherapy,* 55**,** 4935-4936.

Moyes, R. B., Reynolds, J. & Breakwell, D. P. 2009. Differential staining of bacteria: Gram stain. *Current Protocols in Microbiology,* Appendix 3**,** Appendix 3C.

Muniesa, M., Garcia, A., Miro, E., Mirelis, B., Prats, G., Jofre, J. & Navarro, F. 2004. Bacteriophages and diffusion of β-lactamase genes. *Emerging Infectious Diseases,* 10**,** 1134-1137.

Murakami, S., Nakashima, R., Yamashita, E., Matsumoto, T. & Yamaguchi, A. 2006. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. *Nature,* 443**,** 173-179.

Musson, D. G., Majumdar, A., Birk, K., Holland, S., Wickersham, P., Li, S. X., Mistry, G., Fisher, A., Waldman, S., Greenberg, H., Deutsch, P. & Rogers, J. D. 2003. Pharmacokinetics of intramuscularly administered ertapenem. *Antimicrobial Agents and Chemotherapy,* 47**,** 1732-1735.

Naas, T., Cuzon, G., Villegas, M. V., Lartigue, M. F., Quinn, J. P. & Nordmann, P. 2008. Genetic structures at the origin of acquisition of the β-lactamase bla_{KPC} gene. *Antimicrobial Agents and Chemotherapy,* 52**,** 1257-1263.

Naas, T., Nordmann, P., Vedel, G. & Poyart, C. 2005. Plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC in a *Klebsiella pneumoniae* isolate from France. *Antimicrobial Agents and Chemotherapy,* 49**,** 4423-4424.

Nagano, K. & Nikaido, H. 2009. Kinetic behavior of the major multidrug efflux pump AcrB of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 5854-5858.

Nahid, F., Khan, A. A., Rehman, S. & Zahra, R. 2013. Prevalence of metallo-β-lactamase NDM-1-producing multi-drug resistant bacteria at two Pakistani hospitals and implications for public health. *Journal of Infection and Public Health,* 6**,** 487-493.

Nakagawa, J., Tamaki, S. & Matsuhashi, M. 1979. Purified penicillin binding proteins 1bs from *Escherichia coli* membrane showing activities of both peptidoglycan polymerase and peptidoglycan crosslinking enzyme. *Agricultural and Biological Chemistry,* 43**,** 1379-1380.

Nakamura, H. 1968. Genetic determination of resistance to acriflavine, phenethyl alcohol, and sodium dodecyl sulfate in *Escherichia coli*. *Journal of Bacteriology,* 96**,** 987-996.

NAO. 2009. *Reducing healthcare associated infections in hospitals in England* [Online]. Norwich: The Stationery Office. Available: http://www.nao.org.uk/wp-content/uploads/2009/06/0809560es.pdf [Accessed 14th June 2015].

Navon-Venezia, S., Leavitt, A., Schwaber, M. J., Rasheed, J. K., Srinivasan, A., Patel, J. B., Carmeli, Y. & Israeli, K. P. C. K. S. G. 2009. First report on a hyperepidemic clone of KPC-3-producing *Klebsiella pneumoniae* in Israel genetically related to a strain causing outbreaks in the United States. *Antimicrobial Agents and Chemotherapy,* 53**,** 818-820.

Nayler, J. H. C. 1971. Structure-activity relationships in semi-synthetic penicillins. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* 179**,** 357-367.

Nelson, D. E. & Young, K. D. 2000. Penicillin binding protein 5 affects cell diameter, contour, and morphology of *Escherichia coli*. *Journal of Bacteriology,* 182**,** 1714-1721.

Nicas, T. I. & Hancock, R. E. 1983. *Pseudomonas aeruginosa* outer membrane permeability: isolation of a porin protein F-deficient mutant. *Journal of Bacteriology,* 153**,** 281-285.

Nicolau, D. P. 2008. Carbapenems: a potent class of antibiotics. *Expert Opinion on Pharmacotherapy,* 9**,** 23-37.

Nijssen, S., Florijn, A., Bonten, M. J. M., Schmitz, F. J., Verhoef, J. & Fluit, A. C. 2004. β-lactam susceptibilities and prevalence of ESBL-producing isolates among more than 5000 European Enterobacteriaceae isolates. *International Journal of Antimicrobial Agents,* 24**,** 585-591.

Nikaido, H. 1998. Antibiotic resistance caused by Gram-negative multidrug efflux pumps. *Clinical Infectious Diseases,* 27**,** S32-S41.

Nikaido, H. 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Seminars in Cell & Developmental Biology,* 12**,** 215-223.

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews,* 67**,** 593-656.

Nikaido, H., Basina, M., Nguyen, V. & Rosenberg, E. Y. 1998. Multidrug efflux pump AcrAB of *Salmonella* Typhimurium excretes only those β-lactam antibiotics containing lipophilic side chains. *Journal of Bacteriology,* 180**,** 4686-4692.

Nikaido, H. & Rosenberg, E. Y. 1983. Porin channels in *Escherichia coli*: Studies with liposomes reconstituted from purified proteins. *Journal of Bacteriology,* 153**,** 241-252.

Nikaido, H. & Zgurskaya, H. I. 2001. AcrAB and related multidrug efflux pumps of *Escherichia coli*. *Journal of Molecular Microbiology and Biotechnology,* 3**,** 215-218.

Nishino, K., Latifi, T. & Groisman, E. A. 2006. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology,* 59**,** 126-141.

Nordmann, P. 2014. Carbapenemase-producing Enterobacteriaceae: Overview of a major public health challenge. *Medecine et Maladies Infectieuses,* 44**,** 51-56.

Nordmann, P., Dortet, L. & Poirel, L. 2012a. Carbapenem resistance in *Enterobacteriaceae*: Here is the storm! *Trends in Molecular Medicine,* 18**,** 263-272.

