Investigating the Regulatory Mechanisms of Antibiotic Resistance in Gram-negative Pathogens

A Thesis Presented to the National University of Ireland for the Degree of

Doctor of Philosophy

By

Micheál Mac Aogáin B.Sc. (Genetics)

Department of Microbiology
National University of Ireland
Cork

Supervisor: Professor Fergal O'Gara
Head of Department: Professor Gerald Fitzgerald

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Table of contents

Title page ..... **I**
Table of contents ..... **II**
Declaration ..... **III**
Dedication ..... **IV**
Abstract ..... **V**
Peer-review publications ..... **VIII**

General Introduction **1**
1. Introduction 3
2. Antibiotic resistance 5
3. Molecular mechanisms of antibiotic resistance 2
4. The Resistance-Nodulation-Division family of efflux pumps 27
5. Conclusion 41
6. Aim of thesis 43
7. References 44

Chapter 1 **Emergence of Extended-Spectrum β-lactamase and Fluoroquinolone Resistance Genes among Irish Enterobacteriaceae Isolates** **63**
1.1 Abstract 64
1.2 Introduction 65
1.3 Materials and Methods 67
1.4 Results 71
1.5 Discussion 86
1.6 Conclusion 91
1.7 References 92

Chapter 2 **Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa* Isolates from Turkey** **98**
2.1 Abstract 99
2.2 Introduction 100
2.3 Materials and Methods 102
2.4 Results 108
2.5 Discussion 117
2.6 References 121

Chapter 3 **Investigating MexT-mediated Regulation of Antibiotic Resistance, Virulence and the Novel ArsR-family Repressor PA4354 in *Pseudomonas aeruginosa*** **127**
3.1 Abstract 128
3.2 Introduction 129
3.3 Materials and Methods 132
3.4 Results 144
3.5 Discussion 165
3.6 Conclusion 172
3.7 References 173

General Discussion **181**

Acknowledgments **204**

Declaration

I, the undersigned, Micheál Mac Aogáin, declare that I have not obtained a degree from University College Cork, National University of Ireland, Cork or elsewhere on the basis of this Ph. D. thesis and that the results presented in this thesis were derived from experiment undertaken by myself at University College Cork with the following exceptions:

- Typing and PCR screening for resistance genes in *P. aeruginosa* isolates was performed by Dr. Marlies Mooij (Chapter 2, Table 2)
- β-galactosidase assays to determine repression of *exoS* expression by MexT in PAO1 and PAO1Δ*mexEF* included in Chapter 3 were performed by Hazel O'Connor (Chapter 3, Figure 2C, Figure 3C)
- Transcriptome analysis from which data is included in Chapter 3 was performed by Dr. Zhexien Tian (Chapter 3, Table 3)

_____

Micheál Mac Aogáin

May 2011

Do mo thuistí, Eoghan agus Síle dá gcuid ghrá agus tacaíocht, agus i gcuimhne ar m'uncail Paddy.

To my parents, Eoghan and Síle for their love and support and in memory of my uncle Paddy.

Abstract

Resistance to diverse antibiotics has emerged globally in prevalent Gram-negative pathogens including pathogenic members of the Enterobacteriaceae family and *Pseudomonas aeruginosa*. As the treatment of infections caused by these pathogens is dependent on their susceptibility to antibiotics, the emergence of multidrug resistance threatens our ability to treat these infections.

The major selective force behind the emergence of antibiotic resistance is antibiotic use. Ireland and Turkey both have relatively high rates of antibiotic administration and as such, there exists a correspondingly high level of selection for resistant clinical isolates. To investigate how bacterial pathogens are responding to the selective pressure of antibiotic therapy in these regions, multidrug-resistant Enterobacteriaceae from the Mercy University Hospital (MUH, Cork, Ireland) and carbapenem-resistant *Pseudomonas aeruginosa* isolates from Zonguldak Karaelmas University Hospital (ZKUH, Zonguldak, Turkey) were characterized at the molecular level.

In Ireland, similar to other European countries, there is an emerging problem with resistant Enterobacteriaceae which produce Extended-Spectrum β-lactamases (ESBLs). These enzymes hydrolyze β-lactam antibiotics and confer broad-spectrum resistance to bacterial pathogens which cannot be treated with conventional antibiotics or even broad-spectrum agents such as third generation cephalosporins. As levels of cephalosporin resistance have consistently increased at MUH since 2000, this suggested that Enterobacteriaceae harbouring diverse ESBL genes could be emerging in this region, accounting for the observed increase in cephalosporin resistance among Gram-negative isolates. Diverse ESBLs were detected among Irish Enterobacteriaceae including bla_{SHV-12} and $bla_{CTX-M-15}$ confirming the emergence of diverse ESBL-producing strains in Ireland. The transferrable fluoroquinolone resistance genes *aac(6')-IB-cr*, *qnrA*, and *qnrB* were also detected in ESBL-positive isolates for the first time in Irish clinical isolates. ESBLs and transferrable fluoroquinolone resistance genes are commonly found on transferrable multidrug resistance plasmids where they are associated with multiple resistance genes which further narrows treatment options. *Escherichia coli* isolates from MUH

were confirmed to harbour transferrable plasmids conferring resistance to multiple antibiotic classes including narrow and broad-spectrum β-lactam as well as fluoroquinolone and aminoglycoside antibiotics. Among Irish MDR Enterobacteriaceae, carbapenem antibiotics remain a currently effective treatment option as isolates were universally susceptible to this antibiotic class although non-fermentative Gram-negative isolates exhibiting carbapenem resistance were observed.

Carbapenem resistance is a significant threat as carbapenem antibiotics are considered to be agents of last resort and are generally reserved for severe infections. Hence a bacterial infection exhibiting carbapenem resistance may have severely limited treatment options. In Turkey and other countries in the Mediterranean Region, resistance to carbapenem antibiotics is high which poses a serious threat to the treatment of Gram-negative infection in this region. An increased incidence of imipenem-resistant *P. aeruginosa* infections was observed at ZKUH. Molecular investigation revealed the independent emergence of imipenem resistance in diverse *P. aeruginosa* via disruption of the OprD outermembrane porin suggesting that carbapenem therapy is selecting against this porin in *P. aeruginosa* clinical isolates from this region. Strains exhibited resistance profiles which suggested a role for the central transcriptional regulator of antibiotic resistance MexT, which not only induces the MexEF-OprN multidrug efflux system but also downregulates *oprD* hence conferring multidrug resistance. However, it was demonstrated that *mexE* was not induced in imipenem-resistant isolates, which ruled out this hypothesis. This finding is in agreement with other studies which report that induction of MexT in clinical isolates is apparently rare. This suggests that MexT has a physiological role independent of antibiotic detoxification and that its induction in clinical isolates confers a fitness burden which outweighs the benefit of increased antibiotic resistance.

In addition to its ability to mediate antibiotic resistance, MexT also negatively influences virulence. Activation of MexT causes reduced levels of homoserine lactone-dependent virulence traits, including pyocyanin, elastase, rhamnolipids and PQS, reduced expression of the type three secretion system and impaired attachment. Hence MexT regulates global physiological

changes in *P. aeruginosa*. A subset of MexT-associated phenotypes have been attributed to the induction of the MexEF-OprN pump itself which has a pleiotropic effect on the cell. However, a subset of MexT-associated phenotypes which exhibit independence from the MexEF-OprN efflux system was identified suggesting that MexT might directly regulate additional transcriptional targets. Through transcriptome analysis it was demonstrated that MexT is in fact a global transcriptional regulator which directly regulates expression of twelve novel targets in addition to *mexEF-oprN*.

One of the novel MexT-regulated targets identified was the *PA4354-PA4355-xenB* operon. *PA4354* encodes a putative transcriptional repressor, thus adding an extra layer of complexity to the MexT regulatory cascade. This provides a possible regulatory mechanism to explain the negative effects of MexT on diverse targets. The role of PA4354 as an integrated part of the MexT regulon was investigated at the molecular level. PA4354 was demonstrated to be an autorepressor and its autoregulatory binding site was determined. PA4354 did not play a role in MexT-mediated imipenem resistance and was not linked to any of the MexEF-OprN independent virulence phenotypes controlled by MexT. Thus a direct regulatory role for PA4354 in the repression of MexT-associated phenotypes was ruled out. In addition to its regulation by MexT and PA4354, expression of the *PA4354-PA4355-xenB* operon appears to be controlled by other regulatory elements which suggests it may be influenced by other regulatory pathways and may function in divergent physiological processes.

Peer-reviewed puplications associated with this thesis

Chapter 1

Mac Aogáin M, Mooij MJ, Adams C, Clair J, O'Gara F. Emergence of extended-spectrum β-lactamase and fluoroquinolone resistance genes among Irish multidrug-resistant isolates. Diagn Microbiol Infect Dis. 2010 May;67(1):106-9.

Chapter 2

The work carried out in Chapter 2 is to be submitted to for publication to Clinical Microbiology and Infection.

Chapter 3

Tian ZX, **Mac Aogáin M,** O'Connor HF, Fargier E, Mooij MJ, Adams C, Wang YP, O'Gara F. MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. Microb Pathog. 2009 Oct;47(4):237-41.

Tian ZX, Fargier E, **Mac Aogáin M,** Adams C, Wang YP, O'Gara F. Transcriptome profiling defines a novel regulon modulated by the LysR-type transcriptional regulator MexT in *Pseudomonas aeruginosa*. Nucleic Acids Res. 2009 Dec;37(22):7546-59.

Work from Chapter 3 is to be submitted for publication to the Journal of Bacteriology.

General Introduction

Table of contents

1. Introduction **3**

1.1 Nosocomial infection 3
1.2 Infection control 4

2. Antibiotic resistance **5**

2.1 History of antibiotic development 5
2.2 Antibiotic mode of action and structural diversity 6
2.3 Evolution of antibiotic resistance in clinical pathogens 8
2.3.1 Horizontal gene transfer 9
2.3.2 Mobile genetic elements 10
2.5 Prevalence of antibiotic resistance in nosocomial infections 12
2.4 Clinical impact of antibiotic resistance 14
2.6 Resistant Gram-positive nosocomial pathogens 15
2.7 Resistant Gram-negative nosocomial pathogens 15
2.7.1 Enterobacteriaceae 16
2.7.2 Non-fermentative species 17
2.7.3 *Pseudomonas aeruginosa* 18

3. Molecular mechanisms of antibiotic resistance **22**

3.1 Target modification 23
3.2 Enzymatic inactivation 24
3.3 Reduced permeability 25
3.4 Antibiotic efflux 26

4. The Resistance-Nodulation-Division family of efflux pumps **27**

4.1 The RND efflux systems of *P. aeruginosa* 27
4.1.1 MexAB-OprM 29
4.1.2 MexCD-OprJ 30
4.1.3 MexEF-OprN 30
4.1.4 MexXY 31
4.1.5 Other RND efflux systems of *P. aeruginosa* 31
4.2 Distribution of RND systems among diverse bacterial pathogens 32
4.3 Virulence phenotypes associated with RND efflux systems 36
4.4 Transcriptional regulation of resistance 37
4.5 Regulation of RND efflux systems in *P. aeruginosa* 38

5. Conclusion **41**

6 Aim of thesis **43**

7. References **44**

1. Introduction

No matter how potent or sophisticated its mechanisms of action, no medicinal antibiotic can escape the inevitable evolution of counter-acting resistance mechanisms in their target pathogens. In the wake of the golden era of antibiotic drug discovery, the emergence of antibiotic resistance has become an increasing threat as bacterial pathogens expand their resistance profiles while the development of novel antibiotics stagnates [1]. The lack of novel therapeutics active against Gram-negative bacteria is of particular concern as these pathogens increasingly exhibit remarkably limited susceptibility profiles which severely restricts treatment options and leads to inappropriate antimicrobial therapy.

1.1 Nosocomial infection

Nosocomial infections are infections that are contracted in the hospital setting and are generally defined based on the elapsed time between hospital admission and onset of infection. As such, infections which occur more than 48 hours after hospital admission are considered nosocomial or hospital-acquired and infections occurring within 48 hours of admission are considered to be community acquired [2]. Given that many illnesses which require hospitalization result from infections, an aggregation of pathogenic strains are present as they are continually introduced hospital setting by infected patients. Transmission of nosocomial strains in the hospital is facilitated by patients, staff and visitors which can all act as vectors in the transmission of nosocomial strains. This can result in localized outbreaks which are often caused by multidrug resistant (MDR) strains [3]. Thus, hospitalization ironically represents a major risk factor for bacterial infection as hospitals act as reservoirs for infectious bacteria, and a focal point of susceptible cohorts [4].

1.2 Infection control

Given the potential impact of nosocomial infections on patient health, the control of their transmission in the hospital is crucial. Efforts to curtail infection have given rise to the discipline of infection control which is an important component of healthcare management in modern hospitals. Policies such as hand hygiene and disinfection of inanimate surfaces and medical devices are central to modern hospital infection control programs aimed at reducing the transmission of nosocomial infections [5]. Other control measures which reduce nosocomical infection rates include the isolation of infected patients, restriction of factors associated with infection (e.g. use of invasive medical devices) and the management of colonised/potentially colonised individuals through pre-emptive isolation, screening and eradication policies [6-7]. Importantly, local infection control policies should keep pace with data from regional and global surveillance networks, molecular epidemiological studies and innovative research. Surveillance of resistant strains gives insight to molecular factors such as resistance mechanisms which contribute to hospital outbreaks and the global dissemination of nosocomial pathogens. Surveillance data is crucial for the prediction of future trends in nosocomial infection and allows infection control to stay abreast of the continually evolving epidemiology of nosocomial infection [8-9].

The identification of risk factors associated with nosocomial infection is also a crucial part of infection control since better management of patients at risk of infection can reduce infection rates. Epidemiological studies can identify risk factors for colonization/infection with nosocomial pathogens which allows optimization of treatment whilst limiting the risk of cross-contamination. A major risk factor associated with nosocomial infection is antibiotic therapy and this has important implications for infection control [10]. Firstly, antibiotics select for resistant organisms which are refractory to antimicrobial therapy. Resistant strains are maintained under antibiotic selection, thus increasing their prevalence over time resulting in a high rate of antibiotic resistance among nosocomial pathogens [8,11]. Secondly, antibiotic therapy can increase the bioburden of resistant bacteria on the patient

due to the collateral effect of antibiotics on the natural microbial flora. Suppression of the natural flora of the patient allows multiplication of resistant strains which may cause infection and be transmitted within the hospital. Finally, it has been shown that the use of certain antibiotics classes can cause increases in the rates of nosocomial and MDR infections and that in this context, optimal antibiotic policy can have a direct influence on the rate of nosocomial infection [8,12].

2. Antibiotic resistance

2.1 History of antibiotic development

A seminal discovery in the development of antibiotic therapy was the discovery and development of penicillin as a therapeutic agent. The therapeutic potential of penicillin was first demonstrated in 1897 by the French physician Ernest Duchesne who noticed that the mould *Penicillium glaucum*, when injected into animals, protected against normally lethal doses of virulent bacteria. In spite of this, Sir Alexander Fleming is often credited with the discovery of penicillin in 1928 but this was in fact a re-discovery of the work carried out by Duchesne three decades previous which went unnoticed. Indeed, the development of penicillin as therapeutic agent which preceded its introduction into clinical use in 1941 was pioneered by the work of Howard Florey and Ernst Chain who shared the Nobel Prize in Physiology or Medicine with Fleming in 1945 [13]. Regardless of who was chiefly responsible for the discovery, the important underling principle that microbes could produce potentially therapeutically beneficial compounds was realised and this initiated a golden era of antibiotic drug discovery.

The discovery of streptomycin in 1943 by a group led by Selman Waksman highlighted the actinomycetes as a rich source of antibacterial compounds. This is reflected in the fact the most classes of clinical antibiotics in use today originate from these soil-dwelling bacteria [14-15].

This golden era of antibiotic discovery peaked in the 1950s and although many new antibiotic classes were identified, antibiotic drug discovery has waned ever since. Significantly, there has been few truly novel antibiotic classes representing diverse structural scaffolds discovered in the last 20 years. The stagnation in antibiotic discovery has its roots in the associated technical challenges but also in the economics of antibiotic discovery. This is reflected in the decreasing industry focus and antibaterial drug discovery [16-17].

2.2 Antibiotic mode of action and structural diversity

The inhibitory mechanism of an antibiotic compound is defined by its interaction with a primary target (Table 1). Antibiotics interact with different cellular targets and cause disruption of important physiological processes including cell wall synthesis (β-lactams), DNA replication (fluoroquinolones), protein translation (tetracyclines), RNA transcription (rifamycins), folate biosynthesis (sulphonamides) and cell membrane disruption (polymixins). This results in the inhibition of bacterial proliferation which is either bacteriostatic (inhibiting bacterial growth e.g. tetracylcines) or bactericidal (killing bacterial cells, e.g. β-lactams) [1,18].

Table 1. Diverse antibiotic classes and their target pathways. Adapted from Davies and Davies, 2010 [1].

Antibiotic class	Example(s)	Target pathway
β-Lactams	Penicillins (Ampicillin), Cephalosporins (Cephamycin), Carbapenems (Meropenem), Monobactams (Aztreonam)	Peptidoglycan biosynthesis
Aminoglycosides	Gentamicin, Streptomycin, Spectinomycin	Translation
Glycopeptides	Vancomycin, Teicoplanin	Peptidoglycan biosynthesis
Tetracyclines	Minocycline, Tigecycline	Translation
Macrolides	Erythromycin, Azithromicin	Translation
Lincosamides	Clindamycin	Translation
Streptogramins	Synercid	Translation
Oxazolidinones	Linezolid	Translation
Phenicols	Chloramphenicol	Translation
Quinolones	Ciprofloxacin	DNA replication
Pyrimidines	Trimethoprim	Folate synthesis
Sulfonamides	Sulfamethoxazole	Folate synthesis
Rifamycins	Rifampin	Transcription
Lipopeptides	Daptomycin	Cell membrane
Cationic peptides	Colistin	Cell membrane

General Introduction

Of the diverse antibiotic classes known, the most medically significant is the β-lactam family of antibiotics which represent a group of compounds with a broad spectrum of activity against both Gram-positive and Gram-negative pathogens. β-lactam antibiotics are characterized by a lactam ring which represents the skeletal core of this antibiotic class. They function by irreversibly binding and inhibiting the bacterial penicillin binding protein (PBP) which is essential to cell-wall recycling. Due to their comparative efficacy, versatility and safety the β-lactams have become therapeutic workhorses of the clinical setting [19-20]. However, since their introduction to clinical therapy, the efficacy of diverse β-lactams has been repeatedly stifled by the emergence of resistance to this crucial antibiotic class in important pathogens. This represents one of the most well studied examples of the ongoing failure of antibiotic therapy and the danger of over-dependence on a single antibiotic class. [21-22]. Without their first-choice therapeutic agents, clinicians are dependent on alternative antibiotic classes which are less safe, more expensive or less efficient. Moreover, such alternative therapies have come under threat with the emergence of MDR bacteria which are co-resistant to β-lactam and non-β-lactam antibiotics alike. These organisms greatly limit treatment options and are associated with poor treatment outcomes as well as increased hospital stays and costs [23-24]. Coupled to the stagnation of antibiotic drug discovery in the pharmaceutical industry, the emergence of antibiotic resistance is a concern. Although a single antibiotic structure can give rise to multiple derivates of increased antimicrobial activity, resistance to one member of an antibiotic class frequently leads to resistance to the whole class [25-26]. Faced with increasing resistance levels in nosocomial pathogens, our dependence on a relatively limited number of antibiotic structural classes acting on a small number of targets is a major limiting factor to the success of antibiotic therapy in the future.

2.3 Evolution of antibiotic resistance in clinical pathogens

The major driving force behind the emergence of antibiotic resistant pathogens is the selection imposed by human and agricultural/aqua-cultural antibiotic consumption where anthropogenic antibiotics are released in high concentrations into the natural ecological niches inhabited by opportunistic human pathogens [27-28]. Bacteria are forced to adapt to the high concentrations of toxic compounds, which is achieved through deployment of genetically encoded resistance machinery which circumvent the toxic effect of antibiotics [10,29-30].

Anthropogenic antibiotics have their origins in antibiotic-producing microbes and are structurally related to natural antibiotic molecules. Within the microbiome, antibiotics can serve as antagonistic weapons against competing organisms but may also have other roles as hormetic cell-signalling molecules which can effect important physiological traits including virulence and motility at sub-inhibitory concentrations [31]. There is a growing appreciation of the importance of small molecular weight compounds such as antibiotics or structurally related secondary metabolites in bacterial physiology where they may have central functions in diverse processes and affect global changes in physiology [32-33]. In light of their potentially profuse roles in the natural environment, it is important to recall that although antibiotics were discovered by humans, they were 'invented' by microbes. Thus the antibiotics in clinical use today have structural scaffolds which are not new to bacterial evolution. Consequently, bacteria had evolved macromolecular mechanisms for the perception, response, transport and detoxification of diverse small molecules including antibiotics long before the introduction of anthropogenic antibiotic derivatives. Such mechanisms are now commonly identified as primary mediators of antibiotic resistance in clinical pathogens [21,30,34-35].