Nordmann, P., Gniadkowski, M., Giske, C. G., Poirel, L., Woodford, N., Miriagou, V., Akova, M., Naas, T., Seifert, H., Livermore, D., Bogaerts, P., Glupczynski, Y., Canton, R., Rossolini, G. M., Giske, C., Adler, A., Carmeli, Y., Navon-Venezia, S., Samuelsen, O. & Cornaglia, G. 2012b. Identification and screening of carbapenemase-producing Enterobacteriaceae. *Clinical Microbiology and Infection,* 18**,** 432-438.

Norrander, J., Kempe, T. & Messing, J. 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene,* 26**,** 101-106.

Novick, R. P. 1987. Plasmid incompatibility. *Microbiological Reviews,* 51**,** 381-395.

O'Neill, J. 2014. *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations* [Online]. United Kingdom. Available: http://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf [Accessed 14th June 2015].

Oethinger, M., Kern, W. V., Jellen-Ritter, A. S., Mcmurry, L. M. & Levy, S. B. 2000. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. *Antimicrobial Agents and Chemotherapy,* 44**,** 10-13.

Ohnishi, M., Watanabe, Y., Ono, E., Takahashi, C., Oya, H., Kuroki, T., Shimuta, K., Okazaki, N., Nakayama, S. I. & Watanabe, H. 2010. Spread of a chromosomal cefixime-resistant *penA* gene among different *Neisseria gonorrhoeae* lineages. *Antimicrobial Agents and Chemotherapy,* 54**,** 1060-1067.

Ohse, M., Takahashi, K., Kadowaki, Y. & Kusaoke, H. 1995. Effects of plasmid DNA sizes and several other factors on transformation of *Bacillus subtilis* ISW1214 with plasmid DNA by electroporation. *Bioscience, Biotechnology and Biochemistry,* 59**,** 1433-1437.

Opperman, T. J., Kwasny, S. M., Kim, H. S., Nguyen, S. T., Houseweart, C., D'souza, S., Walker, G. C., Peet, N. P., Nikaido, H. & Bowlin, T. L. 2014. Characterization of a novel pyranopyridine inhibitor of the AcrAB efflux pump of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy,* 58**,** 722-733.

Oteo, J., Delgado-Iribarren, A., Vega, D., Bautista, V., Rodríguez, M. C., Velasco, M., Saavedra, J. M., Pérez-Vázquez, M., García-Cobos, S., Martínez-Martínez, L. & Campos, J. 2008. Emergence of imipenem resistance in clinical *Escherichia coli* during therapy. *International Journal of Antimicrobial Agents,* 32**,** 534-537.

Otter, J. A. 2014. What's trending in the infection prevention and control literature? From HIS 2012 to HIS 2014, and beyond. *Journal of Hospital Infection,* 89**,** 229-236.

Ow, D. S. W., Nissom, P. M., Philp, R., Oh, S. K. W. & Yap, M. G. S. 2006. Global transcriptional analysis of metabolic burden due to plasmid maintenance in *Escherichia coli* DH5α during batch fermentation. *Enzyme and Microbial Technology,* 39**,** 391-398.

Pagès, J.-M. & Amaral, L. 2009. Mechanisms of drug efflux and strategies to combat them: Challenging the efflux pump of Gram-negative bacteria. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* 1794**,** 826-833.

Pages, J.-M. & Davin, A. V. 2015. *Enterobacter aerogenes* and *Enterobacter cloacae*; versatile bacterial pathogens confronting antibiotic treatment. *Frontiers in Microbiology,* 6.

Pages, J.-M., James, C. E. & Winterhalter, M. 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nature Reviews Microbiology,* 6**,** 893-903.

Pages, J.-M., Lavigne, J.-P., Leflon-Guibout, V., Marcon, E., Bert, F., Noussair, L. & Nicolas-Chanoine, M.-H. 2009. Efflux pump, the masked side of ß-lactam resistance in *Klebsiella pneumoniae* clinical isolates. *PLoS ONE,* 4**,** e4817.

Pai, H., Kim, J. W., Kim, J., Lee, J. H., Choe, K. W. & Gotoh, N. 2001. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. *Antimicrobial Agents and Chemotherapy,* 45**,** 480-484.

Pannek, S., Higgins, P. G., Steinke, P., Jonas, D., Akova, M., Bohnert, J. A., Seifert, H. & Kern, W. V. 2006. Multidrug efflux inhibition in *Acinetobacter baumannii*: Comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-β-naphthylamide. *Journal of Antimicrobial Chemotherapy,* 57**,** 970-974.

Paphitou, N. I. 2013. Antimicrobial resistance: Action to combat the rising microbial challenges. *International Journal of Antimicrobial Agents,* 42**,** S25-S28.

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A. & Bonomo, R. A. 2011. Carbapenems: Past, present, and future. *Antimicrobial Agents and Chemotherapy,* 55**,** 4943-4960.

Partridge, S. R. 2014. *Tn*4401 Carrying bla_{KPC} is inserted within another insertion in pKpQIL and related plasmids. *Journal of Clinical Microbiology,* 52**,** 4448-4449.

Paterson, D. L. & Bonomo, R. A. 2005. Extended-spectrum β-lactamases: a clinical update. *Clinical Microbiology Reviews,* 18**,** 657-686.

Paulsen, I. T., Skurray, R. A., Tam, R., Saler, M. H., Turner, R. J., Weiner, J. H., Goldberg, E. B. & Grinius, L. L. 1996. The SMR family: A novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. *Molecular Microbiology,* 19**,** 1167-1175.

Paytubi, S., Aznar, S., Madrid, C., Balsalobre, C., Dillon, S. C., Dorman, C. J. & Juárez, A. 2013. A novel role for antibiotic resistance plasmids in facilitating *Salmonella* adaptation to non-host environments. *Environmental Microbiology,* 16**,** 950-962.

Peirano, G. & Pitout, J. D. D. 2010. Molecular epidemiology of *Escherichia coli* producing CTX-M β-lactamases: the worldwide emergence of clone ST131 O25:H4. *International Journal of Antimicrobial Agents,* 35**,** 316-321.

Pérez-Cruz, C., Delgado, L., López-Iglesias, C. & Mercade, E. 2015. Outer-inner membrane vesicles naturally secreted by Gram-negative pathogenic bacteria. *PLoS ONE,* 10.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. & Waddell, J. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *Journal of Antimicrobial Chemotherapy,* 53**,** 28-52.

Piddock, L. J. V. 2006a. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical Microbiology Reviews,* 19**,** 382-402.

Piddock, L. J. V. 2006b. Multidrug-resistance efflux pumps - Not just for resistance. *Nature Reviews Microbiology,* 4**,** 629-636.

Piddock, L. J. V. 2012. The crisis of no new antibiotics—what is the way forward? *The Lancet Infectious Diseases,* 12**,** 249-253.

Piddock, L. J. V., Griggs, D. J., Hall, M. C. & Jin, Y. F. 1993. Ciprofloxacin resistance in clinical isolates of *Salmonella* Typhimurium obtained from two patients. *Antimicrobial Agents and Chemotherapy,* 37**,** 662-666.

Piddock, L. J. V., Traynor, E. A. & Wise, R. 1990. A comparison of the mechanisms of decreased susceptibility of aztreonam-resistant and ceftazidime-resistant Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy,* 26**,** 749-762.

Pittet, D., Mourouga, P., Perneger, T. V. & Infection Control, P. 1999. Compliance with handwashing in a teaching hospital. *Annals of Internal Medicine,* 130**,** 126-130.

Plüss-Suard, C., Pannatier, A., Kronenberg, A., Mühlemann, K. & Zanetti, G. 2011. Hospital antibiotic consumption in Switzerland: comparison of a multicultural country with Europe. *Journal of Hospital Infection,* 79**,** 166-171.

Poirel, L., Lagrutta, E., Taylor, P., Pham, J. & Nordmann, P. 2010. Emergence of metallo-β-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. *Antimicrobial Agents and Chemotherapy,* 54**,** 4914-4916.

Poirel, L., Revathi, G., Bernabeu, S. & Nordmann, P. 2011. Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. *Antimicrobial Agents and Chemotherapy,* 55**,** 934-936.

Polilli, E., Parruti, G., Fazii, P., D'antonio, D., Palmieri, D., D'incecco, C., Mangifesta, A., Garofalo, G., Del Duca, L., D'amario, C., Scimia, M., Cortesi, V. & Fortunato, V. 2011. Rapidly controlled outbreak of *Serratia marcescens* infection/colonisations in a neonatal intensive care unit, Pescara general hospital, Pescara, Italy, April 2011. *Eurosurveillance,* 16.

Poole, K. 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. *Antimicrobial Agents and Chemotherapy,* 44**,** 2233-2241.

Poole, K. 2004. Efflux-mediated multiresistance in Gram-negative bacteria. *Clinical Microbiology and Infection,* 10**,** 12-26.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy,* 56**,** 20-51.

Poole, K. & Lomovskaya, O. 2006. Can efflux inhibitors really counter resistance? *Drug Discovery Today: Therapeutic Strategies,* 3**,** 145-152.

Pope, C. F., Gillespie, S. H., Moore, J. E. & Mchugh, T. D. 2010. Approaches to measure the fitness of *Burkholderia cepacia* complex isolates. *Journal of Medical Microbiology,* 59**,** 679-686.

Potera, C. 1996. Biofilms invade microbiology. *Science,* 273**,** 1795-1797.

Poulou, A., Voulgari, E., Vrioni, G., Koumaki, V., Xidopoulos, G., Chatzipantazi, V., Markou, F. & Tsakris, A. 2013. Outbreak caused by an ertapenem-resistant, CTX-M-15-producing *Klebsiella pneumoniae* sequence type 101 clone carrying an OmpK36 porin variant. *Journal of Clinical Microbiology,* 51**,** 3176-3182.

Pournaras, S., Kristo, I., Vrioni, G., Ikonomidis, A., Poulou, A., Petropoulou, D. & Tsakris, A. 2010. Characteristics of meropenem heteroresistance in *Klebsiella pneumoniae* carbapenemase (KPC)-producing clinical isolates of *K. pneumoniae*. *Journal of Clinical Microbiology,* 48**,** 2601-2604.

Pratt, L. A., Hsing, W., Gibson, K. E. & Silhavy, T. J. 1996. From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. *Molecular Microbiology,* 20**,** 911-917.

Psichogiou, M., Tassios, P. T., Avlamis, A., Stefanou, I., Kosmidis, C., Platsouka, E., Paniara, O., Xanthaki, A., Toutouza, M., Daikos, G. I. & Tzouvelekis, L. S. 2008. Ongoing epidemic of $bla_{\text{VIM-1}}$-positive *Klebsiella pneumoniae* in Athens, Greece: A prospective survey. *Journal of Antimicrobial Chemotherapy,* 61**,** 59-63.

Queenan, A. M. & Bush, K. 2007. Carbapenemases: the versatile β-lactamases. *Clinical Microbiology Reviews,* 20**,** 440-458.

Raimondi, A., Sisto, F. & Nikaido, H. 2001. Mutation in *Serratia marcescens* AmpC β-lactamase producing high-level resistance to ceftazidime and cefpirome. *Antimicrobial Agents and Chemotherapy,* 45**,** 2331-2339.

Ramani, N., Hedeshian, M. & Freundlich, M. 1994. *micF* antisense RNA has a major role in osmoregulation of OmpF in *Escherichia coli*. *Journal of Bacteriology,* 176**,** 5005-5010.