Pathogenic bacteria may harbour intrinsic resistance mechanisms or can acquire resistance through chromosomal mutations which are maintained under antibiotic selection [36]. Acquired resistance can arise through modification of antibiotic-interacting macromolecules

or dis-regulation of genes encoding resistance machinery [36-41]. Such resistance which is chromosomal in origin is referred to as vertically acquired resistance as it is disseminated vertically by clonal expansion. However, the emergence of antibiotic resistance is greatly accelerated by the processes of horizontal gene transfer [42-43]. In contrast to vertically acquired resistance, horizontal gene transfer allows the exchange of resistance between clonally diverse strains and plays a major role in the emergence of resistance (Figure 1A) [42,44].

2.3.1 Horizontal gene transfer

Horizontal gene transfer occurs through three known mechanisms, namely transformation, transduction and conjugation. Transformation involves the uptake of naked DNA from the environment and is exhibited by many bacterial species which exhibit competence; the natural ability to sequester DNA. Transduction is DNA transfer which is mediated by bacteriophages which package bacterial DNA into their protein capsids which is subsequently injected into another bacterial cell during the infective process. DNA fragments sequestered via transformation or transduction are subsequently integrated into the chromosome through homologous recombination at regions of sequence similarity. In conjugation, genetic information is transferred via conjugative pili which form a physical tunnel between donor and recipient strains through which plasmid DNA or Integrative chromosomal elements are transferred [42,45]. Plasmid DNA can autonomously replicate independent of the bacterial chromosome and may harbour many genes which confer beneficial phenotypes which allow adaptation to environmental challenges including antibiotic therapy. Conjugation represents the most significant mechanism of horizontal gene transfer in the dissemination of resistance genes among Gram-negative pathogens. Multidrug resistance plasmids are widespread among resistant clinical isolates and often harbour multiple resistance genes. Within multidrug resistance plasmids, antibiotic resistance genes

of diverse origins are found in mosaic regions in association with mobile genetic elements which facilitate their integration into plasmids [46-47].

2.3.2 Mobile genetic elements

The frequent association of mobile elements with resistance genes reflects their importance in the dissemination of resistance mechanisms as they provide a mechanistic explanation for the mobilization of resistance genes from the chromosome of diverse organisms [48]. Genetic elements such as transposons, insertion sequence elements and integrons are frequently associated with resistance genes and provide the "tool kit" by which exogenous genes can be sequestered from the chromosomes of diverse species (Figure 1B-C). The bla_{TEM-1} β-lactamase gene is one of the most widely distributed resistance genes among the Enterobacteriaceae and has been found in association with the TnpA transposon and TnpR resolvase which are characteristic of Tn3-family transposable elements and which are likely to have facilitated its mobilization from chromosomal origins to diverse plasmids (Figure 1Ci) [47]. Diverse resistance genes are also commonly found in gene capturing systems termed integrons which are often present on multidrug resistance plasmids and can harbour multiple resistance genes cassettes which are captured and integrated into cassette arrays via a site specific recombinase (Figure 1Cii) [49]. Mobile insertion sequences are also important tools of gene mobilisation. When these mobile elements flank a gene they can catalyse its mobilisation through homologous recombination. Some families of insertion sequence such as the IS91-like sequence elements employ a mechanism of rolling circle replication to transpose themselves and adjacent DNA and hence require only a single copy of the element to allow gene mobilization [43]. A major example of this mobilization mechanism is the association of the *ISEcP1* element with the pandemic bla_{CTX-M} cephalosporin resistance genes (Figure 1Ciii). The *ISEcP1* sequence element is capable of self-promoted transfer through rolling circle replication and its ability to mobilize the $bla_{CTX-M-2}$ gene from the chromosome of *Kluyvera ascorbata* and transfer to *E. coli* has been demonstrated in vivo [50]. The

transposition and recombination of mobile elements is increased in response to diverse stresses including the presence of antibiotics which further implicates them in adaptation and places increasing emphasis on their importance in the emergence of antibiotic resistance [11].

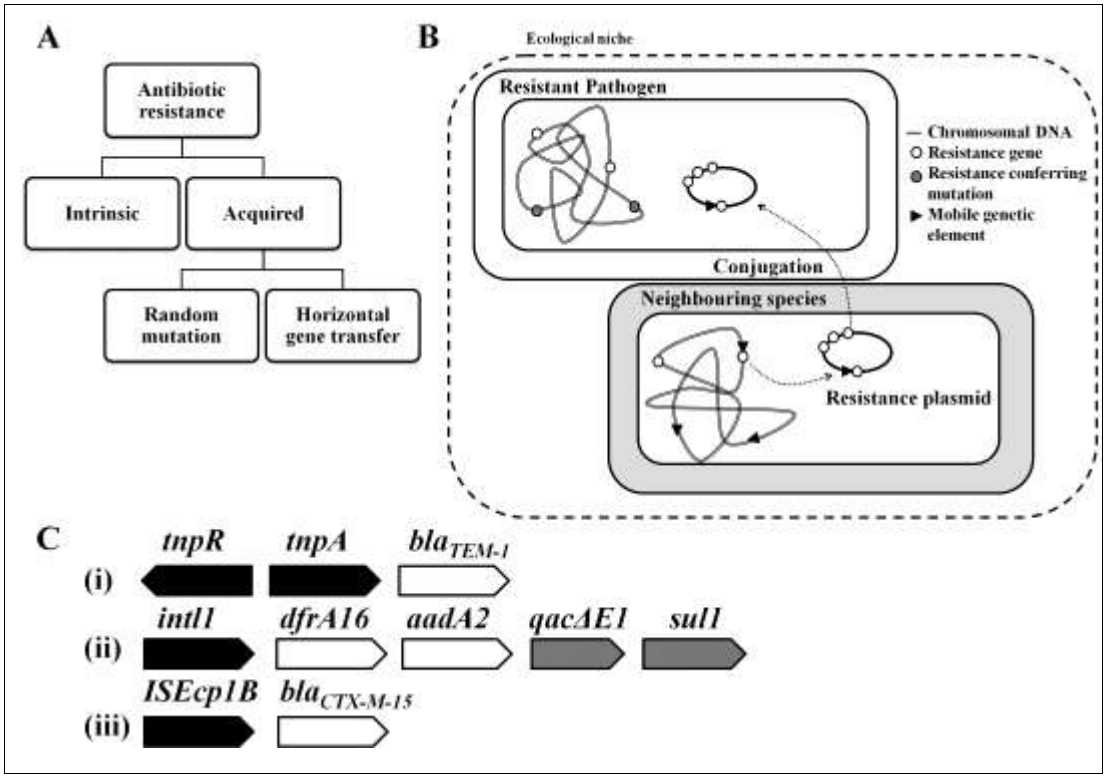

Figure 1. A – Overview of antibiotic resistance mechanisms. Resistance may be intrinsic or acquired. Acquired resistance may occur through random mutation in the chromosome or the horizontal acquisition of exogenous genes. **B – The role of chromosomal and horizontally acquired resistance mechanisms in the emergence of MDR pathogens**. Resistance may result from intrinsic resistance genes within the chromosome or from mutations in the chromosome which cause increased resistance. The acquisition of resistance-conferring plasmids which may harbour multiple resistance genes can significantly contribute to resistance. Resistance genes can be mobilized from the chromosomes of diverse species by association with mobile genetic elements which allows their integration into transferable plasmids which can be transferred via conjugation to opportunistic pathogens from neighbouring species within a common ecological niche. **C – Mobile genetic elements associated with resistance genes.** (i) – the bla_{TEM-1} is frequently associated with a Tn3-family transposase *tnpA* and resolvase *tnpR* which are likely to have facilitated its mobilization (ii) – integrons are gene capturing systems which which can harbour diverse gene arrays such as the trimethoprim resistance gene cassettes *dfrA16 aadA2*. The defective quaternary ammonium compound resistance *qacΔ1E* and *sul1* sulphonamide resistance genes are conserved in class-1 integrons. (iii) – The *ISEcp1* gene is an mobile element of the IS90-like family that can promote its own transposition and has played a role in the global dissemination of the $bla_{CTX-M-15}$ ESBL.

2.4 Prevalence of antibiotic resistance in nosocomial infections

Under antibiotic selection, antibiotic resistance has disseminated via the acquisition of resistance by pathogenic strains which have spread globally [34,51]. The global emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA) has had a major impact on healthcare and is a primary example of the problem inherent in the acquisition of antibiotic resistance by nosocomial pathogens. Within Europe, the prevalence of MRSA and other resistant nosocomial pathogens also exhibits variability with many western and southern European countries including Ireland the UK, Spain and Italy reporting MRSA prevalence of above 25% whereas other regions such as the Netherlands and Scandinavia have rates below 2% (Figure 2A) [52]. These lower rates of antibiotic resistance may reflect the success of "search and destroy" infection control policies implemented by Dutch and Scandinavian healthcare systems which may also explain lower rates of other antibiotic resistant pathogens (Figure 2A - D) [34]. Increased awareness and implementation of infection control policies throughout Europe may explain an apparent overall decline in prevalence rates which have been observed for MRSA and vancomycin-resistant *Enterococcus faecium*, another problematic Gram-positive nosocomial pathogen (Figure 3) [53]. However, in contrast to observed reductions in resistance rates among Gram-positive bacteria, no relent in resistance is observed among Gram-negative pathogens such as *Escherichia coli.* Resistance to diverse antibiotics including broad-spectrum agents such as third-generation cephalosporins and ciprofloxacin continues to increase among strains disseminated throughout Europe (Figure 3) [52].

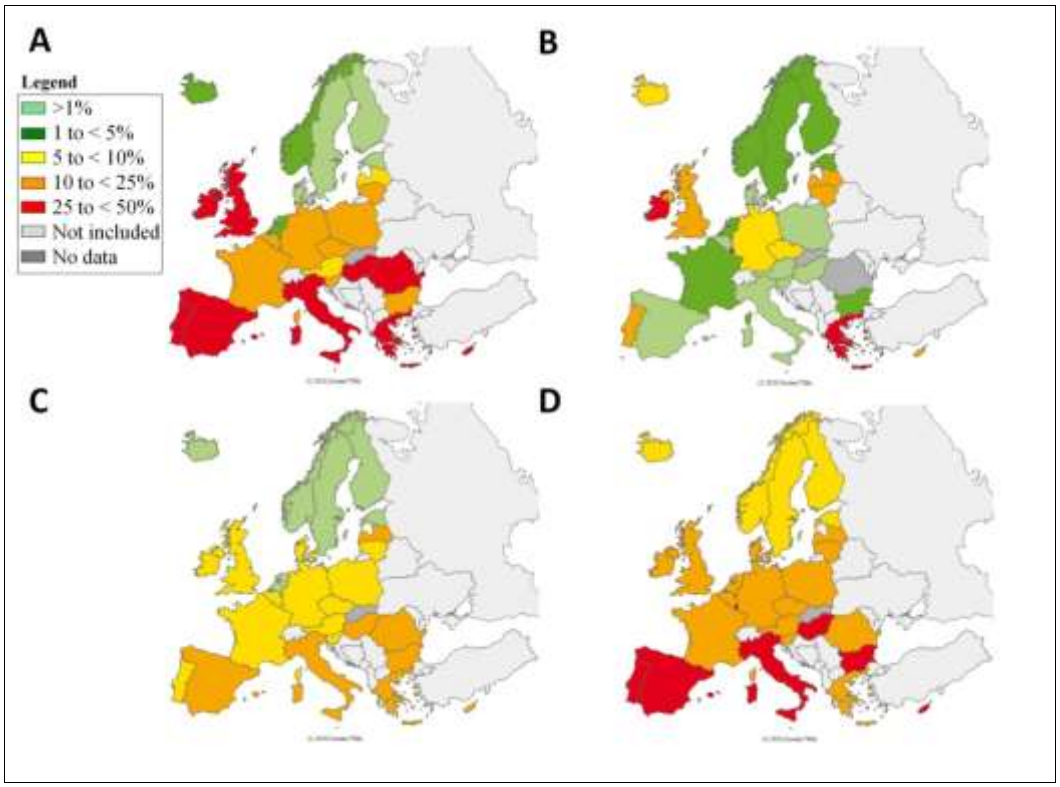

Figure 2. Prevalence of antibiotic resistance among diverse bacteria in Europe. Maps show the percentage brackets of **A** - MRSA **B** - vancomycin resistant *Enterococcus faecium*, **C** - Thrid-generation cephalosporin resistance in *E. coli* and **D** - ciprofloxacin resistant *E. coli* isolates from invasive isolates reported in 2009 by countries participating in the European antimicrobial resistance surveillance network (EARS-net) surveillance scheme.

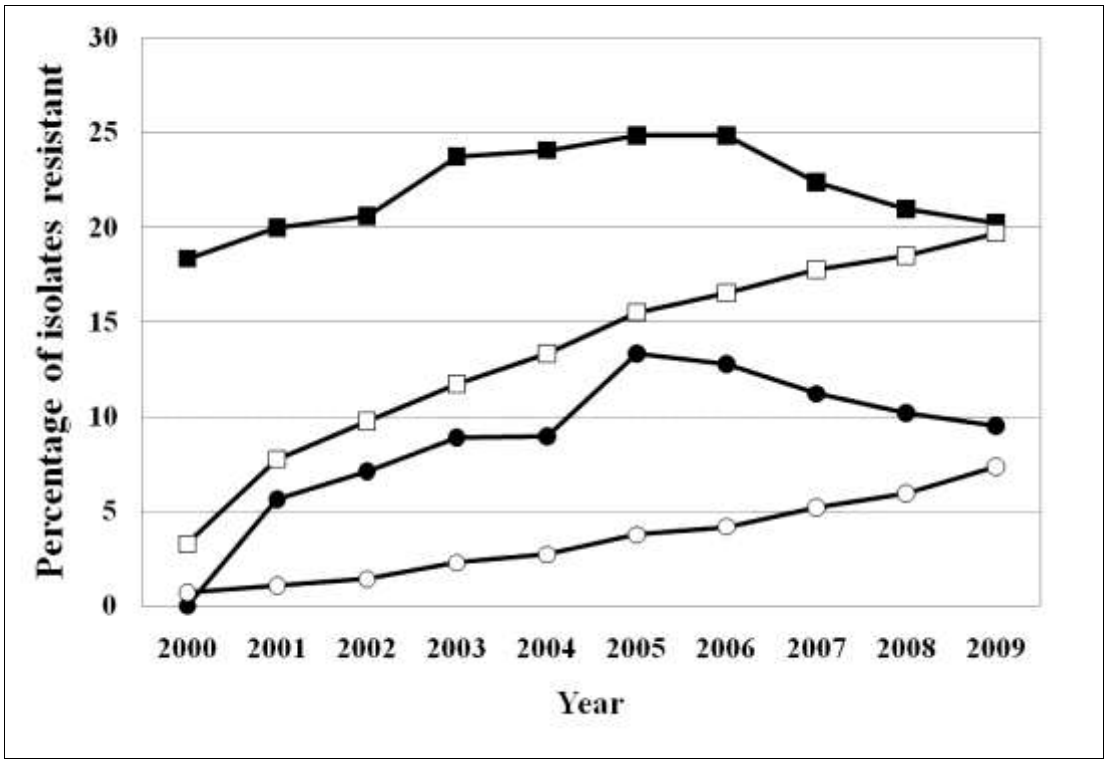

Figure 3. Resistance trends among nosocomial pathogens in Europe. The average percentage of invasive isolates exhibiting antibiotic resistance is shown diverse species isolated in participating countries in the European antimicrobial resistance surveillance network (EARS-net) surveillance scheme [52]. **Black squares** - MRSA, **Black Circles** - vancomycin resistant *Enterococcus faecium*, **White circles** - Thrid-generation cephalosporin resistant *E. coli*, **White squares** - ciprofloxacin resistant *E. coli*.

2.5 Clinical impact of antibiotic resistance

Although antibiotic resistance was once a phenomenon of the hospital environment, MDR strains are now frequently encountered in the community setting. The clinical impact of resistance has been extensively documented among high risk patient groups e.g. patients requiring prolonged hospital stays or antibiotic therapy, the elderly, the critically-ill or those with more severe co-morbidities [21,54-55]. However, in addition to these susceptible cohorts, the lack of orally-administrable antibiotics effective against MDR pathogens complicates routine treatment of outpatients with less severe illnesses [56-57]. Hence, the emergence of multidrug resistance also affects the non-critically ill and may hinder uncomplicated procedures which can increase the risk to the patient if hospitalization or parenteral therapy is required [21,34,58-59]. Outside the hospital setting, the increase in the prevalence of community-acquired infections exhibiting multi-resistance phenotypes continues to rise

[21,34,60-61]. This epidemiological shift is facilitated by the acquisition of resistance by diverse pathogenic bacteria. Furthermore, there is evidence that resistant strains can be maintained even in the absence of antibiotic selection which may account for the aggregation of MDR strains in the community and the accumulation of diverse resistance mechanisms in pathogenic strains [11,28]. The emergence of community-acquired infections suggests an ongoing increase in the prevalence of resistance among opportunistic pathogens and other resident microflora and is indicative of a move toward endemicity by MDR bacterial pathogens which has implications for the broader community as well as those in critical care within the hospital setting.

2.6 Resistant Gram-positive nosocomial pathogens

In addition to MRSA, the emergence of resistance is becoming a significant problem in other Gram-positive species including *Streptococcus pneumoniae*, members of the *Enterococcus* species and *Clostridium difficile* [62-64]. Paradoxically, antimicrobial therapy may enrich for such resistant bacteria and promote infection due to collateral antibiotic selection against non-pathogenic microbial flora. This is exemplified by the emergence *Clostridium difficile* as a major pathogen in antibiotic-associated diarrhea [65-66]. While infections caused by MDR Gram-positive pathogens represent a major burden to healthcare services, there has been some progress in the development of antibiotics for their treatment [67].

2.7 Resistant Gram-negative nosocomial pathogens

In contrast to Gram-positive species, the development of novel antibiotics which are active against Gram-negative bacteria has not been equally fruitful and current treatment options are limited by the comparatively broader resistance profiles of Gram-negative pathogens [68]. The lack of antibiotic alternatives available or in development for the treatment of Gram-negative infection has lead to widespread concern over the emergence of resistance in a number of important Gram-negative species [16,68]. The global stagnation in development of

antibiotics active against Gram-negative pathogens threatens the ability adequately to treat Gram-negative infection in the future.

2.7.1 The Enterobacteriaceae

The most prevalent clinically encountered Gram-negative pathogens belong to the Enterobacteriaceae; a bacterial family which inhabit the intestinal tract of diverse vertebrates including humans where they can exist commensally or cause intestinal and extra-intestinal infection [69]. In humans, the Enterobacteriaceae are causative pathogens of common infection such as urinary tract infections, blood stream infections, pneumonias as well as soft tissue or skin infections [70]. Among the most prevalent members are *E. coli, Enterobacter* spp. *Klebsiella* spp. and members of the Proteeae including *Proteus mirabilis* which are all commonly isolated in the hospital environment as well as the community setting [71]. Other less prevalent pathogenic species include *Serratia marcescens*, *Citrobacter* spp. and *Morganella morganii*. Factors contributing to the emergence of enterobacterial infection include increased life expectancy, numbers of immunocompromised patients as well as an increase in patients requiring long-term care [69].

Given their prevalence as opportunistic human pathogens, the emergence of multidrug resistance in the Enterobacteriaceae represents a significant public health concern. Resistance to important antibiotics has spread globally among the Enterobacteriaceae, notably in prevalent pathogenic species such as *E. coli, Enterobacter* spp. and *Klebsiella* spp. [71-72]. This has been catalysed by the dissemination of resistance genes among prevalent strains via horizontal gene transfer. Of particular concern is the dissemination of transferrable β-lactamases genes as β-lactam antibiotics are the therapeutic agents of choice for the treatment of Gram-negative infection [21]. Moreover, the emergence of β-lactamases with extended hydrolytic profiles termed Extended-Spectrum β-lactamases (ESBLs) has had a major impact on the treatment of Gram-negative infection. ESBLs confer clinical resistance to broad-spectrum β-lactam agents such as third-generation cephalosporins which

were previously relied upon for treatment of Gram-negative infections exhibiting resistance to narrow-spectrum β-lactam antibiotics [72]. ESBLs are frequently encoded on multidrug resistance plasmids which harbour multiple resistance genes and therefore ESBL production is often associated with expression of other resistance genes which further confounds treatment options [73-74]. Fall back therapy for treatment of ESBL-producing Enterobacteria relies heavily on the carbapenems; a class of β-lactams impervious to hydrolysis by ESBLs and to which the Enterobacteriaceae have until recently exhibited universal susceptibility [71]. The emergence of carbapenem resistance among the Enterobacteriacea therefore represents a significant threat to antibiotic treatment of enterobacterial infection. A novel carbapenemase gene, the New-Delhi β-lactamase (bla_{NDM-1}), which was first isolated in a *K. pneumoniae* infection acquired in New-Delhi, has recently been detected among numerous Enterobacteriaceae from disparate geographical locations throughout India, Pakistan and the UK where it has been identified on diverse transferrable plasmids [75-76]. The dissemination of plasmid-mediated resistance genes such as bla_{NDM-1}among the Enterobacteriacea represents a significant problem for the future treatment of infections caused by pathogenic members of this large bacterial family.