Reinert, R. R., Low, D. E., Rossi, F., Zhang, X., Wattal, C. & Dowzicky, M. J. 2007. Antimicrobial susceptibility among organisms from the Asia/Pacific Rim, Europe and Latin and North America collected as part of TEST and the in vitro activity of tigecycline. *Journal of Antimicrobial Chemotherapy,* 60**,** 1018-1029.

Reisner, A., Höller, B. M., Molin, S. & Zechner, E. L. 2006. Synergistic effects in mixed *Escherichia coli* biofilms: Conjugative plasmid transfer drives biofilm expansion. *Journal of Bacteriology,* 188**,** 3582-3588.

Renau, T. E., Léger, R., Flamme, E. M., Sangalang, J., She, M. W., Yen, R., Gannon, C. L., Griffith, D., Chamberland, S., Lomovskaya, O., Hecker, S. J., Lee, V. J., Ohta, T. & Nakayama, K. 1999. Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. *Journal of Medicinal Chemistry,* 42**,** 4928-4931.

Renau, T. E., Léger, R., Yen, R., She, M. W., Flamme, E. M., Sangalang, J., Gannon, C. L., Chamberland, S., Lomovskaya, O. & Lee, V. J. 2002. Peptidomimetics of efflux pump inhibitors potentiate the activity of levofloxacin in *Pseudomonas aeruginosa*. *Bioorganic and Medicinal Chemistry Letters,* 12**,** 763-766.

Ricci, V., Tzakas, P., Buckley, A., Coldham, N. C. & Piddock, L. J. V. 2006. Ciprofloxacin-resistant *Salmonella enterica* serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. *Antimicrobial Agents and Chemotherapy,* 50**,** 38-42.

Richmond, G. E., Chua, K. L. & Piddock, L. J. V. 2013. Efflux in *Acinetobacter baumannii* can be determined by measuring accumulation of H33342 (bis-benzamide). *Journal of Antimicrobial Chemotherapy,* 68**,** 1594-1600.

Richter, S. N., Frasson, I., Franchin, E., Bergo, C., Lavezzo, E., Barzon, L., Cavallaro, A. & Pal, G. 2012. KPC-mediated resistance in *Klebsiella pneumoniae* in two hospitals in Padua, Italy, June 2009-December 2011: Massive spreading of a KPC-3-encoding plasmid and involvement of non-intensive care units. *Gut Pathogens,* 4**,** 7.

Roberts, R. R., Hota, B., Ahmad, I., Scott, R. D., Foster, S. D., Abbasi, F., Schabowski, S., Kampe, L. M., Ciavarella, G. G., Supino, M., Naples, J., Cordell, R., Levy, S. B. & Weinstein, R. A. 2009. Hospital and societal costs of antimicrobial-resistant infections

in a Chicago teaching hospital: Implications for antibiotic stewardship. *Clinical Infectious Diseases,* 49**,** 1175-1184.

Røder, H. L., Hansen, L. H., Sørensen, S. J. & Burmølle, M. 2013. The impact of the conjugative IncP-1 plasmid pKJK5 on multispecies biofilm formation is dependent on the plasmid host. *FEMS Microbiology Letters,* 344**,** 186-192.

Rodríguez, E., Bautista, A. & Barrero, L. 2014. First report of a *Salmonella enterica* serovar typhimurium isolate with carbapenemase (KPC-2) in Colombia. *Antimicrobial Agents and Chemotherapy,* 58**,** 1263-1264.

Roggenkamp, A. 2007. Phylogenetic analysis of enteric species of the family Enterobacteriaceae using the *oriC*-locus. *Systematic and Applied Microbiology,* 30**,** 180-188.

Romanowski, G., Lorenz, M. G. & Wackernagel, W. 1993. Plasmid DNA in a groundwater aquifer microcosm -adsorption, DNAase resistance and natural genetic transformation of *Bacillus subtilis*. *Molecular Ecology,* 2**,** 171-181.

Ropp, P. A., Hu, M., Olesky, M. & Nicholas, R. A. 2002. Mutations in *ponA*, the gene encoding penicillin-binding protein 1, and a novel locus *penC* are requiree for hig-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy,* 46**,** 769-777.

Rosa Gomez-Gil, M., Ramon Pano-Pardo, J., Pilar Romero-Gomez, M., Gasior, M., Lorenzo, M., Quiles, I. & Mingorance, J. 2010. Detection of KPC-2-producing *Citrobacter freundii* isolates in Spain. *Journal of Antimicrobial Chemotherapy,* 65**,** 2695-2697.

Ruiz, E., Ocampo-Sosa, A. A., Rezusta, A., Revillo, M. J., Román, E., Torres, C. & Martínez-Martínez, L. 2012. Acquisition of carbapenem resistance in multiresistant *Klebsiella pneumoniae* strains harbouring $bla_{\text{CTX-M-15}}$, *qnrS1* and *aac(6')-Ib-cr* genes. *Journal of Medical Microbiology,* 61**,** 672-677.

Rupp, M. E. & Fey, P. D. 2003. Extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae - Considerations for diagnosis, prevention and drug treatment. *Drugs,* 63**,** 353-365.

Sacha, P., Wieczorek, P., Hauschild, T., Zorawski, M., Olszanska, D. & Tryniszewska, E. 2008. Metallo-β-lactamases of *Pseudomonas aeruginosa* - a novel mechanism resistance to β-lactam antibiotics. *Folia Histochemica et Cytobiologica,* 46**,** 137-142.

Saier, M. H., Tam, R., Reizer, A. & Reizer, J. 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport *Molecular Microbiology,* 11**,** 841-847.

Salgado, H., Gama-Castro, S., Martínez-Antonio, A., Díaz-Peredo, E., Sánchez-Solano, F., Peralta-Gil, M., Garcia-Alonso, D., Jiménez-Jacinto, V., Santos-Zavaleta, A., Bonavides-Martínez, C. & Collado-Vides, J. 2004. RegulonDB (version 4.0): Transcriptional regulation, operon organization and growth conditions in *Escherichia coli* K-12. *Nucleic Acids Research,* 32**,** D303-D306.

Salje, J. 2010. Plasmid segregation: How to survive as an extra piece of DNA. *Critical Reviews in Biochemistry and Molecular Biology,* 45**,** 296-317.

Samra, Z., Ofir, O., Lishtzinsky, Y., Madar-Shapiro, L. & Bishara, J. 2007. Outbreak of carbapenem-resistant *Klebsiella pneumoniae* producing KPC-3 in a tertiary medical centre in Israel. *International Journal of Antimicrobial Agents,* 30**,** 525-529.

Samuelsen, Ø., Thilesen, C. M., Heggelund, L., Vada, A. N., Kümmel, A. & Sundsfjord, A. 2011. Identification of NDM-1-producing Enterobacteriaceae in Norway. *Journal of Antimicrobial Chemotherapy,* 66**,** 670-672.

San Millan, A., Peña-Miller, R., Toll-Riera, M., Halbert, Z. V., Mclean, A. R., Cooper, B. S. & Maclean, R. C. 2014. Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. *Nature Communications,* 5**,** 5208.

San Millan, A., Toll-Riera, M., Qi, Q. & Maclean, R. C. 2015. Interactions between horizontally acquired genes create a fitness cost in *Pseudomonas aeruginosa*. *Nature Communications,* 6.

Sanders, C. C., Bradford, P. A., Ehrhardt, A. F., Bush, K., Young, K. D., Henderson, T. A. & Sanders Jr, W. E. 1997. Penicillin-binding proteins and induction of AmpC β-lactamase. *Antimicrobial Agents and Chemotherapy,* 41**,** 2013-2015.

Santiviago, C. A., Toro, C. S., Hidalgo, A. A., Youderian, P. & Mora, G. C. 2003. Global regulation of the *Salmonella enterica* serovar Typhimurium major porin, OmpD. *Journal of Bacteriology,* 185**,** 5901-5905.

Savkovic, S. D., Villanueva, J., Turner, J. R., Matkowskyj, K. A. & Hecht, G. 2005. Mouse model of enteropathogenic *Escherichia coli* infection. *Infection and Immunity,* 73**,** 1161-1170.

Scheffers, D. J. & Pinho, M. G. 2005. Bacterial cell wall synthesis: New insights from localization studies. *Microbiology and Molecular Biology Reviews,* 69**,** 585-607.

Schmieger, H. & Schicklmaier, P. 1999. Transduction of multiple drug resistance of *Salmonella enterica* serovar Typhimurium DT104. *FEMS Microbiology Letters,* 170**,** 251-256.

Schneider, E. & Hunke, S. 1998. ATP-binding-cassette (ABC) transport systems: Functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiology Reviews,* 22**,** 1-20.

Schopf, J. W. 1993. Microfossils of the early Archean Apex Chert: New evidence of the antiquity of life. *Science,* 260**,** 640-646.

Schwaber, M. J., Navon-Venezia, S., Schwartz, D. & Carmeli, Y. 2005. High levels of antimicrobial coresistance among extended-spectrum-β-lactamase-producing *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy,* 49**,** 2137-2139.

Scoulica, E. V., Neonakis, I. K., Gikas, A. I. & Tselentis, Y. J. 2004. Spread of $bla_{\text{VIM-1}}$-producing *E. coli* in a university hospital in Greece. Genetic analysis of the integron carrying the $bla_{\text{VIM-1}}$ metallo-β-lactamase gene. *Diagnostic Microbiology and Infectious Disease,* 48**,** 167-172.

Seecoomar, G. D., Marmol, B. C. & Kwon, D. H. 2013. Promoter deletions of *Klebsiella pneumoniae* carbapenemase (KPC)-encoding genes ($bla_{\text{KPC-2}}$) and efflux pump (AcrAB) on β-lactam susceptibility in KPC-producing *Enterobacteriaceae*. *FEMS Microbiology Letters,* 348**,** 120-6.

Sengupta, M. & Austin, S. 2011. Prevalence and significance of plasmid maintenance functions in the virulence plasmids of pathogenic bacteria. *Infection and Immunity,* 79**,** 2502-2509.

Seppälä, H., Klaukka, T., Vuopio-Varkila, J., Muotiala, A., Helenius, H., Lager, K. & Huovinen, P. 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group a streptococci in Finland. *New England Journal of Medicine,* 337**,** 441-446.

Shaheen, B. W., Nayak, R., Foley, S. L., Kweon, O., Deck, J., Park, M., Rafii, F. & Boothe, D. M. 2011. Molecular characterization of resistance to extended-spectrum cephalosporins in clinical *Escherichia coli* Isolates from companion animals in the United States. *Antimicrobial Agents and Chemotherapy,* 55**,** 5666-5675.

Sharan, S. K., Thomason, L. C., Kuznetsov, S. G. & Court, D. L. 2009. Recombineering: a homologous recombination-based method of genetic engineering. *Nature Protocols,* 4**,** 206-223.

Shields, R. K., Nguyen, M. H., Potoski, B. A., Press, E. G., Chen, L., Kreiswirth, B. N., Clarke, L. G., Eschenauer, G. A. & Clancy, C. J. 2015. Doripenem MICs and ompK36 porin genotypes of sequence type 258, KPC-producing *Klebsiella pneumoniae* may predict responses to carbapenem-colistin combination therapy among patients with bacteremia. *Antimicrobial Agents and Chemotherapy,* 59**,** 1797-1801.

Sinha, R. P. & Iyer, V. N. 1971. Competence for genetic transformation and the release of DNA from *Bacillus subtilis*. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis,* 232**,** 61-71.