2.7.2 Non-fermentative Gram-negative bacteria

The propensity of Gram-negative bacteria to develop extensive resistance is prominently illustrated by non-fermentative pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* which may exhibit extreme resistance profiles resulting in very limited treatment options [77-78]. The non-fermentative bacteria encountered clinically are generally considered free-living opportunistic pathogens which can adapt to diverse ecological niches. As such, non-fermentative species harbour in their genomes many potential resistance genes including efflux pumps and degradative enzymes which may reflect the need for these species to protect themselves against the plethora of small bioactive molecules encountered within

diverse environmental niches [79]. Many of these gene can mediate antibiotic resistance in clinical isolates where they are either constitutively expressed, conferring intrinsic resistance, or are quiescent or basally expressed and confer resistance only if they become highly expressed through mutation [38-40,80]. Another distinguishing feature of non-fermentative pathogens is reduced outer membrane permeability. In comparison with *E. coli*, non-fermentative clinical pathogens have markedly reduced permeability and this can be explained by the absence of promiscuous, antibiotic-permissible porins comparable to those present in *E. coli* [81]. The combination of reduced permeability and the presence of many chromosomal resistance genes affords non-fermentative species a greater propensity to develop chromosomal resistance compared to members of the Enterobacteriacea. Such capacity to develop resistance is compounded by the horizontal acquisition of diverse resistance genes which further narrows treatment options for these already therapeutically challenging pathogens [68].

2.7.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the most prevalent non-fermentative Gram-negative opportunistic pathogen encountered clinically [82]. As a human pathogen, it is both invasive and toxigenic, produces multiple virulence factors and can colonise diverse host tissues, inflicting localized tissue damage and causing life-threatening infections [83-87]. *P. aeruginosa* infections have a high associated mortality particularly in ventilator associated pneumonias and bloodstream infections [88-90]. Particular patient cohorts which are at increased risk of infection by *P. aeruginosa* include burn wound victims and cystic fibrosis sufferers [91-92]. *P. aeruginosa* is found ubiquitously in aquatic and soil environments and can colonise important hospital environments such as sinks which may act as reservoirs for outbreak strains [82,93-95].

Several investigations of both environmental and clinical isolates suggests a largely epidemic population structure for *P. aeruginosa* with little influence of clonal expansions in

contrast to pathogens such as *E. coli* which exhibit a clonal population structure in which pathogenic and non-pathogenic lineages can be distinguished based on genetic typing [96].

Antibiotics for first-line treatment of *P. aeruginosa* infections are limited due to the intrinsic resistance of this pathogen. They include potent, broad-spectrum agents such as the carbapenems, aminoglycosides and fluoroquinolones but not all strains are universally susceptible to these antibiotics and resistance frequently emerges during therapy causing relapse [97]. The futility of antibiotic therapy is most notable in the infection of the cystic fibrosis (CF) lung, where even aggressive antimicrobial therapy fails to eradicate chronically colonizing strains particularly following colonization of the lower respiratory tract [92]. Antibiotics are administered in the hope of abating symptoms associated with pulmonary exacerbations rather than in expectation of clearing *P. aeruginosa* strains which chronically colonize the lung and are rarely eradicated [92,98].

In CF patients, chronically colonizing *P. aeruginosa* isolates are known to exhibit great genetic plasticity in adapting to the environment of the lung [92]. As a result of this remarkable adaptation, strains isolated from cystic fibrosis patients can display markedly different phenotypic traits compared to those isolated from other environments [99]. This plasticity is also reflected in the diverse chromosomal genes which mediate antibiotic resistance and survival in the cystic fibrosis lung even under intense antibiotic therapy [39,92,100].

Early infective strains of *P. aeruginosa* are generally highly-virulent, motile, immunostimulatory, planktonic cells whereas chronically colonizing strains form less-virulent, sessile, immuno-evasive, biofilm-forming communities. These changes provide stealth from the host immune system: reduced production of type IV pili, the type III secretion system (TTSS), exotoxins, proteases, phenazines and other immunostimulatory molecules as well as modulation of O-antigen biosynthesis hides the cell from the immune system [101]. Increased production of alginate protects the cell from antibacterial host responses and growth of *P. aeruginosa* as an organised biofilm also provides protection against immune-response [102-103]. Hence, *P. aeruginosa* undergoes a metamorphosis, from a

virulent, immunostimulatory pathogen during early infection, to a recalcitrant, immuno-evasive and highly-resistant pathogen during chronic infection.

This metamorphosis proceeds via diverse chromosomal mutations in the highly regulated genome of *P. aeruginosa*. Mutations in global regulators of virulence such as LasR and MucA are frequently identified in chronically infecting strains resulting in reduced virulence/host response due to their connectivity with multiple immunostimulatory virulence factors [104]. In terms of antibiotic resistance, mutations which cause upregulation of efflux pumps and β-lactamase resistance genes or down regulation of antibiotic-permissible porins are commonly observed in established chronically infecting strains [99,105]. Frequently identified mutations include disruption of *mexZ* which negatively regulates the aminoglycoside and β-lactam efflux system MexXY, mutations in *ampD* which lead to up-regulation of the chromosomal AmpC β-lactamase, and OprD-inactivating mutations which inhibit the entry of carbapenem antibiotics (Figure 4) [99]. The accumulation of such mutations can be accelerated in "mutator" strains which acquire mutations in genes of the DNA mismatch repair system such as *mutS* and *mutL* which causes increased rates of mutation allowing greater scope for adaptation [100,106]. In addition to resistance, antibiotic persistence has recently been demonstrated to emerge in chronically colonizing *P. aeruginosa* strains. In these strains (termed *hip* mutants for "high-persister") a higher number of persister cells survive antibiotic therapy through remaining dormant in the presence of high antibiotic concentrations although their level of resistance is not altered [107]. *P. aeruginosa* switches from a free-living environmental bacteria to a pathogenic lifestyle in the clinical setting. Over the course of this metamorphosis, adaptation to antibiotic therapy is crucial. Antibiotic resistance can be mediated by many chromosomal genes which undergo a functional switch as they adapt to the challenge of antibiotic therapy [39,79].

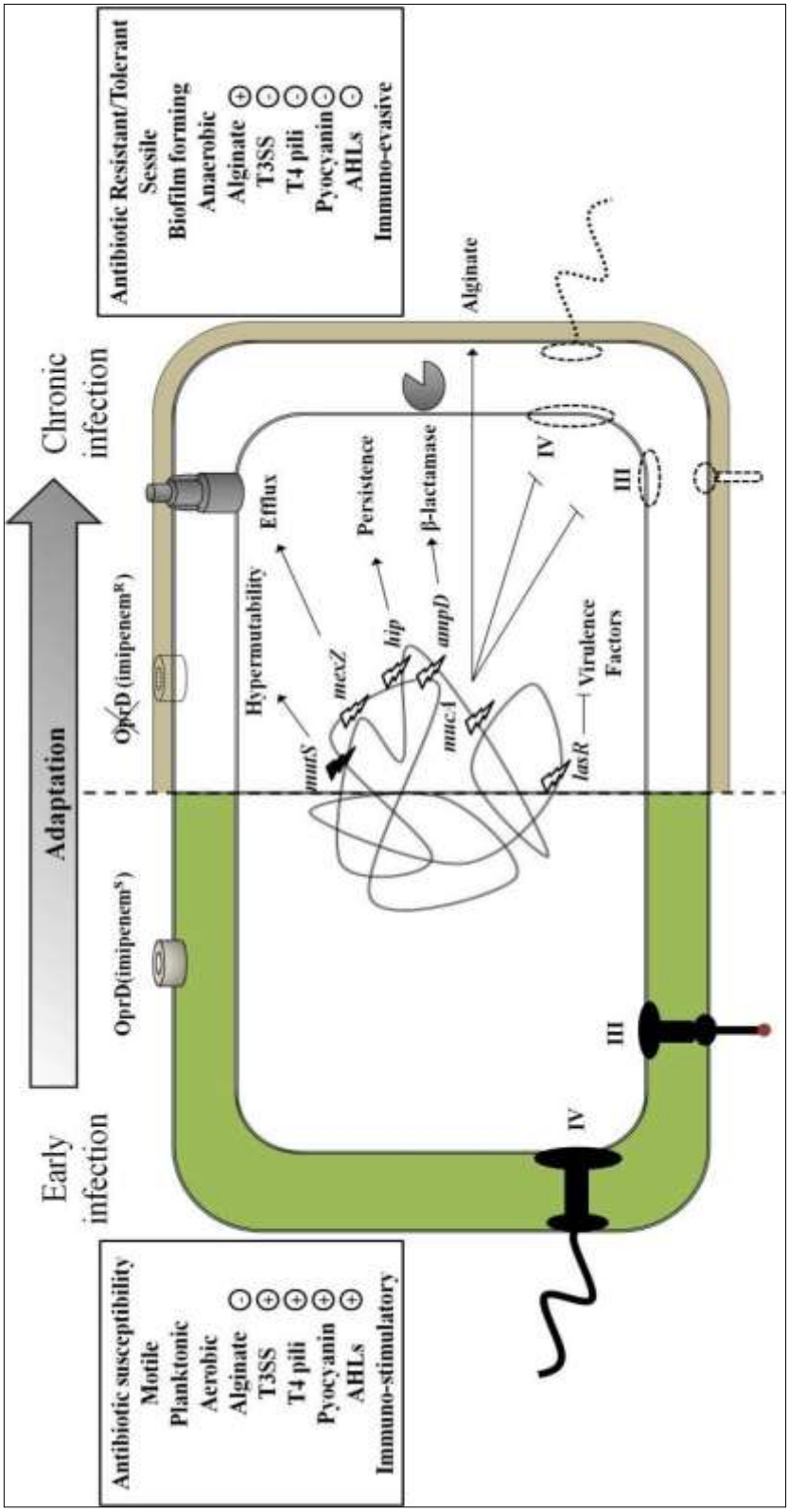

Figure 4. Genetic adaptation of *P. aeruginosa* during chronic infection. *P. aeruginosa* develops increased antibiotic resistance during chronic infection via down-regulation or loss of OprD, and upregulation of resistance genes. *P. aeruginosa* also down-regulates virulence factors including pyocyanin and acylated homoserine lactones (AHLs), expression of type IV pili and the type three secretion system (TTSS). Alginate production is increased and the cell changes to a sessile biofilm forming lifestyle. These changes are caused by diverse chromosomal mutations including *mutS* which causes hypermutability, *mexZ* which regulates efflux expression, *hip* genes which mediate persistence, *ampD* which regulates expression of the AmpC β-lactamase. Global regulatory genes such as *lasR* and *mucA* control diverse virulence factors which are involved in adaptation during chronic infection (adapted from Hogardt and Heesemann 2010 [103]).

3. Molecular mechanisms of antibiotic resistance

Bacteria must overcome the high antibiotic concentration used in antimicrobial therapy to persist as nosocomial pathogens. To do this, strains must circumvent antibiotic toxicity which inhibits vital physiological processes of bacterial proliferation. For bacteria (most evidently among those inhabiting the soil) this is not an novel impasse but one that has been imposed throughout their evolution by naturally occurring antibiotics secreted by niche-cohabiting species [15,27]. Whether natural or anthropogenic, there are four major pathways by which the toxic effect of antibiotics can be surmounted by bacteria (Figure 5) which are discussed below.

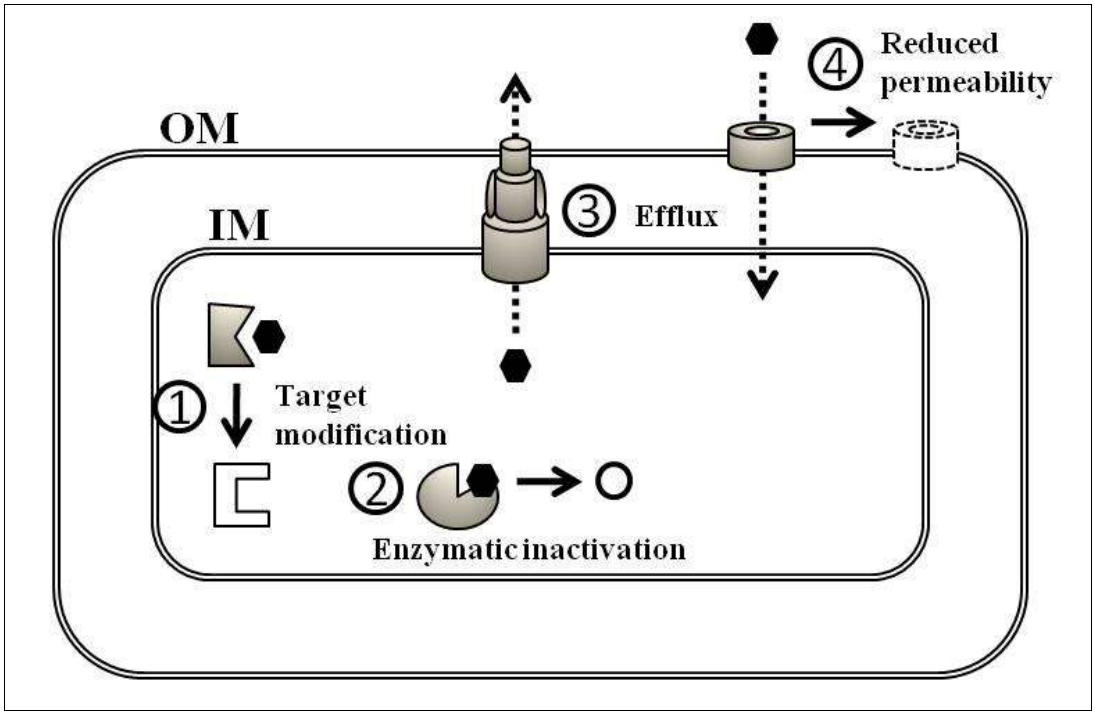

Figure 5. General pathways of antibiotic resistance in Gram-negative bacteria. **1 -** Target modification: mutations occurring in the antibiotic target render the antibiotic inactive as it no longer recognises its target (e.g fluoroquinones/topisomerase). **2 -** Enzymatic inactivation: enzymes which specifically recognise and chemically modify antibiotic structers rendering them inactive (e.g. β-lactam/β-lactamases), **3 -** Active efflux: Resistance-Nodulation-Division (RND) efflux systems recognize and export antibiotics reducing their intracellular concentration and increasing resistance (e.g. AcrAB-TolC/chloramphenicol), **4 -** Porin loss/down regulation: down-regulation or mutational disruption of outermembrane porins through which antibiotics enter the cell results in increased resistance (e.g. OprD/imipenem). **Black hexagons** = antibiotic, **white circle** = modified, inactive antibiotic, **OM** = outer membrane, **IM** = inner membrane.

3.1 Target modification:

Although antibiotics may exert an effect on many cellular components they generally have a primary target with which they are known to directly interact. Resistant strains often incur mutations in these targets which disrupts the affinity between antibiotic and target and negates the effect of the antibiotic on bacterial proliferation. For example, the modification of the bacterial transpeptidases that mediate cross-linking of glycan strands during peptidoglycan assembly are known as 'penicillin binding proteins' (PBPs) as they are the primary targets of β-lactam antibiotics which bind irreversibly, inhibiting cell wall synthesis and resulting in lysis of the bacterial cell [108]. Mutations in PBP-encoding genes which reduce the affinity of the β-lactam for its cognate PBP abrogate the bactericidal effect of β-lactam antibiotics causing resistance [109-110]. Similarly, fluoroquinolone resistance can also emerge through target modification as occurs in topoisomerase II genes *gyrA* and *gyrB* (primarily in Gram-negative bacteria) or topoisomerase IV encoding genes *parC* and *parE* (primarily in Gram-positive bacteria) which represent the targets of fluoroquinolone antibiotics [111]. More recently, the *qnr*-family (quinolone resistance) have been identified which encode small peptides which protect the topoisomoerase from quinolone/fluoroquinolone-mediated inhibition [112-113]. This mechanism may be considered as a sub-class of target modification or more precisely as a strategy of target protection. Another example of this type of resistance strategy is the modification of rRNA by rRNA methylases which modify the 16S rRNA such that aminoglycoside antibiotics which target this molecule can no longer tightly bind [114]. Thus, the bactericidal or bacteriostatic effect of an antibiotic may be countered by target-mutation or target-shielding which disrupts the characteristic antibiotic-target interaction.

3.2 Enzymatic inactivation:

In addition to target modifying mutations, many bacteria produce enzymes which recognise and inactivate clinically administered antibiotics, negating their antimicrobial activity. This occurs predominantly through hydrolysis and group transfer [30]. The β-lactamases, which hydrolyse β-lactam antibiotics are one of the most significant enzyme classes which contribute to resistance particularly in Gram-negative bacteria [115]. These enzymes are structurally related to bacterial PBPs and the molecular interaction between β-lactam and β-lactamase parallels that of the β-lactam antibiotic with the PBP with the exception that the β-lactam-PBP interaction is covalent and irreversible whereas the β-lactam-β-lactamase interaction is hydrolytic and short-lived [109]. The β-lactamase enzymes can be divided into 4 classes based on protein sequence: class A, C and D are characterized by serine residues at their active sites and class B β-lactamases are metallo-proteins which require at least one active-site zinc ion to facilitate β-lactam hydrolysis [116]. Other enzymes which confer resistance through antibiotic hydrolysis include macrolide esterases and fosfomycin epoxidases [30,117]. However their clinical significance pales in comparison with the β-lactamases. This may be due to the relatively lower levels of administration of these antibiotics and the contribution of other resistance mechanisms to macrolide and fosfomycin resistance [20,118-119].

Group-transfer is another major enzymatic mechanism responsible for conferring resistance to multiple antibiotics including aminoglycosides, macrolides and chloramphenicol [30]. Group transfer results in the covalent modification of antibiotics such that they no longer bind effectively to their targets. Aminoglycoside resistance is achieved by the transfer of chemical groups via aminoglycoside phosphotransferases (APHs), aminoglycoside nucleotidyltransferases (ANTs), and aminoglycoside acetyltransferases (AACs) and represents the predominant mechanism of aminoglycoside resistance in bacteria [114,120]. The most prevalent aminoglycoside resistance genes is *aac(6')-Ib* and interestingly a variant of this gene encodes an enzyme which can also acetylate fluoroquinolones [121]. This is a

significant finding as it suggests that two antibiotic selective forces have converged on a single enzyme resulting in the selection of a resistance gene which confers resistance to two distinct antibiotic classes. This observation represents the first incidence of a single enzyme conferring multiple resistance to diverse clinical antibiotic classes and its efficiency as a mediator of resistance is supported by its global dissemination [122]. This may set a precedent for the future evolution of bi-functional antibiotic resistance mechanisms.

3.3 Reduced permeability:

Many antibiotics must first traverse the cell wall to enter the cytoplasm or periplasmic space where they mediate their antibiotic effects. In Gram-positive bacteria the cell wall is permeable and does not restrict the entry of antibiotics into the cell except in certain strains which produced thickened cell walls [123]. In Gram-negative bacteria, the presence of an outer-membrane contributes to intrinsic resistance as antibiotics are impeded by the gel-like structure of the LPS and the selectivity of outer membrane porins [124]. This effect is well illustrated in the case of β-lactam antibiotics whose targets, the PBPs, are exposed at the cell surface in Gram-positive bacteria but shielded by the outer membrane in Gram-negative bacteria which makes Gram-positive species intrinsically more susceptible to many β-lactam antibiotics although they can develop resistance to cell wall-targeting antibiotics such as vancomycin through thickening of the cell-wall which represents a barrier to permeability [125]. In Gram-negative bacteria, entry of many hydrophilic antibiotics including β-lactams, aminoglycosides, tetracyclines, chloramphenicol and some fluoroquinolones is restricted to outer membrane porins which are water-filled protein channels in the outer membrane [39,126]. Thus, species which lack antibiotic-permissible porins or which down-regulate such porins through mutation exhibit decreased outer-membrane permeability which increases antibiotic resistance [81]. This can have an additive effect on resistance when coupled to other resistance mechanisms such as enzymatic degradation or efflux [124,127].

3.4 Antibiotic efflux

The extrusion of antibiotics by efflux systems which recognise and transport antibiotics out of the cell can allow bacteria to survive in the presence of high antibiotic concentrations by reducing the intracellular antibiotic concentration [124]. Antibiotics which target intracellular machinery must reach inhibitory concentrations in the cell before impacting on bacterial growth or viability. Below these concentrations antibiotics may have limited impact on bacterial survival or mediate other effects which may even be beneficial to the bacterial population [31]. Antibiotic extrusion is achieved by five families of efflux system in bacteria which are known to extrude antimicrobials namely; [i] the ATP-binding cassette (ABC) family, [ii] the major facilitator super-family (MFS), [iii] the resistance-nodulation-division (RND) family, [iv] the multidrug and toxic metabolite extrusion (MATE) family and [v] the small multidrug resistance (SMR) family [37,124]. While the ABC family of transporters are primary transporters, the majority of transporters involved in drug-resistance in Gram-positive and Gram-negative bacteria rely on secondary transport [37]. In Gram-negative bacteria the most significant family of transporter is the RND-family as these cytoplasmic transporters link synergistically with outermembrane porins (OMPs) via membrane fusion proteins (MFPs) to create a tri-partite channel which can export antibiotics from the cytoplasmic membrane directly to the extracellular space [124,128]. The remaining four families of efflux systems appear to play a less prominent role in the development of resistance in Gram-negative bacteria and are implicated in resistance more frequently in Gram-positive species. In Gram-negative bacteria, tri-partite RND efflux systems confer high level resistance and have broad substrate profiles which include diverse antibiotics [124].

4. The Resistance-Nodulation-Division family of multidrug efflux pumps

RND efflux pumps are the most clinically significant transporters associated with antibiotic resistance in Gram-negative bacteria [124]. Gram-negative species may contain a variable number of RND efflux pumps in their genomes; *E. coli* harbours seven RND systems whereas *Burkholderia cenocepacia* harbours sixteen [129-130]. In addition to antibiotic resistance, these systems can also contribute to diverse physiological functions [131]. The RND efflux systems of *Pseudomonas aeruginosa* have been extensively characterized due to their contribution to the formidable antibiotic resistance profile of this important human pathogen [39]. The *P. aeruginosa* genome contains 12 RND efflux pumps each of which have been investigated at the molecular level and can contribute to resistance to diverse compounds including antibiotics, detergents and metals (Table 2) [39,124,132-138]. *P. aeruginosa* is therefore an excellent model organism for the study RND efflux systems.

4.1 The RND efflux systems of *P. aeruginosa*

Of the 12 RND efflux pumps encoded in the genome of *P. aeruginosa* seven are located in operons with their cognate MFP, OMP components which comprise tripartite pumps. Other RND and MFP-encoding operons including *mexJK*, *mexMN*, *mexXY*, *mexVW* and *triABC* are not situated beside OMP-encoding genes. In order to extrude substrates beyond the outer membrane, these bi-partite efflux systems can associate with OMPs which are disparately located in the genome including native OMPs of diverse tripartite RND systems (Figure 6). The ability of RND components to form promiscuous associations with diverse OMPs may allow them scope to extrude diverse substrates such as different antibiotic classes [39,131]. Variations on the bi- or tri-modular composition of the RND efflux system are also encountered in *P. aeruginosa* including the TriABC system which incorporates two MFPs (TriB and TriC) which interact with OmpH [139]. Additionally, the *mexG* gene located in the *mexGHI-opmD* operon encodes a predicted membrane protein of unknown function which

may contribute to pump function whilst the *muxABC* operon encodes two RND systems [133,135] (Figure 6).

Table 2. Resistance profiles associated wuth diverse RND-efflux systems in *P. aeruginosa*.

RND pump	Antibiotic structural classes extruded[a]	Other extruded compounds[a]
MexAB-OprM	Aminoglycoside, β-lactam, Cerulenin, Chloramphenicol, Fluoroquinolone, Macrolide, Novobiocin, Sulphonamide, Tetracycline, Trimethoprim	Acriflavine, Crystal violet, Ethidium bromide, Homoserine lactones, Irgasan, Indole scaffolds [140b], Rhodamine 6G, SDS, Tetraphenyl phosphonium, Triclosan
MexCD-OprJ	β-lactam, Chloramphenicol, Fluoroquinolone, Macrolide, Novobiocin, Tetracycline, Trimethoprim	Acriflavine, Crystal violet, Ethidium bromide, Rhodamine 6G, SDS, Tetraphenyl phosphonium, Triclosan
MexEF-OprN	Chloramphenicol, Fluoroquinolone, Trimethoprim	Triclosan,
MexGHI-OpmD	Fluoroquinolone	Acriflavine, Ethidium bromide, Phenazines [141 *b*], Rhodamine 6G, Vanadium
MexJK-OprM	Fluoroquinolone, Macrolide, Tetracycline	triclosan
MexMN-OprM	Chloramphenicol, Thiamphenicol	
MexPQ-OpmE	Chloramphenicol, Fluoroquinolone, Macrolide, Tetracycline	Copper [136 *b*]
MexVW-OprM	Fluoroquinolone, Chloramphenicol, Macrolide, Tetracycline	Acriflavine, Ethidium bromide
MexXY-OprM	Aminoglycoside, β-lactam, Fluoroquinolone, Macrolide, Tetracycline	
MuxABC-OpmB [135 *b*]	Aminglycoside, β-lactam, Chloramphenicol, Macrolide	
TriABC-OpmH	-	Triclosan
CzcAB-CzcC [138 *b*]	-	Cadmium, Zinc

a **–** Inferred based on resistance profiling of strains which overexpressing or with mutated RND systems.

b **–** This table is adapted from two excellent, in-depth reviews on RND efflux pumps by Poole 2004 and Lister *et al* 2009 [39,142]. Additional data included from other sources is indicated.

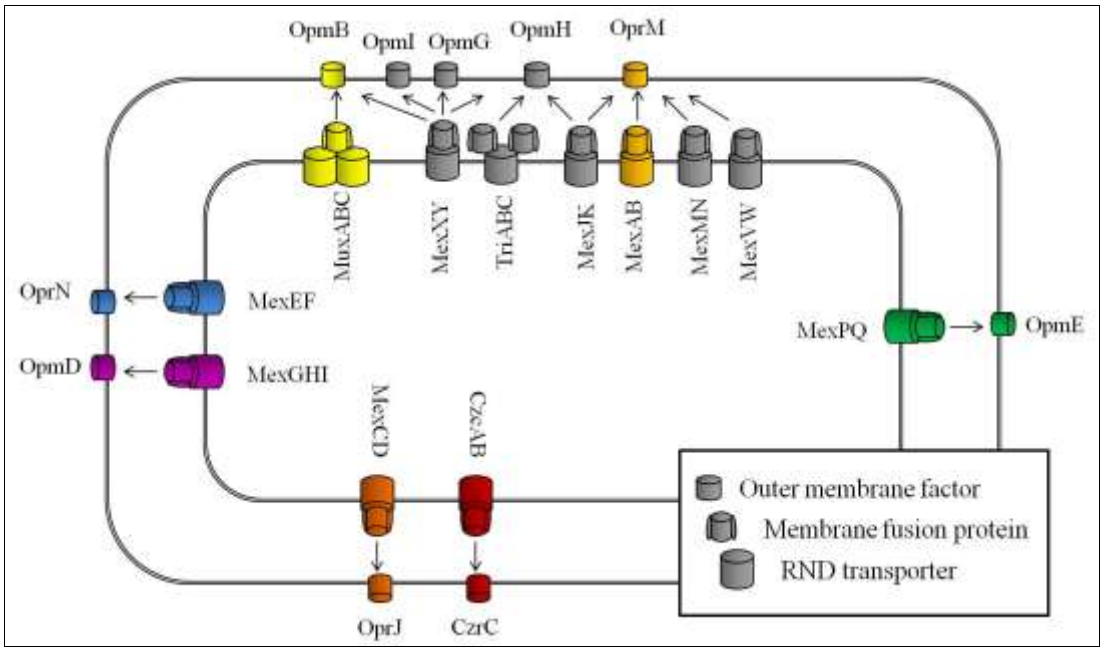

Figure 6. The modular nature of RND efflux systems in *P. aeruginosa*. The RND and MFP components of the tripartite system combine with an OMP to form a functional tripartite pump capable of extruding diverse compounds (*cf.* Table 1). Tripartite pumps for which cognate OMPs are disparately located on the chromosome are coloured in grey. Tripartite systems which are co-located in the genome are coloured as follows; MuxABC-OpmB (yellow), MexAB-OprM (gold), MexPQ-OpmE (green), CzcAB-CzcC (red), MexCD-OprJ (orange), MexGHI-OpmD (purple), MexEF-OprN (blue). The OMP component utilized by each RND system is indicated by an arrow.

4.1.1 MexAB-OprM

The MexAB-OprM efflux system is the most extensively characterized RND system in *P. aeruginosa* and has the widest antibiotic substrate profile (Table 1). It extrudes diverse antibiotics and is constitutively expressed contributing to intrinsic resistance as demonstrated by knock-out mutants which exhibit hyper-susceptibility to known pump substrates [39,143]. MexAB-OprM is strongly associated with clinical resistance to numerous antibiotics and also extrudes biocides and detergents as well as other chemically diverse compounds [142-143]. The OprM porin is essential for the resistance associated not only with MexAB but also MexJK, MexMN and MexVW suggesting that although OprM is located in an operon with MexA and MexB, other RND/MFP components can associate with this porin. This is also evidenced by the presence of MexAB-independent promoter elements upstream of OprM which is analogous to the situation in *E. coli* in which the TolC OMP functions as an outer-membrane outlet in tandem with diverse RND pumps [124,144]. In addition to its

contribution to aminoglycoside, β-lactam, chloramphenicol, trimethoprim and tetracycline resistance in planktonic culture, MexAB-OprM also contributes to biofilm-specific tolerance to the AMP colistin in flow-cell grown biofilms [145]. MexAB-OprM expression is elevated in the presence of chlorinated phenols including triclosan which is itself a substrate of the pump [146].

4.1.2 MexCD-OprJ

The MexCD-OprJ substrate profile is not as broad as MexAB-OprM in terms of known antibiotic substrates. Notably, MexCD-OprJ extrudes a narrower array of β-lactam antibiotics by comparison with MexAB-OprM which is important in terms of the clinical impact of this pump. Interestingly, overexpression of MexCD-OprJ is associated with hyper-susceptibility to a number of antibiotics including certain β-lactam and aminoglycoside antibiotics which is explained by the existence of interplay between RND-efflux systems which are apparently differentially regulated dependant on the expression status of other pumps [147]. MexCD-OprJ is induced by a number of membrane disruptive compounds which is in line with is role in mediating resistance to membrane-targeting biocides [148-149].

4.1.3 MexEF-OprN

The known substrate profile of the MexEF-OprN efflux system is narrower than that of MexCD-OprJ and similarly to this pump, induction of MexEF-OprN also is associated with β-lactam hyper-susceptibility. This has been shown to be mediated by a down-regulation of the MexAB-OprM system mediated by interruption of cell-signalling [150]. This highlights the compensatory changes in expression that occur between MexAB-OprM, MexCD-OprJ and MexEF-OprN. This may also occur between other efflux systems which potentially confounds interpretation of the precise substrates excluded by specific RND pumps based on susceptibility profiling. Induction of efflux systems may also alter resistance profiles through indirect effects on other resistance machinery and MexEF-OprN has increased

clinical relevance because of its association with carbapenem resistance. This carbapenem resistance is not due to MexEF-OprN expression but to concomitant repression of the OprD porin which is inversely regulated to MexEF-OprN. This highlights the existence of co-regulation of between resistance mechanisms in *P. aeruginosa* via intersecting regulatory pathways which has implications for antibiotic therapy [39,143]. The MexEF-OprN efflux system has been shown to be induced by chloramphenicol and nitrosative stress [151].

4.1.4 MexXY

The MexXY system which forms a promiscuous tripartite system with OpmB, OpmG, OpmH or OpmI OMPs mediates resistance to diverse compounds including fluoroquinolone, β-lactam, aminoglycoside, tetracycline, chloramphenicol and macrolide antibiotics and is frequently identified as being upregulated in clinical isolates of *P. aeruginosa* leading to antibiotic resistance [152-153]. This is frequently observed in isolates from CF patients which are often administered aminoglycosides. Induction of MexXY occurs in response to ribosome disrupting antimicrobials such as aminoglycosides and oxidative stress [154]. The high prevalence of aminoglycoside therapy among CF patients coupled to the high levels of oxidative stress encountered by *P. aeruginosa* in the lung could explain the frequency of MexXY-overproducing mutants in clinical isolates.

4.1.5 Other RND efflux systems of *P. aeruginosa*

Other RND pumps including MexJK, MexGHI-OpmD, MexMN-OprM, MexPQ-OpmE, MexVW-OprM and TriABC-OpmH are rarely if ever associated with resistance in clinical isolates although most pumps can mediate resistance to antibiotics and diverse toxic compounds [39,155]. The CzcABC RND system was identified in a *P. aeruginosa* strain isolated from metal contaminated water and confers resistance to cadmium, cobalt and zinc [138]. MexGHI-OpmD and MexPQ-OpmE have been implicated in resistance to vanadium and copper respectively suggesting that RND systems may be involved in metal homeostasis in

P. aeruginosa. Thus RND efflux systems of *P. aeruginosa* exhibit specific substrate profiles defined by their macromolecular protein structure which include clinically and environmentally important compounds such as antibiotics, biocides and metals. This provides clues as to the natural chemical scaffolds recognised by these pumps which is central to their natural physiological function. The elucidation of associated RND resistance profiles can also potentially be exploited clinically to link resistance profiles to specific resistance mechanisms in clinical isolates which could have therapeutic implications [156].

4.2 Distribution of RND systems among diverse bacterial pathogens

Many Gram-negative pathogens harbour RND-efflux systems homologous to those characterized in *P. aeruginosa*. This implies that these systems may contribute to multidrug resistance in these pathogens. Clinical studies have revealed RND systems homologous to those present in *P. aeruginosa* to be involved in resistance in diverse nosocomial pathogens. The relationship at the sequence level between RND pump components distributed among opportunistic pathogens *P. aeruginosa, E. coli, S. marcescens, A. baumannii*, and *B. cenocepacia* is illustrated in Figure 7. Sequence comparison of these RND pumps reveals that each of these pathogenic species harbour a complement of diverse RND efflux pumps which have been demonstrated to or could potentially contribute to antibiotic resistance. RND pumps present in these pathogenic species cluster into five groups which contain at least one group member which has been characterized. This may allow inference of the substrate profiles and function of uncharacterized groups based on sequence homology with characterized pumps.

Group I contains pumps similar with MexB and AcrB of the AcrAB-TolC tripartite system in *E. coli* which has homology with the MexAB-OprM pump and similarly has a broad substrate profile consistent with the general detoxification functions of these pumps [143,157-158]. Group II contains both MexF and MexQ and other characterized pumps which commonly transport chloramphenicol in these pathogenic species. Group III includes RND pumps

which are found in operon arrangement similar to MuxABC including SmeDE, MdtBC and BCAL1079-BCAL180. Group IV contains triclosan transporters MexK and TriB and Group V contains RND pumps characterized by their ability to transport metals.

The substrate profiles of characterized pumps within the tree are listed in Table 3. Group I and II seem to have the broadest substrate profiles which may suggest that they are general transporters which can accommodate diverse compound structures including antibiotics, giving them greater clinical relevance. The majority of Group I RND members have been characterized and are involved in the extrusion of antibiotics in clinical isolates. This may reflect the ability of these pumps to mediate resistance to clinical antibiotics due to common sequence features which contribute to their broad substrate profiles.

Smaller groups such as III and IV appear to have narrower substrate ranges which may suggest a more specific function. Additionally, MexV and MexI exhibit sequence homology to each other but do not cluster to any of the larger groups which could suggest a *Psuedomonas*-specific function for these RND pumps. In support of this the MexGHI-OpmD has been implicated in the transport of antibiotic phenazines which are produced by *P. aeruginosa* [133,141]. Finally some pumps exhibit sequence divergence from characterized RND transporters and may exhibit divergent functions and substrates profiles from characterized pumps.

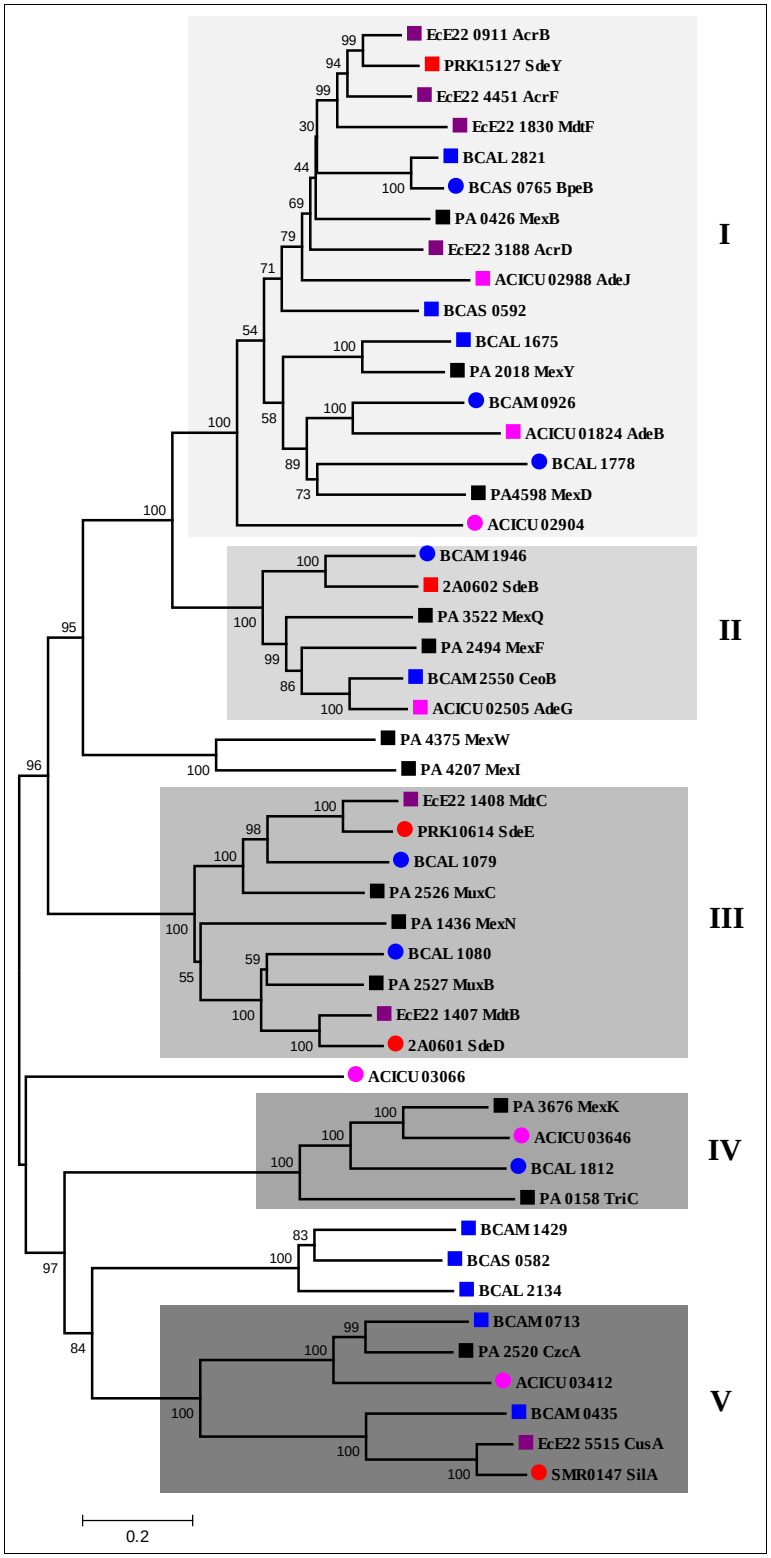

Figure 7. Neighbour-joining tree of RND efflux systems in *P. aeruginosa* and other Gram-negative pathogens. The protein sequence of RND pumps present in *P. aeruginosa* PAO1 (black symbols), *E. coli* O103:H2 (purple symbols), *Serratia marcescens* ATCC 13800 (red symbols), *A. baumannii* ACICU (pink symbols) and *B. cenocepacia* J2315 (blue symbols) were aligned using the clustalW algorithm [159]. A neighbour-joining tree of the sequence alignment was generated using MEGA 4.1[160]. RND efflux pumps whose associated resistance profiles have been determined are represented by squares and pumps which have not been characterized experimentally are represented by circles. The percentage of 1000 replicates that support each branch are indicated. The genetic distance (number of amino acid substitutions per site) is indicated below the dendrogram.

Table 3: Substrate profiles of RND efflux pumps of *P. aeruginosa* and other Gram-negative species.