Smith, H. E. & Blair, J. M. A. 2014. Redundancy in the periplasmic adaptor proteins AcrA and AcrE provides resilience and an ability to export substrates of multidrug efflux. *Journal of Antimicrobial Chemotherapy,* 69**,** 982-987.

Smith, M. A. & Bidochka, M. J. 1998. Bacterial fitness and plasmid-loss: The importance of culture conditions and plasmid size. *Canadian Journal of Microbiology,* 44**,** 351-355.

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G. & Edwards Jr, J. E. 2004. Trends in antimicrobial drug development: Implications for the future. *Clinical Infectious Diseases,* 38**,** 1279-1286.

Spratt, B. G. 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. *Proceedings of the National Academy of Sciences of the United States of America,* 72**,** 2999-3003.

Sreevatsan, S., Pan, X., Stockbauer, K. E., Williams, D. L., Kreiswirth, B. N. & Musser, J. M. 1996. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrobial Agents and Chemotherapy,* 40**,** 1024-1026.

Stapleton, P., Shannon, K. & Phillips, I. 1995. The ability of β-lactam antibioties to select mutants with derepressed β-lactamase synthesis from *Citrobacter freundii*. *Journal of Antimicrobial Chemotherapy,* 36**,** 483-496.

Stone, B. J. & Miller, V. L. 1995. *Salmonella enteritidis* has a homologue of tolC that is required for virulence in BALB/c mice. *Molecular microbiology,* 17**,** 701-12.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K. S., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E. W., Lory, S. & Olson,

M. V. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature,* 406**,** 959-964.

Symmons, M. F., Bokma, E., Koronakis, E., Hughes, C. & Koronakis, V. 2009. The assembled structure of a complete tripartite bacterial multidrug efflux pump. *Proceedings of the National Academy of Sciences,* 106**,** 7173-7178.

Szabó, D., Silveira, F., Hujer, A. M., Bonomo, R. A., Hujer, K. M., Marsh, J. W., Bethel, C. R., Doi, Y., Deeley, K. & Paterson, D. L. 2006. Outer membrane protein changes and efflux pump expression together may confer resistance to ertapenem in *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy,* 50**,** 2833-2835.

Tang, H. J., Chen, Y. T., Chiang, T., Fung, C. P., Chuang, Y. C. & Kristopher Siu, L. 2014. Identification of the first imported KPC-3 *Klebsiella pneumoniae* from the USA to Taiwan. *International Journal of Antimicrobial Agents,* 44**,** 431-435.

Thanassi, D. G., Cheng, L. W. & Nikaido, H. 1997. Active efflux of bile salts by *Escherichia coli*. *Journal of Bacteriology,* 179**,** 2512-2518.

Thomas, J. R., Denap, J. C. B., Wong, M. L. & Hergenrother, P. J. 2005. The relationship between aminoglycosides' RNA binding proclivity and their antiplasmid effect on an IncB plasmid. *Biochemistry,* 44**,** 6800-6808.

Thomson, K. S. 2010. Extended-spectrum-β-lactamase, AmpC, and carbapenemase issues. *Journal of Clinical Microbiology,* 48**,** 1019-1025.

Tijet, N., Macmullin, G., Lastovetska, O., Vermeiren, C., Wenzel, P., Stacey-Works, T., Low, D. E., Patel, S. N. & Melano, R. G. 2013. Verona integron-encoded metallo-β-lactamase 1 in Enterobacteria, Ontario, Canada. *Emerging Infectious Diseases,* 19**,** 1156-1158.

Timmerman, T., Dewulf, J., Catry, B., Feyen, B., Opsomer, G., Kruif, A. D. & Maes, D. 2006. Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium. *Preventive Veterinary Medicine,* 74**,** 251-263.

Tipper, D. J. & Stroming, J. L. 1965. Mechanism of action of penicillins - a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proceedings of the National Academy of Sciences of the United States of America,* 54**,** 1133-1141.

Tsai, Y. K., Fung, C. P., Lin, J. C., Chen, J. H., Chang, F. Y., Chen, T. L. & Siu, L. K. 2011. *Klebsiella pneumoniae* outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. *Antimicrobial Agents and Chemotherapy,* 55**,** 1485-1493.

Tuomanen, E., Gilbert, K. & Tomasz, A. 1986. Modulation of bacteriolysis by cooperative effects of penicillin-binding protein-1a and -3 in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy,* 30**,** 659-663.

Turnidge, J., Kahlmeter, G. & Kronvall, G. 2006. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clinical Microbiology and Infection,* 12**,** 418-425.

Tzouvelekis, L. S., Miriagou, V., Kotsakis, S. D., Spyridopoulou, K., Athanasiou, E., Karagouni, E., Tzelepi, E. & Daikos, G. L. 2013. KPC-producing, multidrug-resistant *Klebsiella pneumoniae* sequence type 258 as a typical opportunistic pathogen. *Antimicrobial Agents and Chemotherapy,* 57**,** 5144-5146.

Umesaki, Y., Setoyama, H., Matsumoto, S. & Okada, Y. 1993. Expansion of alpha-beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. *Immunology,* 79**,** 32-37.

Van Bambeke, F., Pagès, J. M. & Lee, V. J. 2006. Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. *Recent patents on anti-infective drug discovery,* 1**,** 157-175.