Species	Group	RND pump	Resistance Profile[a]
Escherichia coli	I	AcrB	AC BA BL BS CM CV EB FQ FA FU LN ML MX NV PO SDS TC TS TX
Serratia marcescens	I	SdeY	AC BA EB FQ ML TC RD
Escherichia coli	I	AcrF	AC BL BS FQ LN ML RD SDS TC TP
Escherichia coli	I	MdtF	BA BL BS CV DOX EB NV ML RD SDS
Burkholderia cenpcepacia	I	BCAL2821	AG BL CM EB FQ
Pseudomonas aeruginosa	I	MexB	AC AG BL CL CM CV EB FQ HL IR IN ML NV QS RD SM TC TP TS TTP
Escherichia coli	I	AcrD	AG BL BS NV SDS
Acinetobacter baumannii	I	AdeJ	ACR BL CM FU FQ LS ML NV PY RF SA SDS TC TP
Burkholderia cenpcepacia	I	BCAL1675	HL NA
Pseudomonas aeruginosa	I	MexY	AG BL FQ ML TC
Acinetobacter baumannii	I	AdeB	AG BL CM FQ ML TC TP
Pseudomonas aeruginosa	I	MexD	AC BL FQ CM CV EB ML NV SDS RD TC TP TTP
Serratia marcescens	II	SdeB	CM EB FQ NX SDS
Pseudomonas aeruginosa	II	MexQ	CM Cu FQ ML TC
Pseudomonas aeruginosa	II	MexF	CM FQ TP TS
Burkholderia cenocepacia	II	CeoB	CM FQ TP
Acinetobacter baumannii	II	AdeG	ACR CM EB FQ LS TC TP SDS SM SO
Pseudomonas aeruginosa		MexW	AC CM EB FQ ML TC
Pseudomonas aeruginosa		MexI	AC EB FQ PZ RD Vn
Escherichia coli	III	MdtC	BL BS NV SDS
Pseudomonas aeruginosa	III	MuxC	AG BL CM ML
Pseudomonas aeruginosa	III	MexN	CM TM
Pseudomonas aeruginosa	III	MuxB	AG BL CM ML
Escherichia coli	III	MdtB	BL BS NV SDS
Pseudomonas aeruginosa	IV	MexK	CM FQ ML TC TS
Pseudomonas aeruginosa	IV	TriC	TS
Pseudomonas aeruginosa	V	CzcA	Cd Zn

[a] AC - acriflavine; ACR - acridines; AG - aminoglycosides; BA - benzalkonium; BL - β-lactams; BS - bile salts; CL - cerulenin; CM - chloramphenicol; CP - ciprofloxacin; Cd – cadmium; Cu - copper; CV - crystal violet; DOX - doxyrubicin; EB - ethidium bromide; FA - fatty acids; FU - fusaric acid; FQ - fluoroquinolones; HL - homoserine lactones; IN - Indole scaffolds; IR - irgasan; LN - linezolid; ML - macrolides; MX - methotrexate; NAL - nalidixic acid; NV - novobiocin; NX - n-hexane; PO - pine oil; PY - pyrosine; PZ - phenazines; Quorum sensing intermediates; RD - rhodamine; RF - rifampicin; SA - Safranin - SDS - sodium dodecyl sulphate; SM - sulphonamides; TC - tetracycline; TO - toluene; TS - triclosan; TP - trimethoprim; TPP - tetraphenyl phosphonium; TX - Triton X-100; Vn - vanadium, Zn – zinc.

4.3 Virulence phenotypes associated with RND efflux systems

Many bioactive secondary metabolites are toxic and bacteria go to great lengths both to synthesize and protect against their toxic properties [30,161]. In antibiotic-producing bacteria, resistance genes which protect the population from the lethal effects of endogenous antibiotics are essential [162-163]. Thus, RND efflux systems such as MexGHI-OpmD which transport toxic phenazines can modulate virulence through controlling the secretion of these endogenously produced toxins. MexGHI-OpmD is also implicated in modulating the levels of virulence-related molecules including acylated homoserine lactones and the Pseudomonas quinolone signalling molecule (PQS) [141,164]. In line with this, disruption of the MexGHI-OpmD system leads to reduced virulence in a plant and mammalian infection models [165]. Conversely, efflux systems can also protect from exogenous virulence factors produced by neighbouring organisms as well as from host toxins such as bile salts, plant isoflavinoids or mammalian antimicrobial peptides [131]. This appears to be a common pathway by which RND efflux pumps contribute to virulence although RND systems are also proposed to influence virulence via cell signal trafficking and changes in central metabolism [141,166-168]. MexAB-OprM is implicated in regulating diverse phenotypes including virulence, epithelial invasion and cell-signal trafficking [169]. Hence a dual function is suggested for this efflux system in co-ordinating pathogen-host interactions during infection but also in directing population behaviour through trafficking of small-molecule regulators of quorum-sensing [150,170]. MexEF-OprN, whose induction causes resistance to diverse clinical antibiotics, has also been linked to reduced levels of quorum sensing-regulated virulence traits, including pyocyanin, elastase and rhamnolipid production which is speculated to result from interruption of the quorum sensing signalling cascade which commits the cell to a state of reduced virulence [167]. MexEF-OprN also has a negative effect on expression of the TTSS, a trait it shares with another multidrug efflux system MexCD-OprJ which also elicits global metabolic changes when induced by mutation. MexCD-OprJ induction is also associated with impaired production of siderophores, rhamnolipids, secreted proteases, and pyocyanin

[168,171]. Hence RND efflux systems have appear to have diverse roles outside of bacterial stress response to antibiotics or other toxic metabolites and can exert pleiotropic effects on the cell.

The impact of efflux on diverse physiological processes is not a phenomenon restricted to *P. aeruginosa*. The AcrAB-TolC pump of *E. coli* has been well characterized and is involved in excretion of bile salts [131]. Homologous AcrAB-TolC systems have been identified in diverse bacterial species and TolC plays a crucial role in intestinal colonization by *Salmonalla enterica* and *Vibrio cholera* [155,172]. Virulence is also directly correlated with RND efflux expression in diverse pathogenic species including *Burkholderia pseudomallei, S. maltophilia* and diverse physiological functions have been proposed for bacterial efflux systems including regulation of chromosome condensation and segregation and modulation of host immunity [155]. As the diverse physiological processes in which resistance machinery is involved become evident, the collateral effect of antibiotic selection on pathogen physiology is highlighted. The regulation of resistance determinants must be highly co-ordinated given the diverse physiological processes in which they are implicated. This is supported by the diverse transcriptional regulators which have been identified to regulate resistance machinery and which are often perturbed in MDR clinical isolates which exhibit high-level expression of resistance genes.

4.4 Transcriptional regulation of resistance

To ensure execution of an optimal gene expression programme, bacteria have evolved complex networks of transcriptional regulators which perceive and co-ordinate gene expression in response to changing environmental and intracellular signals [173-174]. Transcriptional regulators which govern expression of antibiotic resistance genes may co-regulate other targets and, in this context, antibiotic selection can have a collateral effect on bacterial physiology through its impact on global transcriptional regulators [175].

4.5 Regulation of RND efflux systems in *P. aeruginosa*

P. aeruginosa provides a well studied example of how diverse efflux systems can be interwoven into complex regulatory networks in nosocomial pathogens [155]. The MexAB-OprM pump can contribute to high level resistance when mutations occur in several described transcriptional repressors. Regulators found to directly regulate MexAB-OprM included MexR, NalC, NalD, AmrR and the response regulator RocA2 [176-180]. All exhibit direct negative regulation of the *mexA* promoter apart from ArmR which is a small protein anti-repressor of MexR (Figure 8). Similarly to MexAB-OprM, the MexCD-OprJ pump is negatively regulated by the LacI-family repressor NfxB and MexJK is regulated by the TetR-family repressors MexL. No other targets of NfxB or MexL been defined however MexCD-OprJ appears also to be regulated by the alternative sigma factor AlgU which is a global regulator of virulence in *P. aeruginosa* [104,149].

MexEF-OprN and MexGHI-OpmD are regulated by MexT and SoxR respectively, both of which are positive transcriptional regulators which regulate diverse gene targets in addition to MexEF-OprN and MexGHI-OpmD [181-182]. MexT is a LysR-family transcriptional regulator known to directly regulate MexEF-OprN as well as mediating indirect down-regulation of the outermembrane porin OprD which is involved in resistance to carbapenem antibiotics. The *mexS* gene, which encodes a putative oxidoreductase of unknown function, is divergently transcribed from the *mexT*. MexS negatively regulates MexT activity indirectly and mutations in *mexS* lead to activation of MexT in clinical isolates[183]. Work presented in this thesis has investigated whether MexT acts a a global regulator [181]. This could explain how MexT mediates down-regulation of the OprD porin and virulence determinants exhibited by *mexEF-oprN* overexpressing strains via its global regulator effect on diverse targets. Similarly SoxR is a MerR-family regulator which positively activates expression of *mexGHI-oprM*, *PA2274* and *PA3718*. Another positive activator of RND efflux expression is CueR which not only regulates expression from the *mexPQ-OpmD*

promoter but also from four other promoters regulating a total of 12 genes involved in response to copper stress [136,182].

The local repressor of MexXY, MexZ, is indirectly influenced by *PA5471* which encodes a protein of unknown function and by the two-component system ParRS. ParRS also regulates the ArnB-F operon involved in LPS modification and polymyxin resistance and negatively regulates OprD mediating resistance to carbapenems [184]. Thus, similar to MexT, ParRS is a central regulator of antibiotic resistance which illustrates how transcriptional regulators can interlink diverse resistance mechanisms. OprD is also downregulated by the copper responsive CopRS two-component system which co-regulates the metal resistance efflux pump CzcABC and PtrA: a repressor of the TTSS. CzcABC is also regulated by another two-component system CzcRS which also regulates OprD and is thus linked to carbapenem resistance [138]. MexEF-OprN and MexCD-OprJ also mediate negative effects on the TTSS and are also co-regulated with other virulence factors. Thus, genes involved in diverse traits such as virulence can be influenced under antibiotic selection by virtue of their co-regulation with resistance genes via global transcriptional regulators.

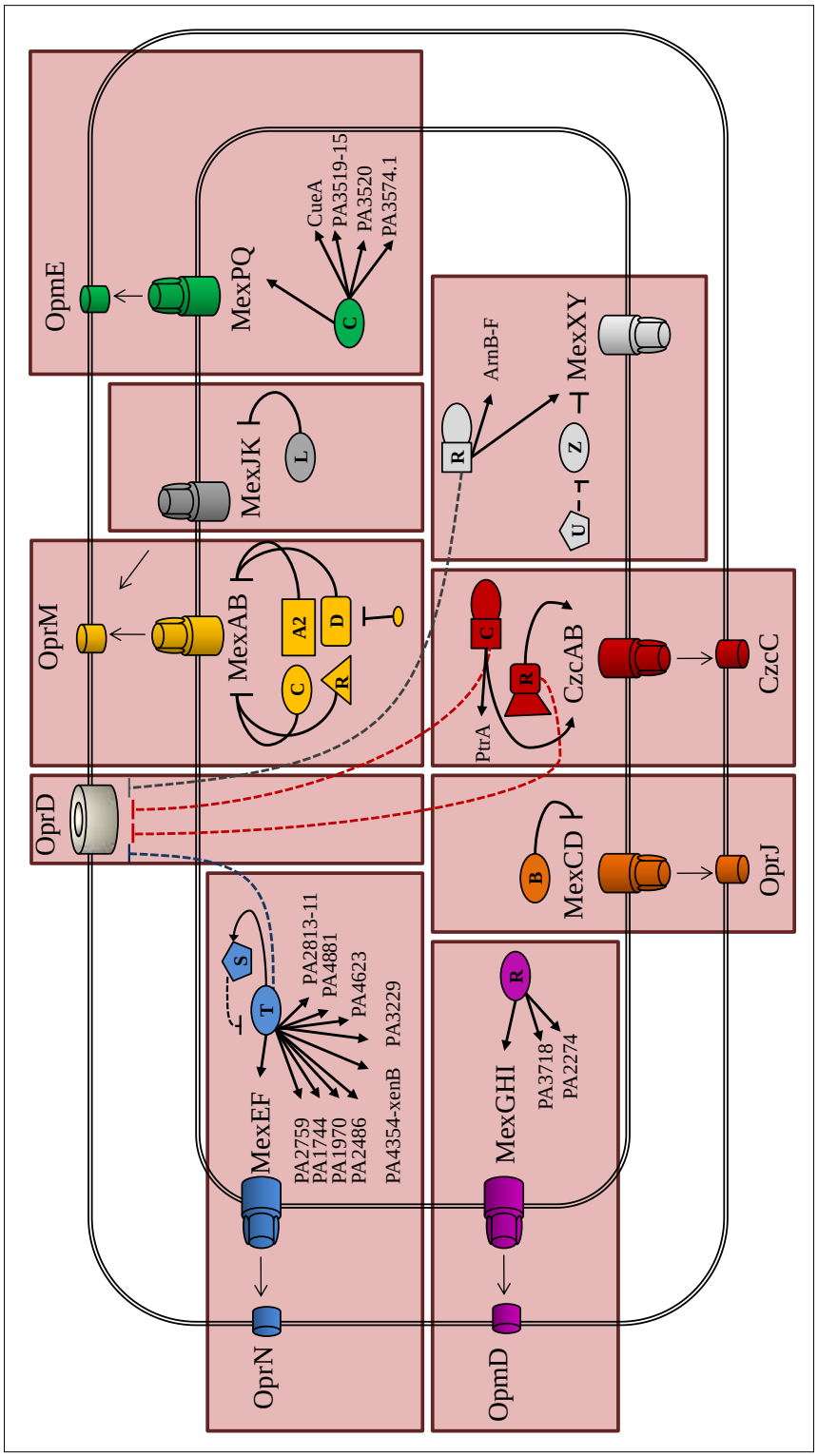

Figure 8. Transcriptional regulation of efflux pump expression in *P. aeruginosa*. **MexEF-OprN (blue):** T – MexT, S – MexS. **MexAB-OprM (gold):** C – NalC, A2 – RocA2, R – MexR, D – NalD, small ellipse – ArmR. **MexJK (dark Grey):** L – MexL, **MexPQ-OpmE (green):** C – CueR. **MexXY (light grey):** R – ParRS, U - PA5471, Z – MexZ. **CzcABC (red):** C – CopRS, R – CzcRS. **MexCD-OprJ(orange):** B – NfxB. **MexGHI-OpmD (purple):** R – SoxR.

5. Conclusion

Antibiotics possess unique properties which makes them invaluable in the treatment of bacterial infection. However, their efficacy is threatened by the emergence of antibiotic-resistant bacteria. The current stagnation in the development of novel antibiotics heightens the clinical impact of this emerging global problem.

Molecular epidemiology and surveillance networks have a critical role in curtailing the dissemination of antibiotic resistant bacteria and in detecting emerging resistance mechanisms in clinical pathogens. The identification of key factors contributing to resistance has led to changes in infection control policies and antibiotic stewardship which aim to halt escalating resistance levels. Molecular investigation of the diverse resistance mechanisms which have emerged in Gram-negative pathogens has also given insight into the causes of antibiotic resistance in bacterial pathogens.

For many chromosomal resistance genes, antibiotic detoxification is an adaptive role which has evolved under the selective pressure of antibiotic therapy. This may mask the natural physiological function of these genes. For example, the role of RND efflux systems in virulence suggest they are not merely detoxification systems and play central roles in co-ordinating central physiological processes such as cell-signal trafficking and metabolic homeostasis which are important to pathogenesis.

Transcriptional regulators of chromosomal resistance genes are frequently implicated in the emergence of resistance. The co-regulation of diverse resistance genes such as MexEF-OprN and OprD highlights the potential of transcriptional regulators to co-ordinate multidrug resistance. Global transcriptional regulators which mediate resistance can integrate resistance machinery such as RND efflux systems into broader physiological processes such as infection. The elucidation of the transcriptional networks into which resistance mechanisms are integrated thus provides insight into their physiological roles as part of complex gene expression programs. In the case of RND efflux systems, this offers insight into the co-regulation of two clinically important phenomena: antibiotic resistance and

virulence. Such insight may uncover novel therapeutic strategies for the treatment of MDR Gram-negative pathogens.

6. Aims of thesis

This thesis aims to investigate emerging resistance mechanisms among Gram-negative clinical pathogens in response to broad-spectrum antimicrobial therapy. It also aims to investigate the role of transcriptional regulators in *P. aeruginosa* mediating antibiotic resistance as well as other physiological phenomena related to pathogenesis.

Chapters 1 and 2 focus on the molecular epidemiology and emerging resistance mechanisms of MDR Gram-negative clinical isolates as revealed by molecular typing, DNA sequencing and expression analysis of resistance genes. Chapter 3 focuses on the precise physiological impact of the MexEF-OprN RND efflux system on *P. aeruginosa* physiology as directed by the transcriptional regulator MexT. The global regulatory scope of MexT is investigated revealing that MexT directly regulates a previously uncharacterized regulon which includes the non-canonical ArsR-family transcriptional repressor PA4354. The PA4354 repressor was characterized at the molecular level to gain insight into its regulatory function as part of the MexT regulon which controls antibiotic resistance and virulence in *P. aeruginosa*.

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Chapter 1

Emergence of Extended-Spectrum β-lactamase and Fluoroquinolone Resistance Genes among Irish Enterobacteriaceae Isolates

Chapter 1

1.1 Abstract

This study sought to identify mechanisms behind resistance to third-generation cephalosporins and ciprofloxacin in Irish multidrug-resistant Enterobacteriaceae isolated at the Mercy University Hospital, Cork. An increase in the isolation of resistant Gram-negative bacteria prompted the molecular characterization of prevalent multidrug-resistant strains and the resistance mechanisms they harboured. Among resistant Enterobacteriaceae, extended-spectrum β-lactamase (ESBL) producing strains were prevalent. The most prevalent ESBL genes identified were bla_{SHV-12} and $bla_{CTX-M-15}$. These were associated with the fluoroquinolone resistance genes *aac(6')-IB-cr*, *qnrA*, and *qnrB* not previously reported in Irish isolates.

1.2 Introduction

The emergence of multidrug-resistant (MDR) Gram-negative pathogens is an increasing global healthcare problem and a major challenge to the adequate treatment of bacterial infection [1-2]. The increasing levels of antibiotic resistance among Gram-negative bacteria has been attributed to the dissemination of transferrable plasmids harbouring multiple antibiotic resistance genes particularly among pathogenic members of the Enterobacteriaceae [2-3]. The acquisition of such plasmids by 'successful' pathogenic Gram-negative pathogens promotes their clonal expansion increasing the prevalence of resistance. Additionally, the increased numbers of plasmid-harbouring strains raises the probability of horizontal transfer of resistance plasmids among diverse pathogenic strains which further promotes resistance [4]. This phenomenon has been reported in many studies and is believed to have contributed significantly to global increases in MDR infections in the hospital and community setting [2,5-7].

Ireland has a high rate of antibiotic consumption in comparison with other European countries which places increased selective pressure on bacterial pathogens to develop resistance [8]. Over the last number of years, resistance to antibiotics including broad-spectrum agents such as third-generation cephalosporins and fluoroquinolones has consistently increased among Irish Gram-negative isolates [9]. However, studies reporting the epidemiology and molecular mechanisms of resistance among MDR Gram-negative bacteria in the Republic of Ireland are relatively limited.

To gain insight into currently emerging resistance trends in Irish Gram-negative isolates, a surveillance study was undertaken at the Mercy University Hospital (MUH), Cork, a 350-bed teaching hospital in the south of the Ireland. The prevalence of resistance to diverse antibiotics was determined among total Gram-negative bacteria. Isolates exhibiting multidrug-resistance were the subject of further investigation. MDR isolates were investigated at the molecular level to identify the genetic relationship between isolates and

the causative resistance mechanisms contributing to resistance among MDR Gram-negative bacteria at this hospital.

1.3 Materials and methods

1.3.1 Strain collection

Over three, six-month surveillance intervals (period A; 01.01.2004-30.06.2004, period B; 01.01.2006-30.06.2006, period C; 01.10.2006-31.03.2007), 3910 Gram-negative bacteria were isolated from patients at the Mercy University Hospital (MUH), a university teaching hospital in Cork, Ireland as part of routine diagnostic procedures.

1.3.2 Identification of MDR Gram-negative isolates

To identify Gram-negative isolates exhibiting multidrug resistance, antibiotic disks (Oxoid) containing ampicillin (10μg), amoxicillin-clavulanic acid (20μg -10μg), cephalothin (30μg), ciprofloxacin (5μg), gentamicin (10μg), ceftazidime (30μg), cefotaxime (30μg), amikacin (30μg), meropenem (10μg) and pipericillin-tazobactam (100μg-10μg) were used to determine resistance profiles by disc diffusion in accordance with the guidelines of the Clinical and Laboritory Standards Institute (CLSI) [10]. Isolates exhibiting non-susceptibility to $\geq$ 5 antibiotics were considered multidrug-resistant and stocked at -70°C for further analysis. MDR isolates were identified at the species level using the API 20E and 20NE bacterial identification strips (bioMerieux, France) and ESBL-production was assayed in testable species using Etest® strips (AB Biodisk, Sweden) in accordance with the manufacturers guidelines.

1.3.3 Genomic DNA extraction

Total genomic DNA was isolated by chloroform extraction to provide a template for strain typing and PCR screening of resistance genes. Overnight cultures of MDR isolates were pelleted and re-suspended in 400μl Lysis buffer consisting of 40mM Tris-acetate (pH7.8), 20mM sodium acetate, 1mM EDTA and 1% SDS. 5M NaCl was added to cell lysates which were centrifuged. DNA was extracted from supernatants in 500μl of chloroform. DNA was precipitated in 1ml 96% ethanol and pelleted by centrifugation. DNA was washed twice with

70% ethanol, air-dried and re-suspended in sterile de-ionized water. DNA concentrations were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) and adjusted a concentration of 100ng/µl, and stored at -20°C.

1.3.4 Molecular typing of isolate DNA

Strain diversity among prevalently identified species including *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. was assessed by Randomly Amplified Polymorphic DNA (RAPD) analysis as previously described [11]. The genetic relatedness of a subset of MDR *E. coli* isolates which exhibited similar RAPD fingerprints was further resolved using BOX, ERIC and REP-specific primers [12]. Ploymerase chain reaction (PCR) products from all typing reactions were separated on 2% agarose gels containing EtBr (1µg/mL) at 80V for 1-2 hours. DNA products were visualised by UV transillumination. Gel images were captured and analyzed using GelSnap version 6.04.00, and GeneTools version 3.05.03, (Syngene, UK)

1.3.5 PCR analysis

Detection for resistance gene in clinical isolates was performed by PCR using previously described primer sets to detect diverse $bla_{CTX\text{-}M}$, bla_{AmpC} and *qnr* genes by multiplex PCR. Simplex PCR was employed to detect bla_{TEM}, bla_{SHV}, bla_{OXA}, β-lactamase genes and *aac(6')-Ib* and *qepA* aminoglycoside and fluoroquinolone resistance genes [13-20]. In selected isolates, published primers specific for bla_{PER}, bla_{VEB}, bla_{GES} were employed to check for the presence of these genes [21-23]. PCR reactions were carried out using GoTaq® MasterMix (Promega, UK). Cycling conditions, primer and template concentrations were as previously described [13-23].

1.3.6 Sequence analysis

Platinum® High-fidelity proof-reading polymerase (Invitrogen, UK) was used to amplify resistance genes which were subsequently sequenced with previously described primers [11,13,15,20,24]. The bla_{TEM} and bla_{SHV} DNA sequences obtained were compared to a reference database of ESBL type-specific amino acid mutations [25] using the online ESBL Genotyping Tool (EGT) [26]. The sequences of bla_{CTX-M} and bla_{AmpC} resistance genes were compared to published sequence data using the NCBI Basic local alignment tool (BLAST). Sequence analysis of the cloned inserts in pBBR1MCS5 was performed using primers which flanked the multiple cloning site of pBBR1MCS5 and subsequent primer walking.

1.3.7 Conjugation

Isolates were incubated with a rifampicin resistant *E. coli* J53 recipient strain in a 10:1 ratio in LB broth or by filter mating on LB agar for 6-24 hours. Transconjugants were selected on LB agar containing cefotaxime (5μg/ml) and rifampicin (300μg/ml) incubated at 37°C for 24-48 hours. Resistant colonies were verified as *E. coli* J53 transconjugants by RAPD. Transconjugants were screened by disk diffusion for resistance to cefotaxime and rifampicin confirm the transfer of resistance. The co-transfer of bla_{CTX-M} *and aac(6')-Ib* was confirmed by PCR as described above. The ESBL phenotype of transconjugants TC01 and TC13 was assayed using Etest strips (AB Biodisk, Sweden) in accordance with the manufacturer's guidelines.

1.3.8 Plasmid extraction

Plasmid DNA was isolated from overnight cultures of clinical isolates and transconjugants following the standard alkaline lysis protocol [27]. Isolated plasmids were run on a 0.8% agrose gel containing EtBr (1μg/mL) for at 80V for 2-3 hours. DNA was visualised by UV transillumination. To obtain sufficient plasmid DNA concentrations for cloning of resistance genes, large-scale plasmid isolations were performed [27]. Isolated pBBR1MCS5 constructs

harbouring restricted fragment inserts were isolated from cefotaxime-resistant clones using the Qiagen® plasmid MINI-kit (Qiagen, UK).

1.3.9 Generation and screening of plasmid-fragment library

A restriction library of isolated plasmid DNA from transconjugants TC01 and TC13 was generated using three different endonucleases; *Hin*dIII, *Bam*HI, and *Eco*RI (Roche, Applied Sciences, UK). Digested fragments were ligated using T4 DNA ligase (Roche, Applied Sciences, UK) into respective *Hin*dIII, *Bam*HI, and *Eco*RI-digested, SAP-treated (Promega, UK) pBBR1MCS5 vector [28]. The ligation reaction contained an approximate vector to insert ratio of 3:1 based on the average restriction fragment size. The reaction was incubated overnight at 16°C and the ligation mixture was transformed into One Shot® Top10 chemically competent *E. coli* (Invitrogen, UK) by heat shock in accordance with manufacturer's recommendations. Transformed cells were screened on cefotaxime (20μg/ml) and gentamicin (20μg/ml) LB agar plates containing 40μg/ml Xgal. Colourless colonies exhibiting gentamicin and cefotaxime were sub cultured and their plasmids were isolated and digested to confirm plasmid fragments had been successfully ligated into pBBR1MCS5. Plasmid DNA was used as a template for subsequent sequence analysis of cloned inserts.

1.4 Results

1.4.1 Prevalence of antibiotic resistance among Gram-negatives at MUH

A major rise in the prevalence of antibiotic resistance among Gram-negative isolates was observed at the MUH between 2000 and 2007 (Figure 1). This included an increased prevalence of resistance to β-lactam and β-lactam inhibitor combinations (ampicillin, ceflaothin, cefotaxime, ceftazidime, meropenem, amoxicillin-clavulanic acid and pipericillin-tazobactem) as well as non-β-lactam antibiotics (ciprofloxacin, gentamicin and amikacin). Among the antibiotics surveyed, Gram-negative isolates exhibited a high rate of susceptibility to both meropenem and amikacin for which the prevalence of resistance was below 1%.

A surveillance study to investigate the mechanisms of resistance in Gram-negative bacteria was undertaken at MUH. Over three 6-month surveillance intervals, all Gram-negative bacteria exhibiting multidrug-resistance phenotypes (defined as non-susceptibility to ≥ 5 antibiotics) were collected for further characterization. Seventy-two non-replicate MDR bacteria were isolated over three collection intervals (period A - 21 strains, period B - 18 strains, period C - 33 strains). MDR strains were recovered from urine samples, tissue swabs, sputum samples, catheters, percutaneous endoscopic gastrostomy (PEG) sites, feces, blood culture and other specimens and were taken from patients located in hospital wards, intensive care, surgical wards, casualty, nursing homes as well as from outpatients (Table 1). On average, 1.8% (72/3910) of all Gram-negative bacteria isolated at MUH over the three collection intervals exhibited MDR profiles.

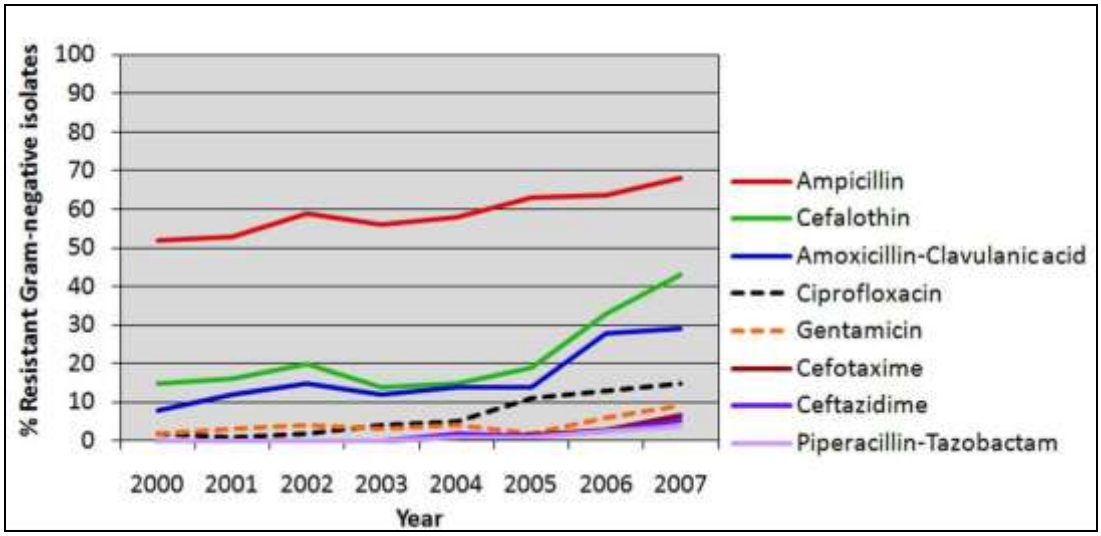

Figure 1: Increasing prevalence of resistance to commonly administered antibiotics among Gram-negative isolates at MUH (2000-2007). Levels of resistance to amikacin and meropenem are not shown as the percentage of isolates resistant to these antibiotics was below 1%.

Table 1: Distribution of MDR isolates among specimen types and patient location

Specimen Type	**N**	**%**	**Patient location**	**N**	**%**
Urine	19	26.4	Hospital ward	37	51.4
Tissue swab	17	23.6	Intensive Care Unit	15	20.8
Sputum	13	18.1	Casualty	6	8.3
Urinary catheter	6	8.3	Surgical ward	5	6.9
PEG site	5	6.9	Outpatient	4	5.6
Catheter	5	6.9	Nursing home residents	4	5.6
Nasal swab	2	2.8	Unknown	1	1.4
Feces	2	2.8			
Blood culture	1	1.4			
Other	1	1.4			
Unknown	1	1.4			
Total	**72**	100		**72**	100

1.4.2 Characterization of MDR isolates

Gram-negative bacteria exhibiting a MDR phenotype primarily belonged to the Enterobacteriaceae family (66/72, 92%) of which *Escherichia coli* was the most frequently identified species (38%, 25/66) followed by *Enterobacter cloacae* (32%, 21/66) and *Klebsiella pneumoniae* (11%, 7/66). All MDR isolates exhibited non-susceptibility to third generation cephalosporins, ceftazidime or cefotaxime suggesting the production of broad-spectrum β-lactamases such as ESBLs by these isolates. This was confirmed phenotypically in 53% (35/66) of Enterobacteriaceae which exhibited an ESBL production by Etest. Non-fermentative isolates were isolated at a low prevalence (Table 2) and were therefore excluded from further characterization. Molecular characterization focused on the most prevalent species among MDR Enterobacteriaceae namely, *E. coli*, *Enterobacter* spp., and *Klebsiella* spp. which accounted for >80% of total MDR Gram-negative isolates (59/72).

Table 2: Distribution of antibiotic resistance and ESBL phenotypes across diverse MDR Gram-negative species

Species	No. Isolates	ESBL positive[a]	No. of non-susceptible isolates[b]							
			AMC	CTX	CAZ	CIP	TZP	GM	AK	MEM
Enterobacteriaceae										
Escherichia coli	25	20 (80)	25 (100)	25 (100)	23 (92)	23 (92)	10 (40)	9 (36)	1 (4)	0
Enterobacter cloacae	21	7 (33)	21 (100)	21 (100)	21 (100)	12 (57)	16 (76)	7 (33)	0	0
Enterobacter spp.	3	0	3 (100)	3 (100)	3 (100)	1 (33)	2 (66)	1 (33)	0	0
Klebsiella pneumoniae	7	7 (100)	1 (14)	7 (100)	7 (100)	7 (100)	1 (14)	7 (100)	0	0
Klebsiella oxytoca	3	1 (33)	2 (66)	0	2 (66)	1 (33)	2 (66)	2 (66)	0	0
Serratia marcescens	4	0	4 (100)	4 (100)	2 (50)	4 (100)	4 (100)	0	0	0
Morganella morganii	2	ND[c]	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	0	0	0
Raoultella ornithinolytica	1	ND	1 (100)	0	0	1 (100)	1 (100)	0	0	0
Total (Enterobacteriaceae)	66 (92)	35 (53)	59 (89)	62 (94)	60 (91)	51 (77)	37 (56)	26 (39)	1 (2)	0
Non-Enterobacteriaceae										
Stenotrophomonas maltophilia	4	ND	4 (67)	4 (67)	4 (67)	4 (67)	2 (33)	4 (67)	3 (50)	4 (67)
Burkholderia cepacia	1	ND	1 (17)	1 (17)	1 (17)	0	1 (17)	1 (17)	1 (17)	0
Pseudomonas aeruginosa	1	ND	1 (17)	1 (17)	1 (17)	1 (17)	1 (17)	1 (17)	0	1 (17)
Total (Non-Enterobacteriaceae)	6 (8)	ND	6 (100)	6 (100)	6 (100)	5 (83)	4 (67)	6 (100)	4 (67)	5 (83)
Total (All Gram-negative)	**72**	**35 (49)**	**65 (90)**	**68 (94)**	**66 (92)**	**56 (78)**	**41 (57)**	**32 (44)**	**5 (7)**	**5 (7)**

a Identified phenotypically using Etest ESBL strips (AB Biodisk, Sweden).

b AMC amoxicillin + clavulanic acid, CTX cefotaxime, CAZ ceftazidime, CIP ciprofloxacin, TZP piperacillin + tazobactam, GM gentamicin, CPM cefepime, AK amikacin, MEM meropenem. Percentages are given in brackets. All MDR isolates were also resistant to both ampicillin and cephalothin.

c Not determined.

1.4.3 Molecular typing of MDR Enterobacteriaceae

Strain diversity among MDR *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. was assessed by Randomly Amplified Polymorphic DNA (RAPD) analysis to identify whether hospital transmission of outbreak strains had occurred. The diversity of RAPD profiles generated showed heterogeneity among *Enterobacter* spp. and *Klebsiella* spp. isolates thereby excluding a clonal outbreak of MDR strains (Table 3). Several smaller (<3 isolates - Groups II, III, V), but also one larger related group (12 isolates - Group I) was identified among *E. coli* isolates. However, further typing using BOX, ERIC and REP-specific primers identified differences between the 12 strains, excluding the presence of a recurrent hospital-transmitted clone (Table 3 and Figure 2). Moreover, six of these strains were isolated from patients within 48 hours of admission suggesting community acquisition and inter-strain antibiotic resistance profiles differed. Additionally, strains were isolated on disparate dates spanning all three collection intervals. Therefore, over-representation of resistance genes due to the presence of clonal, hospital-transmitted strains in this collection is unlikely.

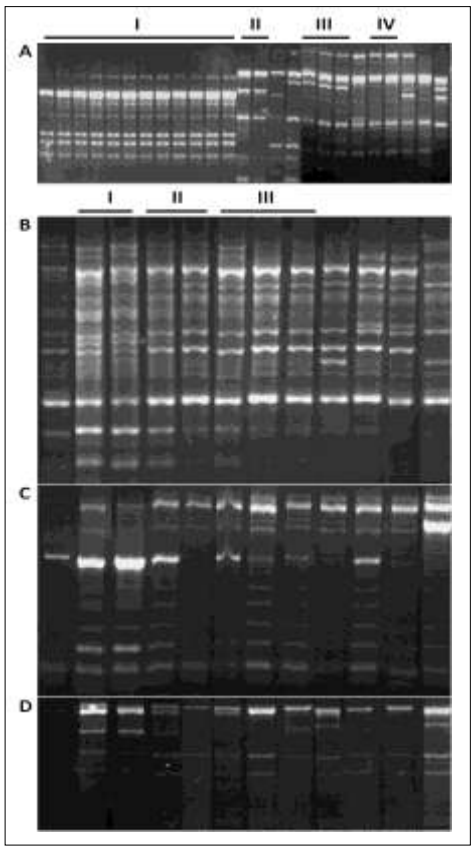

Figure 2. RAPD and REP typing of MDR *E. coli* isolates. (A) RAPD banding patterns observed for MDR *E. coli* illustrating genetic relatedness. Strains are grouped (I-IV) based on observed similarity in banding profiles. Strains with no similarity to any group are unassigned. Twelve strains (group I) had indistinguishable profiles. (B-D) REP typing profiles of the 12 isolates with indistinguishable RAPD profiles using alternate primers specific for REP (B), BOX (C) and ERIC (D) interspersed repetitive elements.

Table 3. Summary of strain diversity among same-species isolates

	No. of isolates within groups							
RAPD banding group	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**Unique[a]**
E. coli	12	2	3	2	-	-	-	6
E. cloacae	3	3	2	2	2	2	2	5
K. pneumonia	2	-	-	-	-	-	-	5
REP-typing pattern	**I**	**II**	**III**					**Unique[a]**
E. coli group I (N = 12)	2	2	3	-	-	-	-	5

[a] Number of strains exhibiting unique banding profiles with no similarity to any other isolates.

Table 4. Distribution of β-lactam and fluoroquinolone resistance genes among MDR Enterobacteriaceae

	Species					
	E. coli	***Enterobacter* spp.**	***Klebsiella* spp.**	**Other**	**Total**	**% of MDR Enterobacteriaceae**
Total isolates	25	24	10	7	66	-
non-clonal isolates[a]	17	18	9	ND[b]	-	-
ESBL-positive isolates	20	7	8	ND	35	53
β-lactam resistance genes[c]						
$bla_{\text{TEM-1}}$	3(1)	2(0)	1(0)	ND	6(1)	9.1
$bla_{\text{TEM-2}}$	0	1(0)	0	ND	1(0)	1.5
$bla_{\text{TEM-30}}$	1(0)	1(0)	0	ND	2(0)	3.0
$bla_{\text{TEM-39}}$	1(0)	0	0	ND	1(0)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{OXA-1}}$	0	0	1(0)	ND	1(0)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-11}}$	1(1)	0	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-12}}$	1(1)	3(1)	2(2)	ND	6(4)	9.1
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-12}}$ $bla_{\text{CMY-2}}$	1(1)	0	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-12}}$ $bla_{\text{OXA-1}}$	0	0	3(3)	ND	3(3)	4.6
$bla_{\text{SHV-12}}$	0	4(2)	0	ND	4(2)	6.1
$bla_{\text{SHV-12}}$ $bla_{\text{CTX-M-25}}$	0	1(1)	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{CTX-M-15}}$	4(4)	0	0	ND	4(4)	6.1
$bla_{\text{TEM-1}}$ $bla_{\text{CTX-M-15}}$ $bla_{\text{OXA-1}}$	1(1)	0	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{CMY-2}}$	1(0)	0	0	ND	1(0)	1.5
None	0	8(0)	0	ND	8(0)	12.1
β-lactam + FQ resistance genes[c]						
$bla_{\text{TEM-1}}$ $bla_{\text{CTX-M-15}}$ $bla_{\text{OXA-1}}$ *aac(6')-Ib-cr*	9(9)	0	0	ND	9(9)	13.6
$bla_{\text{TEM-1}}$ $bla_{\text{CTX-M-15}}$ *aac(6')-Ibcr*	1(1)	0	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{CTX-M-15}}$ $bla_{\text{OXA-1}}$ *aac(6')-Ib-cr qnrA*	1(1)	0	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-12}}$ *qnrA*	0	3(2)	0	ND	3(2)	4.6
$bla_{\text{SHV-12}}$ *qnrA*	0	1(1)	0	ND	1(1)	1.5
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-12}}$ $bla_{\text{OXA-1}}$ *qnrB*	0	0	2(2)	ND	2(2)	3.0
$bla_{\text{TEM-1}}$ $bla_{\text{SHV-12}}$ *qnrB*	0	0	1(1)	ND	1(1)	1.5

a Number of unique genomic DNA fingerprints observed among each species (cf. Table 3).

b Not determined.

c The number of phenotypically ESBL-positive isolates is indicated in brackets. Identified ESBL genes

1.4.4 Detection of resistance genes

The high prevalence of cephalosporin resistance and phenotypic ESBL-production among MDR Enterobacteriaceae suggested that broad-spectrum β-lactamase resistance genes including ESBLs were present in these isolates. In order to identify which specific genes were present among prevalent MDR isolates, all *E. coli*, *Enterobacter* spp. and *Klebsiella* spp. isolates were screened for the presence of known β-lactamase genes encoding TEM, SHV, CTX-M, AmpC and OXA-family β-lactamases. This revealed a high prevalence of TEM, SHV and CTX-M-family β-lactamase genes distributed among the Enterobacteriacea with SHV-family genes being found primarily in *Enterobacter* spp. and *Klebsiella* spp. and a high prevalence of CTX-M-family genes detected among MDR *E. coli* isolates. Sequence analysis of bla_{TEM} amplicons revealed the absence of TEM-family ESBLs, since only non-ESBL variants including $bla_{\text{TEM-1}}$, $bla_{\text{TEM-2}}$, $bla_{\text{TEM-30}}$ and $bla_{\text{TEM-39}}$ were identified (Table 4). However, non-ESBL bla_{TEM} variants were frequently associated with $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-15}}$ ESBLs in MDR Enterobacteriacea. SHV- and CTX-M-family β-lactamases represented the predominant ESBLs detected (Table 4). In 33 of the 35 ESBL-positive isolates identified phenoytpically, a causative ESBL gene was identified.