Van De Sande-Bruinsma, N., Grundmann, H., Verloo, D., Tiemersma, E., Monen, J., Goossens, H., Ferech, M., Mittermayer, H., Metz, S., Koller, W., Hendrickx, E., Markova, B., Tambic-Andrasevic, A., Francetic, I., Kalenic, S., Bagatzouni, D., Dvorak, P., Urbaskova, P., Monnet, D., Anker Nielsen, A., Naaber, P., Huovinen, P., Paakkari, P., Lyytikainen, O., Nissinen, A., Maugendre, P., Guillemot, D., Coignard, B., Jarlier, V., Kern, W., Schroeder, H., Witte, W., Heckenbach, K., Giamarellou, H., Antoniadou, A., Tsakris, A., Vatopoulos, A., Ternak, G., Fuzi, M., Kristinsson, K., Smyth, E., Cunney, R., Igoe, D., Murphy, O., Raz, R., Cornaglia, G., Pantosti, A., D'ancona, P., Berzina, S., Balode, A., Valenteliene, R., Miciulevicience, J., Hemmer, R., Bruch, M., Borg, M., Zarb, P., Janknegt, R., Filius, M., De Neeling, H., Tiemermsa, E., Degener, J., Salvesen Blix, H., Hoiby, A., Simonsen, G., Hryniewicz, W., Grzesiowski, P., Caldeira, L., Canica, M., Codita, I., Foltan, V., Tesar, T., Langsadl, L., Cizman, M., Mueller-Premru, M., Kolman, J., Campos, J., Baquero, F., Cars, O., Skoog, G., Liljequist, B., Kahlmeter, G., Unal, S., Gür, D., Davey, P., Johnson, A., Hill, R., Hughes, H. & Coyne, M. 2008. Antimicrobial drug use and resistance in Europe. *Emerging Infectious Diseases,* 14**,** 1722-1730.

Villa, L., Capone, A., Fortini, D., Dolejska, M., Rodri'guez, I., Taglietti, F., De Paolis, P., Petrosillo, N. & Carattoli, A. 2013. Reversion to susceptibility of a carbapenem-resistant clinical isolate of *Klebsiella pneumoniae* producing KPC-3. *Journal of Antimicrobial Chemotherapy,* 68**,** 2482-2486.

Visalli, M. A., Murphy, E., Projan, S. J. & Bradford, P. A. 2003. AcrAB multidrug efflux pump is associated with reduced levels of susceptibility to tigecycline (GAR-936) in *Proteus mirabilis*. *Antimicrobial Agents and Chemotherapy,* 47**,** 665-669.

Voulgari, E., Zarkotou, O., Ranellou, K., Karageorgopoulos, D. E., Vrioni, G., Mamali, V., Themeli-Digalaki, K. & Tsakris, A. 2013. Outbreak of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* in Greece involving an ST11 clone. *Journal of Antimicrobial Chemotherapy,* 68**,** 84-88.

Waksman, S. A. 1973. History of the word 'Antibiotic'. *Journal of the History of Medicine and Allied Sciences,* XXVIII**,** 284-286.

Wand, M. E., Mccowen, J. W. I., Nugent, P. G. & Sutton, J. M. 2013. Complex interactions of *Klebsiella pneumoniae* with the host immune system in a *Galleria mellonella* infection model. *Journal of Medical Microbiology,* 62**,** 1790-1798.

Wang, Z., Xiang, L., Shao, J., Wegrzyn, A. & Wegrzyn, G. 2006. Effects of the presence of ColE1 plasmid DNA in *Escherichia coli* on the host cell metabolism. *Microbial Cell Factories,* 5**,** 34.

Wanner, B. L., Gottesman, M. & Gottesman, M. 1983. Overlapping and separate controls on the phosphate regulon in *Escherichia coli* K12. *Journal of Molecular Biology,* 166**,** 283-308.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. & Copeland, N. G. 2005. Simple and highly efficient BAC recombineering using *galK* selection. *Nucleic Acids Research,* 33**,** 1-12.

Webber, M. & Coldham, N. 2010. Measuring the activity of active efflux in Gram-negative bacteria. *In:* GILLESPIE, S. H. & MCHUGH, T. D. (eds.) *Antibiotic Resistance Protocols.* Humana Press.

Webber, M. A., Bailey, A. M., Blair, J. M. A., Morgan, E., Stevens, M. P., Hinton, J. C. D., Ivens, A., Wain, J. & Piddock, L. J. V. 2009. The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. *Journal of Bacteriology,* 191**,** 4276-4285.

Wei, Y., Havasy, T., Mcpherson, D. C. & Popham, D. L. 2003. Rod shape determination by the *Bacillus subtilis* Class B penicillin-binding proteins encoded by *pbpA* and *pbpH*. *Journal of Bacteriology,* 185**,** 4717-4726.

Weindorf, H., Schmidt, H. & Martin, H. H. 1998. Contribution of overproduced chromosomal β-lactamase and defective outer membrane porins to resistance to extended-spectrum β-lactam antibiotics in *Serratia marcescens*. *Journal of Antimicrobial Chemotherapy,* 41**,** 189-195.

WHO. 2011. *World Health Statistics* [Online]. Geneva: World Health Organization. Available: http://www.who.int/whosis/whostat/EN_WHS2011_Full.pdf [Accessed 14th June 2015].

WHO. 2014. *Antimicrobial Resistance - Global Report on Surveillance* [Online]. France: World health Organization. Available: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf [Accessed 14th June 2015].

Williams, J. J. & Hergenrother, P. J. 2008. Exposing plasmids as the Achilles' heel of drug-resistant bacteria. *Current Opinion in Chemical Biology,* 12**,** 389-399.

Willmott, C. J. R. & Maxwell, A. 1993. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. *Antimicrobial Agents and Chemotherapy,* 37**,** 126-127.

Wolter, D. J., Hanson, N. D. & Lister, P. D. 2004. Insertional inactivation of *oprD* in clinical isolates of *Pseudomonas aeruginosa* leading to carbapenem resistance. *FEMS Microbiology Letters,* 236**,** 137-143.

Woo, P. C. Y., To, A. P. C., Lau, S. K. P. & Yuen, K. Y. 2003. Facilitation of horizontal transfer of antimicrobial resistance by transformation of antibiotic-induced cell-wall-deficient bacteria. *Medical Hypotheses,* 61**,** 503-508.

Woodford, N., Dallow, J. W. T., Hill, R. L. R., Palepou, M. F. I., Pike, R., Ward, M. E., Warner, M. & Livermore, D. M. 2007. Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. *International Journal of Antimicrobial Agents,* 29**,** 456-459.