A high proportion of the Enterobacteriaceae (77%) exhibited fluoroquinolone resistance including 92% of MDR *E. coli.* Fluoroquinolone resistance is primarily associated with chromosomal target-altering mutations in topoisomerase encoding genes, however, transferrable fluoroquinolone resistance genes have recently been identified among globally disseminated strains of Enterobacteriacea [29]. To assess whether such genes were present among Irish MDR Enterobacteriacea, all isolates were screened for the *qnrA*, *qnrB*, *qnrS* and *qepA* fluoroquinolone resistance genes, and the *aac(6')-Ib*-family acetyltransferase genes. The *qnr* genes, *qnrA* and *qnrB* were detected in association with $bla_{\text{SHV-12}}$ in *Enterobacter* (4/24, 17%) and *Klebsiella* spp. (3/11, 27%) isolates respectively. One *E. coli* isolate was found to harbour *qnrA* and *qepA* was not detected in any isolate. Eleven isolates harboured AAC(6')-Ib-family aminoglycoside resistance genes, all of which were found to encode the

fluoroquinolone-modifying variant *aac(6')-Ib-cr* by amplicon sequencing. The *aac(6')-Ib-cr* gene was detected exclusively in *E. coli* (11/25, 44%) invariably in association with $bla_{CTX-M-15}$.

1.4.5 Transferrable resistance in clinical isolates.

The transfer of cefotaxime resistance from donor *E. coli* isolates to a cefotaxime-sensitive/rifampicin-resistant recipient was demonstrated in 13/20 ESBL-positive isolates confirming the transferrable nature of resistance in these strains. Susceptibility profiling of transconjugants revealed that resistance to diverse antibiotics was co-transferred with cefotaxime resistance (Table 5). This suggested the presence of multidrug-resistance plasmids in ESBL-positive isolates.

Table 5. Co-transfer of antibiotic resistance/resistance genes from MDR isolates

Transconjugant	**Resistance phenotype**[a]	**Transferred resistance genes**[b]
TC03	AM AMC KF CTX GN TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC04	AM AMC KF CTX TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC05	AM KF CTX	$bla_{CTX-M-15}$
TC06	AM AMC KF CTX GN TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC07	AM AMC KF CTX TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC09	AM AMC KF CTX TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC10	AM AMC KF CTX GN TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC14	AM KF CTX TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC16	AM AMC KF CTX	$bla_{CTX-M-15}$
TC17	AM AMC KF CTX	$bla_{CTX-M-15}$
TC18	AM AMC KF CTX TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*
TC19	AM AMC KF CTX	$bla_{CTX-M-15}$
TC20	AM AMC KF CTX TO KM cip	$bla_{CTX-M-15}$, *aac(6')-Ib-cr*

[a] AM ampicillin, AMC amoxicillin + clavulanic acid, KF cefalothin, CTX cefotaxime, GM gentamicin, TO tobramycin, KM kanamycin, CIP ciprofloxacin (lower case indicates low-level resistance).

[b] Transconjugants were screened for the presence of bla_{CTX-M} and *aac(6')-Ib* by PCR. The presence of specific variants ($bla_{CTX-M-15}$ and *aac(6')-Ib-cr*) is inferred from sequence analysis of these genes in donor isolates.

1.4.6 Investigating linkage of *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr* on transferred MDR-resistance plasmids.

The linkage of *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr* on common plasmids disseminated among *E. coli* isolates from diverse foci has been reported [30-33]. Eleven MDR *E. coli* isolates from MUH harboured both *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*. For nine of these strains, the co-transfer of both genes to a susceptible recipient strains was observed and imparted multidrug resistance to transconjugants. To determine if *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr* were co-located on a single plasmid, plasmid extractions were performed on the nine *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr* co-harbouring transconjugants. Seven transconjugants harboured a single plasmid, confirming the presence of both genes on a single genetic entity. The other two transconjugants harboured two plasmids, confounding interpretation of the linkage between *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr* in these strains.

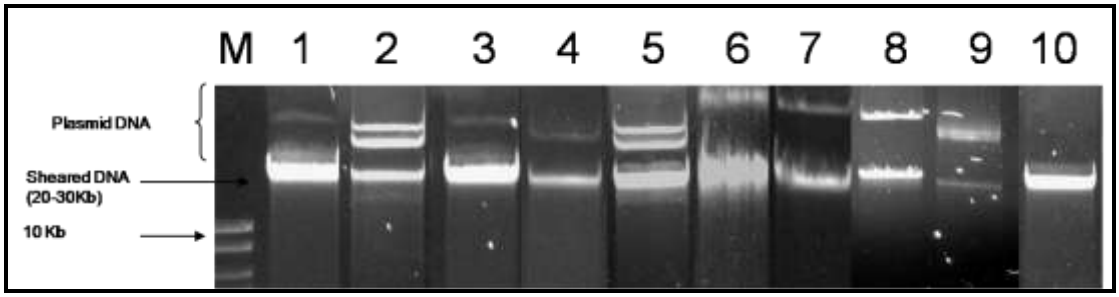

Figure 3. Isolation of plasmids from resistant transconjugants harbouring both *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*. Plasmids can be identified as larger DNA fragments seen to migrate to the upper regions of the gel above sheared DNA at approximately 20-30Kb (Lanes 1-9). Lane M is 1kb DNA ladder (Promega, UK) and lane 10 contains sheared genomic DNA isolated from the plasmid-less *E. coli* J53 recipient

1.4.7 Characterization of transferable resistance in EC 78879 and EC 41660

For two of the 35 ESBL positive isolates identified (EC 41660 and EC 78879), a gene responsible for the observed ESBL phenotype was not detected. Although, both were phenotypically ESBL-positive, no ESBL resistance gene could be identified in either strain by PCR using previously described primers specific for diverse ESBL genes [13-15,17,21-23]. The non-ESBL β-lactamase gene $bla_{\text{TEM-1}}$ was identified in both isolates and isolate EC 78879 also harboured another non-ESBL β-lactamase gene $bla_{\text{SHV-11}}$. The cause of cephalosporin resistance in these isolates, was the subject of further investigation. To determine if the cephalosporin resistance observed in EC 78879 and EC 41660 was transferrable, conjugations were set up for each strain with a cephalosporin susceptible *E. coli* recipient (*E. coli* J53 Rif^{R}). This yielded transconjugant strains TC01 and TC13 respectively to which cephalosporin resistance had been transferred. However, the ciprofloxacin and gentamicin resistance observed in EC 78879 and EC 41660 was not transferrable (Table 6). The level of cefotaxime resistance was confirmed in both isolates and transconjugants by micro-broth dilution and the ESBL phenotype observed in donors was confirmed in transconjugants by Etest (Table 7, Figure 4). The ESBL Etest confirmed the transfer of a ESBL-like resistance gene which was evidenced by the presence of "phantom" zone (Figure 4, D). These zones result from the inhibition of β-lactamase-mediated resistance by clavulanic acid and their appearance is indicative of ESBL production and correlated to ESBL production in clinical isolates [34]. This confirmed the transfer of a phenotypically ESBL-like cephalosporin resistance gene to both TC01 and TC13.

Table 6. Resistance profiles of Isolates and transconjugants.

	Antibiotic resistance[a]									
Strain	AMP	AMC	KF	CIP	GM	AK	CAZ	CTX	MEM	TZP
Recipient (E. coli J53 Rif^R)	S	S	S	S	S	S	S	S	S	S
EC 78879 (donor)	**R**	**I**	**R**	**R**	S	S	S	**R**	S	S
TC 01 (transconjugant)	**R**	**I**	**R**	S	S	S	S	**R**	S	S
EC 41660 (donor)	**R**	**I**	**R**	**R**	**R**	S	S	**R**	S	S
TC 13 (transconjugant)	**R**	S	**R**	S	S	S	S	**I**	S	S

[a] AMP ampicillin, AMC amoxicillin + clavulanic acid, KF cephalothin, CIP ciprofloxacin, GM gentamicin, AK amikacin , CAZ ceftazidime, CTX cefotaxime, TZP piperacillin+ tazobactam, MEM meropenem. R = resistant I = intermediate, S = susceptible determinined by disk diffusion according to CLSI guidelines [10].

Table 7. Minimum inhibitory concentration (MIC) of cefotaxime in isolates and transconjugants.

Strain	**Cefotaxime MIC (µg/ml)**
Recipient (*E. coli* J53 Rif^R)	<0.5
Donor 01	>64
Transconjugant 01	>64
Donor 13	>64
Transconjugant 13	8

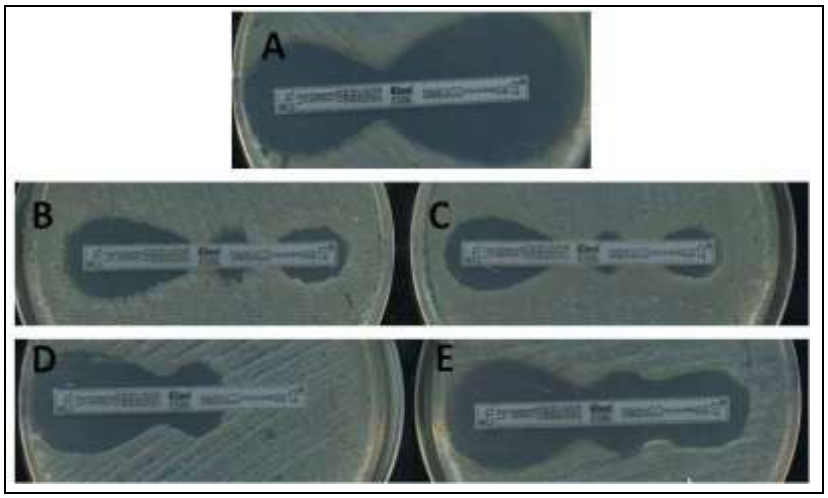

Figure 4. Confirmation of ESBL phenotype in *E. coli* isolates and transconjugants. The relative inhibition by cefotaxime (right side of strip "CT") and cefotaxime in the presence of clavulanic acid (left side of strip CTL) determines ESBL production. **A** - The recipient strain E. coli J53 Rif^R is highly susceptible to cefotaxime in the presence and absence of clavulanic acid (as indicated by large inhibitory zones around each half of the Etest® strip) and is therefore ESBL negative. **(B + C)** - Both donors (**B:** EC 78879 **C:** EC 41660) exhibit cefotaxime resistance and ESBL-positive phenotypes. **(D + E)** - Both transconjugants (**D:**TC01, **E:**TC13) exhibit increased cefotaxime resisitance compared to **A** and are ESBL positive as indicated by the presence of "phantom" zones at the centre of the Etest® strip.

1.4.8 Cloning of the causative ESBL resistance gene from TC01 and TC13

In order to identify the causative ESBL-like resistance gene which had been transferred to transconjugants TC01 and TC13 from EC 78879 and EC 41660, plasmids were isolated from each transconjugant, digested and cloned into the pBBR1MCS5 vector. Initial isolation of plasmids revealed the presence of two plasmids in TC01 and a single plasmid in TC13 (Figure 5). Digestion of plasmids with diverse restriction enzymes revealed enzyme-specific fragment patterns for *Eco*RI, *Hin*dIII and *Bam*HI. Although the patterns for plasmid DNA from each transconjugant were not identical, certain similarities were evident (Figure 5). Digested fragments were ligated into pBBR1MCS5 and were transformed into Top10 chemically competent *E. coli*. Screening for cephalosporin-resistant clones yielded candidates for *Hin*dIII-digested TC01 plasmid DNA and for *Eco*RI digested plasmid fragments of TC13. Sub-culturing and plasmid extraction was performed on each candidate and plasmids were re-transformed into new *E. coli* cells. Introduction of the isolated plasmids into *E. coli* conferred resistance to gentamicin and cefotaxime confirming the successful cloning of a ceftotaxime resistance gene through ligation into pBBR1MCS5. The pBBR1MCS5 plasmid construct generated from *Hin*dIII-restricted TC01 plasmid DNA (pBBR-TC01/*Hin*dIII) and the plasmid generated from *Eco*RI-restricted TC13 plasmid DNA (pBBR-TC13/*Eco*RI) were further analysed to determine causative cephalosporin resistance genes.

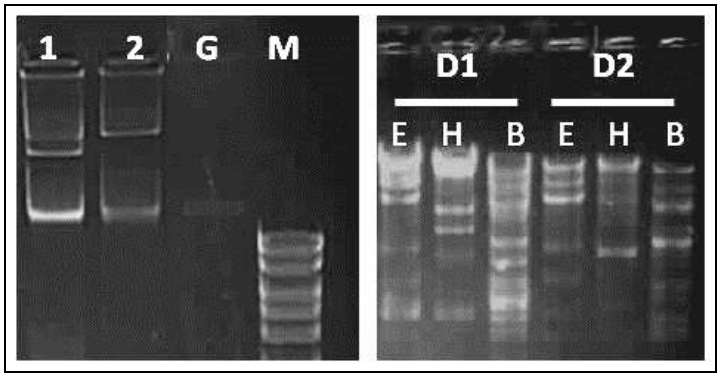

Figure 5. Isolation and digestion of plasmid DNA from transconjugants. On the left, Lanes **1** and **2** show plasmid DNA isolated form TC01 and TC13 respectively run beside genomic DNA (**G**) and a DNA ladder (**M**, top rung = 10kb) for comparison. Two plasmids are present in TC01 and TC13 harbours a single plasmid. The digestion profiles of plasmid DNA isolated from TC01 (**D1**) and TC13 (**D2**), following digestion *Eco*RI (**E**) *Hin*dIII (**H**) and *Bam*HI (**B**) are shown on the right.

1.4.9 Sequence analysis of cloned fragments

Restriction digests of pBBR-TC01/*Hin*dIII and pBBR-TC13/*Eco*RI revealed inserts of approximately 2 and 6.5 kb respectively. Sequencing of the cloned insert in pBBR-TC01/*Hin*dIII identified the presence of the previously described $bla_{CTX\text{-}M\text{-}9}$ cefotaxime resistance gene and a partial sequence of the downstream putative recombinase *orf513* which were present in a 1.8kb inserted fragment. Sequencing of the larger fragment by primer walking also identified these genes and revealed the wider genetic context of the $bla_{CTX\text{-}M\text{-}9}$ including the previously described Orf3-like coding region and a partial sequence of the IS3000 insertion sequence encoded by *orf1005*. Both fragments aligned to genbank accession AF174129.3 which contains a partial sequence of the pMSP071 plasmid in which $bla_{CTX\text{-}M\text{-}9}$ is present within the class-1 integron In60 (Figure 3) [35]. Thus the cause of cephalosporin resistance in isolates EC 78879 and EC 41660 was the presence of the $bla_{CTX\text{-}M\text{-}9}$ which was not detected in by PCR screening for CTX-M-family β-lactamase genes.

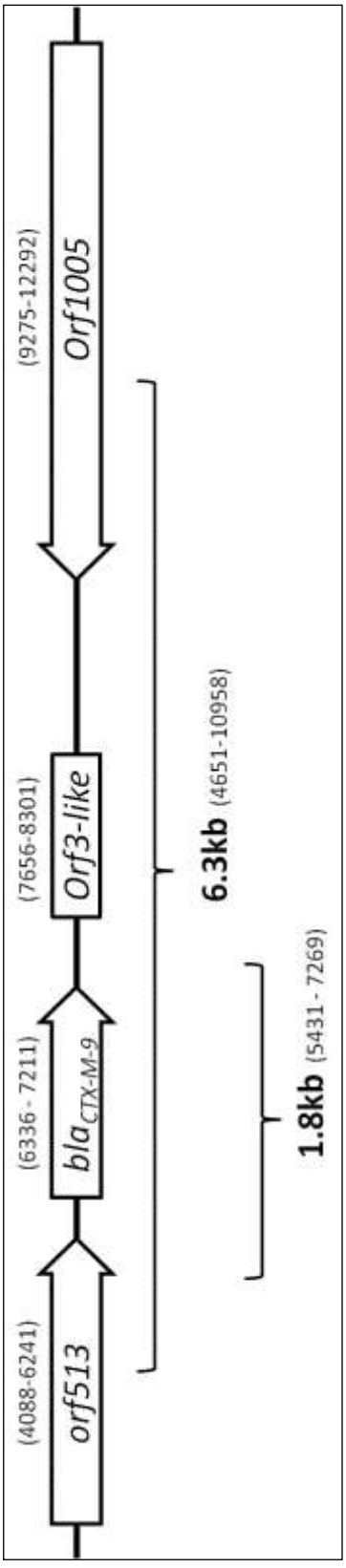

Figure 6. Alignment of cloned fragments with AF174129.3. The $bla_{CTX-M-9}$ is located within a class-1 integron structure. Brackets indicate the regions on AF174129.3 to which restricted plasmid fragments cloned from TC01 (1.8kb) and TC13 (6.3kb) aligned. Bracketed numbers represent the nucleotide positions relative to the AF174129.3 accession.

1.5 Discussion

1.5.1 Emergence of resistance among the Enterobacteriaceae in Europe.

The frequency of antibiotic resistance among Gram-negative bacteria has increased steadily at MUH in recent years. The prevalence of resistance to broad-spectrum antibiotics including ceftazidime, cefotaxime, ciprofloxacin and gentamicin all rose significantly, between 2004 and 2007. This increase is similar to previously published data of other groups in Europe; including a recent study by Livermore *et al*. in which a dramatic shift among Enterobacteriaceae is observed, with sharply rising non-susceptibility to cephalosporins, ciprofloxacin and gentamicin in England, Wales and Northern Ireland [3,9,36].

The average prevalence of multidrug-resistance among Gram-negatives isolated across all three collection intervals was 1.8% according to our selection criteria. Definitions of multidrug-resistance vary widely among different studies and can differ depending on the bacterial species/group under study and may be affected by the specific antibiotics used in susceptibility screening [1,37-38]. In this study, any strain exhibiting non-susceptibility to 5 out of 10 routinely tested antibiotics for resistance profiling of Gram-negative isolates at MUH was considered multidrug resistant. This differs, for example, from the definition of the European Antimicrobial Resistance Surveillance System (EARSS) which defines multi-resistance in *E. coli* and *K. pneumonia*e as "resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides, irrespective of aminopenicillin susceptibility." The definition of the EARSS may have been restrictive in this study which focussed on a relatively small collection of resistant isolates. Less than 1% (27/3910) Gram-negative bacteria isolated at MUH during this study meet the EARSS criteria for multi-resistance. Nonetheless, our selection criteria provided a sample of Gram-negative clinical isolates with markedly reduced susceptibility to multiple clinically relevant antibiotics which accounted for 1.8% (72/3910) of total Gram-negative bacteria isolated over the study interval.

1.5.2 Diversity of ESBL-producing Enterobacteriaceae in Ireland.

The diversity of resistance genes identified among MDR Gram-negatives at MUH reflects the tendency of Gram-negative bacteria to become increasingly resistant in response to antimicrobial therapy. Under persistent antibiotic selection, efficient resistance genes associated with mobile elements become widespread through their integration into transferrable resistance plasmids which are maintained. Such plasmids are found to be associated with dominant clonal complexes of clinical pathogens resulting in a shift to endemicity by MDR strains [4,39]. Antibiotic consumption is a key factor which drives the emergence of antibiotic resistance and is high in Ireland compared to other European countries [8]. High strain diversity among ESBL-producing strains at MUH reflects the independent acquisition of resistance plasmids by diverse strains which might be due to the widespread use or misuse of antibiotics in Ireland.

1.5.3 Emergence of *bla*$_{CTX\text{-}M\text{-}15}$ in Irish *E. coli* isolates.

Among *E. coli*, *bla*$_{CTX\text{-}M\text{-}15}$ was the most prevalently detected ESBL gene and the second most prevalent among MDR Enterobacteriaceae. This gene has spread globally by association with diverse plasmid scaffolds which have allowed its acquisition by prevalent strains such as the ST131 clonal complex which have driven its dissemination [7,33]. The incidence of *bla*$_{CTX\text{-}M\text{-}15}$-positive *E. coli* increased across the three collection intervals of this study (A: 1 isolate, B: 3 isolates, C: 11 isolates). Even though 12 *E. coli* strains exhibited strong banding similarities, differences in REP banding patterns and epidemiological data suggest these strains were not hospital-transmitted clones. However, the similarities observed between their RAPD and REP profiles may indicate that they belong to a common lineage such as ST131 (within which strains are known to differ in typing profiles [33]) or may represent another locally prevalent strain type [4]. The *bla*$_{CTX\text{-}M\text{-}15}$ gene was also present in *E. coli* with markedly different RAPD profiles confirming that diverse strains in this region harbour *bla*$_{CTX\text{-}M\text{-}15}$ and that *bla*$_{CTX\text{-}M\text{-}15}$ -producing *E. coli* may be have become endemic as a

result of the expansion of diverse clonal complexes harbouring this gene. In line with this, a retrospective nationwide study by Morris *et al*. reported the first detection of a bla_{CTX-M}-positive isolate in Ireland in 2000. In 2007 that number had risen to 101 (falling from 168 the previous year) suggesting an endemic situation in which the presence of CTX-M-family genes in diverse clonal lineages of *E. coli* was also noted [40].

1.5.6 The bla_{SHV-12} ESBL in Irish *Enterobacter* spp. and *Klebsiella* spp. isolates.

The bla_{SHV-12} was the most prevalent ESBL detected with highest prevalence among *Enterobacter* spp. and *Klebsiella* spp. isolates. There is no nationwide study of bla_{SHV-12}-enzymes in Ireland hence it is difficult to conclude whether an endemic situation exists regarding bla_{SHV-12}-producing Enterobacteriaceae though they are prevalent throughout Europe [2]. The clonal diversity of bla_{SHV-12}-producing strains detected suggests that this gene is prevalent in *Enterobacter* spp. and *Klebsiella* spp. strains from this region.