Woodford, N., Tierno, P. M., Young, K., Tysall, L., Palepou, M.-F. I., Ward, E., Painter, R. E., Suber, D. F., Shungu, D., Silver, L. L., Inglima, K., Kornblum, J. & Livermore, D. M. 2004. Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing

class A β-lactamase, KPC-3, in a New York Medical Center. *Antimicrobial Agents and Chemotherapy,* 48**,** 4793-4799.

Yamaguchi, Y. & Inouye, M. 2011. Regulation of growth and death in *Escherichia coli* by toxin–antitoxin systems. *Nature Reviews Microbiology,* 9**,** 779-790.

Yamamoto, T., Takano, T., Iwao, Y. & Hishinuma, A. 2011. Emergence of NDM-1-positive capsulated *Escherichia coli* with high resistance to serum killing in Japan. *Journal of Infection and Chemotherapy,* 17**,** 435-439.

Yang, F. C., Yan, J. J., Hung, K. H. & Wu, J. J. 2012. Characterization of ertapenem-resistant *Enterobacter cloacae* in a Taiwanese University Hospital. *Journal of Clinical Microbiology,* 50**,** 223-226.

Yang, Y. J., Bhachech, N. & Bush, K. 1995. Biochemical comparison of imipenem, meropenem and biapenem: permeability, binding to penicillin-binding proteins, and stability to hydrolysis by β-lactamases. *Journal of Antimicrobial Chemotherapy,* 35**,** 75-84.

Yaron, S., Kolling, G. L., Simon, L. & Matthews, K. R. 2000. Vesicle-mediated transfer of virulence genes from *Escherichia coli* O157:H7 to other enteric bacteria. *Applied and Environmental Microbiology,* 66**,** 4414-4420.

Yigit, H., Queenan, A. M., Anderson, G. J., Domenech-Sanchez, A., Biddle, J. W., Steward, C. D., Alberti, S., Bush, K. & Tenover, F. C. 2001. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 45**,** 1151-1161.

Yigit, H., Queenan, A. M., Anderson, G. J., Domenech-Sanchez, A., Biddle, J. W., Steward, C. D., Alberti, S., Bush, K. & Tenover, F. C. 2008. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 52**,** 809.

Yoneyama, H. & Katsumata, R. 2006. Antibiotic resistance in bacteria and its future for novel antibiotic development. *Bioscience Biotechnology and Biochemistry,* 70**,** 1060-1075.

Yoneyama, H. & Nakae, T. 1993. Mechanism of efficient elimination of protein D2 in outer membrane of imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy,* 37**,** 2385-2390.

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K. & Walsh, T. R. 2009. Characterization of a new metallo-β-lactamase gene, *bla*$_{\text{NDM-1}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella*

pneumoniae sequence type 14 from India. *Antimicrobial Agents and Chemotherapy,* 53**,** 5046-5054.

Yoshida, T. 1980. Structural requirements for antibacterial activity and β-lactamase stability of 7β-arylmalonylamino-7α-methoxy-1 -oxacephems. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences,* 289**,** 231-237.

Yoshimura, F. & Nikaido, H. 1985. Diffusion of β-lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrobial Agents and Chemotherapy,* 27**,** 84-92.

Younes, H., Coudray, C., Bellanger, J., Demigne, C., Rayssiguier, Y. & Remesy, C. 2001. Effects of two fermentable carbohydrates (inulin and resistant starch) and their combination on calcium and magnesium balance in rats. *British Journal of Nutrition,* 86**,** 479-485.

Zalewska-Piątek, B., Wilkanowicz, S., Bruździak, P., Piątek, R. & Kur, J. 2013. Biochemical characteristic of biofilm of uropathogenic *Escherichia coli* Dr^+ strains. *Microbiological Research,* 168**,** 367-378.

Zaslaver, A., Mayo, A. E., Rosenberg, R., Bashkin, P., Sberro, H., Tsalyuk, M., Surette, M. G. & Alon, U. 2004. Just-in-time transcription program in metabolic pathways. *Nature Genetics,* 36**,** 486-491.

Zavascki, A. P., Falci, D. R., Da Silva, R. C. F., Dalarosa, M. G., Ribeiro, V. B., Rozales, F. P., Luz, D. I., Magagnin, C. M., Vieira, F. J., Sampaio, J. M. & Barth, A. L. 2014. Heteroresistance to carbapenems in New Delhi metallo-β-lactamase-1-producing isolates: A challenge for detection? *Infection Control and Hospital Epidemiology,* 35**,** 751-752.

Zavascki, A. P., Machado, A. B. M. P., De Oliveira, K. R. P., Superti, S. V., Pilger, D. A., Cantarelli, V. V., Pereira, P. R., Lieberkmecht, A. C. & Barth, A. L. 2009. KPC-2-producing *Enterobacter cloacae* in two cities from Southern Brazil. *International Journal of Antimicrobial Agents,* 34**,** 286-288.

Zhang, W., Robertson, D. C., Zhang, C., Bai, W., Zhao, M. & Francis, D. H. 2008. *Escherichia coli* constructs expressing human or porcine enterotoxins induce identical diarrheal diseases in a piglet infection model. *Applied and Environmental Microbiology,* 74**,** 5832-5837.

Zilberberg, M., Shorr, A., Micek, S., Vazquez-Guillamet, C. & Kollef, M. 2014. Multi-drug resistance, inappropriate initial antibiotic therapy and mortality in Gram-negative severe sepsis and septic shock: a retrospective cohort study. *Critical Care,* 18**,** 596.

Zünd, P. & Lebek, G. 1980. Generation time-prolonging R plasmids: Correlation between increases in the generation time of *Escherichia coli* caused by R plasmids and their molecular size. *Plasmid,* 3**,** 65-69.