A high prevalence of bla_{SHV-12} among *Enterobacter* spp. is problematic as its presence in this genus can confound detection of ESBL production [34]. Although half of the *Enterobacter* spp. isolates harboured bla_{SHV-12}, only 29% were phenotypically ESBL-positive. This may be explained by the fact that up-regulation of the chromosomal AmpC β-lactamase of *Enterobacter* spp. isolates can also result in resistance to third-generation cephalosporins which can confound detection of ESBLs and is a controversial issue in this genus with various levels of sensitivity and specificity reported [34,41-43]. Thus, MDR *Enterobacter* spp. represent a significant problem at this institution, in terms of their prevalence and their ability to confound selection of pertinent treatment options based on interpretation of susceptibility data.

1.5.7 Transferrable fluoroquinolone resistance genes in Irish Enterobacteriaceae isolates

Of the 16 *bla*$_{CTX-M-15}$-positive *E. coli* identified, 11 harboured the aminoglycoside and flouroquinolone resistance gene *aac(6')Ib-cr*. The combination of these genes has been documented previously in diverse *E. coli* isolates from Europe and they have been frequently identified together on common plasmids [30-33]. It was verified that this is also the case in seven *E. coli* strains identified at MUH as the co-location of *bla*$_{CTX-M-15}$ and *aac(6')Ib-cr* on a single plasmid from each strain was demonstrated by conjugation.

In addition to *aac(6')Ib-cr* which was detected exclusively among *E. coli* isolates, the *qnr* genes *qnrA* and *qnrB* were detected in association with *bla*$_{SHV-12}$ in *Enterobacter* spp. and *Klebsiella* spp. isolates, respectively. Although *acc(6')Ib-cr* and *qnr* genes confer low-level ciprofloxacin resistance, it has been proposed that the low level of fluoroquinolone resistance conferred by these genes increases the chance of selection for high-level resistance-conferring chromosomal mutations. In agreement with this all, *E. coli* isolates harbouring *aac(6')ib-cr* were highly ciprofloxacin resistant. In contrast, not all *qnr*-harbouring isolates exhibited high-level flouroquinolone resistance as two *E. cloacae* isolates harbouring *qnrA* were ciprofloxacin susceptible. Nonetheless, these isolates would be expected to have an increased capacity to acquire higher flouroquinolone resistance in comparison with non-*qnr* producing strains by virtue of the fact that they do have reduced fluoroquinolone susceptibility albeit below the breakpoint for clinical resistance [29].

1.5.8 Cloning of *bla*$_{CTX-M-9}$ from cephalosporin resistant *E. coli*

In two ESBL-positive *E. coli* isolates, no causative ESBL resistance gene was detected by PCR analysis suggesting a novel resistance mechanism might be present. Following the cloning of plasmid fragments containing a cephalosporin resistance gene from these isolates, the *bla*$_{CTX-M-9}$ ESBL gene was identified and was present in a previously described integron platform. In these isolates, the *bla*$_{CTX-M-9}$ is associated within the complex class 1 integron

structure, In60, which was initially described in Spanish clinical *E. coli* isolates in 2002 (AF174129.3), where the $bla_{CTX\text{-}M\text{-}9}$ ESBL was first identified [35].

The failure to detect $bla_{CTX\text{-}M\text{-}9}$ in these isolates raises questions about the multiplex PCR detection method employed and highlights the importance of optimization of PCR screening protocols particularly for multiplex approaches. It has been shown that 'Hot-start' technologies can lower the probability of mis-priming/dimerization during the lower temperatures of PCR setup. This could be employed to increase the efficiency of the $bla_{CTX\text{-}M}$-family multiplex reaction but would increase the cost. In addition, PCR-screening of clinical isolates by a 'gene-by-gene' approach is labour intensive and may be subject to error. It is certainly not a method that could be readily employed in the clinical setting by non-specialised hospital laboratories. More high-throughput approaches include the use of DNA microarrays for typing ESBL and other resistance genes. This represents a potentially cheaper and more practical alternative to screening multiple resistance genes in clinical isolate and has been shown to correlate strongly with PCR-based detection methods. Indeed, a recent study investigating array-based detection of resistance genes noted the failure to detect $bla_{CTX\text{-}M}$-family genes using the same multiplex PCR-based detection method employed here in seven *E. coli* isolates, four of which harboured $bla_{CTX\text{-}M\text{-}9}$. This highlights the increased accuracy of the array-based approach [44].

1.6 Conclusion

The prevalence of resistance among Gram-negative bacteria at MUH is in line with Irish and European data which show an increasing prevalence of cephalosporin resistance among Gram-negative bacteria [9,36,45-46]. The observed increase in cephalosporin resistance among Gram-negative bacteria coincides with the detection of bla_{CTX-M}-producing *E. coli* at MUH and other Irish studies [40]. The presence of $bla_{CTX-M-15}$ in diverse *E. coli* strains including those isolated from community-acquired infections suggests $bla_{CTX-M-15}$-producing *E. coli* are endemic in this region. Among *Enterobacter* spp. and *Klebsiella* spp., the bla_{SHV-12} and ESBL genes was most prevalent and detected in diverse strains excluding hospital transmission which also suggests increasing endemicity.

Throughout Europe, fluoroquinolone resistance has also risen at a dramatic rate [9]. This has been associated with the emergence of transferrable fluoroquinolone resistance genes among the Enterobacteriaceae [29]. Diverse strains of $bla_{CTX-M-15}$-positive *E. coli* identified at MUH also harboured the *aac(6')-Ib-cr* transferrable fluoroquinolone resistance gene. In the majority of these strains both genes were present on a common transferrable plasmid. This association has been observed in other studies and exemplifies the potential of resistance plasmids to accumulate diverse resistance genes [7,30,33,47]. The spread of such plasmids between dominant clonal lineages leads to the emergence of multidrug resistant strains such as those isolated at MUH which have limited treatment options [4]. Other transferrable fluoroquinolone resistance genes, *qnrA* amd *qnrB*, were also detected among the MDR Enterobacteriaceae isolated at MUH and this study represents the first detection of transferrable fluoroquinolone resistance genes in Irish clinical isolates.

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Chapter 2

Mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Turkey

Chapter 2

2.1 Abstract

The increased isolation of carbapenem-resistant *Pseudomonas aeruginosa* isolates at Zonguldak Karaelmas University Hospital (Zonguldak, Turkey) was investigated. Imipenem -resistant strains were typed using Amplified fragment length polymorphism PCR (AFLP) revealing that imipenem-resistant isolates were largely non-clonal. No transferrable carbapenemase genes were identified suggesting that resistance was primarily due to chromosomally encoded resistance mechanisms. Although the majority of imipenem-resistant strains were chloramphenicol co-resistant, congruent with the induction of MexEF-OprN, the induction of *mexE* was not observed in clinical isolates precluding the involvement of MexT in imipenem and chloramphenicol resistance. Sequence analysis of *oprD* revealed the emergence of diverse mutations in this outer membrane porin-encoding gene in the majority of strains. Downregulation of *oprD* and induction of the AmpC β-lactamase was also observed in a small group of imipenem-resistant isolates which harboured intact *oprD* genes.

2.2 Introduction

Pseudomonas aeruginosa is a leading cause of nosocomial infections and as such is associated with hospital acquired pneumonia as well as urinary tract, surgical site and bloodstream infections [1-2]. Infections caused by *P. aeruginosa* are associated with high levels of morbidity particularly among susceptible cohorts such as ventilated patients and those in intensive care [3-4]. Other groups with increased susceptibility to *P. aeruginosa* infection include thermally injured patients and cystic fibrosis suffers [5-6].

Infections caused by *P. aeruginosa* often respond poorly to antimicrobial therapy due the ability of this pathogen to develop multidrug resistance via chromosomally encoded resistance mechanisms [1]. Multidrug resistance and even pan-drug resistance has been reported in this species which severely limits treatment options [7-8]. The use of broad-spectrum antimicrobial agents such as the carbapenem antibiotics is recommended for suspected *P. aeruginosa* infections as they have excellent activity against this species [9-10]. However, resistance to carbapenem antibiotics such as imipenem has risen among *P. aeruginosa* and can also emerge during therapy causing treatment failure [11]. This is especially evident in nosocomial pneumonia were approximately 15% of *P. aeruginosa* infections exhibit imipenem resistance prior to initiation of treatment and where an additional ~40% develop resistance during therapy [11]. This has a significant clinical impact given the high prevalence and associated mortality of *P. aeruginosa* in ventilator assisted pneumonia and the fact that inappropriate therapy increases the risk of death two-fold [11-12]. Across diverse infection types, the prevalence of carbapenem resistance in *P. aeruginosa* isolates varies geographically with isolates in the US exhibiting resistance rates of approximately 20% whereas several European countries now report rates above 25% including Greece which consistently reported rates of above 40% between 2006-2009 [7,13-14]. In Turkey, carbapenem resistance in *P. aeruginosa* reached 30% in 2008 which has been attributed to a rise in antibiotic consumption which is now comparatively higher than in many other countries [15-16].

Carbapenem resistance can emerge in *P. aeruginosa* via perturbation of complex regulatory pathways which cause upregulation of chromosomally encoded resistance mechanisms or downregulation of antibiotic permissible porins [1]. In addition, *P. aeruginosa* may acquire resistance genes horizontally, further increasing its resistance profile [17].

Given the widespread use of carbapenem antibiotics in the treatment of *P. aeruginosa* infection, the surveillance of resistance rates to this antibiotic class is important. The identification of specific resistance mechanisms which contribute to resistance in *P. aeruginosa* is also informative in determining optimal treatment strategies [18-19]. The aim of this study is to characterize the mechanisms contributing to imipenem resistance in *P. aeruginosa* clinical isolates from Turkey, a region where carbapenem resistance rates are high.

2.3 Materials and methods

2.3.1 Bacterial strains

P. aeruginosa clinical strains were isolated at Zonguldak Karaelmas University Hospital (ZHUK - Zonguldak, Turkey), a tertiary care teaching hospital with 260 inpatient and 40 ICU beds. Between 2003 and 2006 all imipenem resistant strains were isolated for further analysis.

2.3.2 AFLP analysis of strain diversity

DNA was isolated from *P. aeruginosa* isolates with a MagNA Pure LC Instrument using the MagNA Pure DNA Isolation Kit III for bacteria and fungi (Roche, The Netherlands) in accordance with manufacturer's instructions. DNA was dissolved in 100ul TE buffer (10mM Tris, 1mM EDTA, pH 8) and stored at -20°C. Following restriction and amplification, DNA fragments were separated on an ABI Prism® 3100 Genetic Analyser (Applied Biosystems, UK) and data were analysed with the Pearson correlation coefficient and clustered by unweighted pair-group matrix analysis (UPMGA) using Bionumerics software, v. 3.0 (Applied maths, Belgium).

2.3.3 Antibiotic susceptibility testing

The resistance of *P. aeruginosa* isolates to imipenem and chloramphenicol was assessed using antibiotic disks containing imipenem (10μg) and chloramphenicol (30μg) (Oxoid, UK). Assays were performed and interpreted in accordance with CLSI guidelines [20]. Etest® MBL test trips (AB Biodisk, Sweden) were used for the detection of metallo-β-lactamase production in imipenem resistant isolates in accordance with the manufacturer's instructions.

2.3.4 PCR analysis of resistance genes

PCR screening to detect known transferrable carbapenemase resistance genes *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-51}$, *bla*$_{OXA-58}$, *bla*$_{GIM-1}$, *bla*$_{SIM-1}$, *bla*$_{SPM-1}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ as previously described using isolated bacterial DNA as a template [21].

2.3.5 Sequencing of *OprD*

DNA from clinical isolates was used as a template for amplification of the *oprD* gene. The *oprD* gene was amplified from all isolates using flanking primers 5'-ACTAGCCGTCACTGCGGC-3' and 5'-CCTGGTGGCCCATGTCAC-3' with Accuzyme™ High-fidelity master mix (Bioline, UK). PCR cycling parameters were as follows; initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 50°C annealing for 30 s and 68°C elongation for 3 min. This was proceeded by a final 10 min elongation step at 68°C. Amplified products were purified using the QIAquick® PCR purification kit (Qiagen, UK) and sequenced using previously described sequencing primers complementary to either end of the purified *oprD* fragment (pDF2 and pDR2) [22]. In addition, primers internal to *oprD* (pDF2 and pDR2) were also used for sequencing [22].

2.3.6 Sequence alignment of *oprD*

The sequence of *oprD* determined in *P. aeruginosa* isolates was compared to *oprD* sequences in the Pseudomonas Genome Database which contains *oprD* alleles identified in a collection of 328 *P. aeruginosa* of diverse geographical origins [23-24]. Sequence data for *oprD* genes identified in this study were aligned with the sequences in the Pseudomonas Genome Database using the ClustalW algorithm [25]. Minmal gap opening penalties were selected to reduce distortions caused by deletions or insertions in *oprD* as observed in some isolates. A dendrogram representing the multiple sequence alignment was generated by UPMGA clustering using MEGA 4.1 [26]. Sequences were analysed for the presence of defective OprD mutations (DOMs) such as frame shifts or deletions which would disrupt expression of the OprD porin.

2.3.7 RNA isolation and cDNA synthesis

RNA was extracted from *P. aeruginosa* isolates to serve as a template from quantitative reverse-transcriptase PCR (qRT-PCR) analysis of resistance genes expression. Isolates were grown on LB plates at 37°C for 16h. Fifty micro litres of RNAprotect (Qiagen, UK) was pipetted onto the plate and a 10µl aliquot of cell suspension was transferred into 200µl RNA protect using volume-standardized inoculating loops (Sarstedt, UK). Pellets were stored at -70°C and subsequently used as starting material for RNA extraction using the Qiagen RNAeasy® RNA extraction kit (Qiagen, UK). RNA was extracted from three biological replicates. Chromosomal DNA was eliminated from isolated RNA using RQ1 RNase-free DNase I (Promega, UK) as verified by the failure to detect PAO1 genomic DNA in RNA samples by PCR. DNA-free RNA was used as a template for cDNA synthesis using random primers and AMV reverse tanscriptase in accordance with manufacturer's instructions (Promega, UK). Successful reverse transcription of cDNA was confirmed by PCR.

2.3.8 Expression analysis of resistance genes by qRT-PCR

Gene expression levels of *mexE, ampC* and *oprD* relative to *proC* were determined in clinical isolates and compared to their expression levels in *P. aeruginosa* model organism PA14. A spontaneously generated *mexEF-oprN* overexpressing mutant (PA14*nfxC*) was used as a positive control for *mexE* induction. Fluorescently-labelled locked nucleic acid (LNA^{TM}) probes specific for *mexE*, *ampC oprD* and *proC* were selected from the Roche Universal ProbeLibrary^{TM} (Roche Applied Biosystems, UK). Suitable probes and primer sets were designed using the online Roche assay design algorithm [27]. Ten micro litres of 1:10 diluted cDNA was used as a template in each qRT-PCR reaction to make up a final reaction volume of 25ul in FastStart TaqMan® Probe Master amplification mix containing 200nM of probe and 200nM of each primer. Primer and probe sequences are listed in Table 1. Real-time PCR analysis was performed using the Chromo4^{TM} Real-time PCR system (MJ Research, UK) using the following cycling parameters; 95°C for 10 min followed by 50 cycles of 95°C for 15s, 60°C for 1 min. Fluorescence was detected after each cycle using a Sybr® Green I excitation emissions filter. Data was analysed using the MJ Opticon Monitor® Analysis software (v3.1). All fluorescence signals were normalised to the *proC* housekeeping gene and relative expression values of *mexE, ampC* and *oprD* were determined for each sample.

Table 1. Probes and primers used in qRT-PCR analysis

Target gene	UPL probe[a]	Primers[b]
proC	#20 (5'-CCAGCCAG-3')	5'-CTTCGAAGCACTGGTGGAG-3'
		5'-TTATTGGCCAAGCTGTTCG-3'
mexE	#61 (5'-CTGGGCAA-3')	5'-CACCCTGATCAAGGACGAAG-3'
		5'-CCAGGACCAGCACGAACT-3'
oprD	#132 (5'-TGCTGCTC-3')	5'-GGGTTCATCGAAGACAGCAG-3'
		5'-GCCGTCACGGTTGAAATAGT-3'
ampC	#23 (5'-GGGCTGGG-3')	5'-CCATCGCGGTTACTACAAGG-3'
		5'-GAGATCGGCCAGTCGTAGG-3'

[a] Probes from the universal probe library labelled at the 5'-terminal with fluorescein (*FAM*) and 3'-proximal with a dark *quencher* dye

[b] Specific primers for probe hydrolysis reaction

2.4 Results

2.4.1 Increased imipenem resistance in non-clonal *P. aeruginosa* isolates

Seventy-four *P. aeruginosa* isolates exhibiting imipenem resistance were identified at ZKUH between 2003-2006 with increasing prevalence in their isolation over the study interval. The imipenem-resistant strains belonged to 27 divergent clonal lineages indicating strain diversity. However, two larger clonal groups were identified: group I - 20 isolates and group X - 10 isolates (Table 2) which contributed to the increase in numbers in 2005 and 2006 which could represent the hospital transmission of imipenem resistant strains. Nonetheless, the clonal diversity observed confirms the independent emergence of imipenem resistance in diverse strains. To investigate the mechanisms of imipenem resistance in diverse *P. aeruginosa* clinical isolates from this region, a single representative isolate from each of the 27 AFLP clonal groups identified was chosen for further characterization.

Table 2. Imipenem-resistant *P. aeruginosa* isolates isolated at ZKUH

Isolate no.	AFLP group[a]	Year	Clinical specimen
TY 5007	II	2003	Urine
TY 5008	II	2003	Wound
TY 5005	IX	2003	Wound
TY 5006	IX	2003	Wound
TY 5003	u	2003	Wound
TY 5016	III	2004	Wound
TY 5011	XII	2004	Wound
TY 5009	u	2004	Urine
TY 5010	u	2004	Broncho alveoler lavage
TY 5014	VII	2004	Tracheal aspirate
TY 5015	VII	2004	Wound
TY 5024	I	2005	Urine
TY 5025	I	2005	Tracheal aspirate
TY 5026	I	2005	Urine
TY 5029	I	2005	Wound
TY 5032	I	2005	Tracheal aspirate
TY 5020	III	2005	Wound
TY 5021	IV	2005	Urine
TY 5022	IV	2005	Broncho alveoler lavage
TY 5028	IX	2005	Tracheal aspirate
TY 5027	u	2005	Sputum
TY 5019	XII	2005	Tracheal aspirate
TY 5017	u	2005	Wound
TY 5018	u	2005	Wound
TY 5030	u	2005	Blood
TY 5031	u	2005	Broncho alveoler lavage
TY 5034	u	2005	Blood
TY 5036	u	2005	Tracheal aspirate

TY 5033	XI	2005	Blood
TY 5035	XI	2005	Wound
TY 5038	I	2006	Tracheal aspirate
TY 5041	I	2006	Tracheal aspirate
TY 5042	I	2006	Blood
TY 5044	I	2006	Blood
TY 5045	I	2006	Tracheal aspirate
TY 5048	I	2006	Wound
TY 5049	I	2006	Wound
TY 5055	I	2006	Urine
TY 5058	I	2006	Wound
TY 5059	I	2006	Tracheal aspirate
TY 5060	I	2006	Tracheal aspirate
TY 5061	I	2006	Sputum
TY 5069	I	2006	Blood
TY 5074	I	2006	Wound
TY 5078	I	2006	Tracheal aspirate
TY 5046	IV	2006	Wound
TY 5047	IV	2006	Urine
TY 5082	IX	2006	Tracheal aspirate
TY 5039	u	2006	Wound
TY 5040	u	2006	Urine
TY 5053	u	2006	Tracheal aspirate
TY 5054	u	2006	Tracheal aspirate
TY 5067	u	2006	Sputum
TY 5056	V	2003-2006	Unknown
TY 5068	V	2006	Sputum
TY 5072	V	2006	Abdomial fluid
TY 5043	VI	2006	Wound
TY 5050	VI	2006	Wound
TY 5073	VIII	2006	Blood
TY 5076	VIII	2006	Abdomial fluid
TY 5077	VIII	2006	Abdomial fluid
TY 5037	X	2006	Tracheal aspirate
TY 5051	X	2006	Tracheal aspirate
TY 5052	X	2006	Wound
TY 5057	X	2006	Tracheal aspirate
TY 5062	X	2006	Tracheal aspirate
TY 5063	X	2006	Wound
TY 5064	X	2006	Wound
TY 5065	X	2006	Blood
TY 5071	X	2006	Tracheal aspirate
TY 5075	X	2006	Wound
TY 5079	X	2006	Tracheal aspirate
TY 5066	XI	2006	Sputum
TY 5070	XI	2006	Broncho alveoler lavage

[a] Isolate exhibiting AFLP patterns with >90% similarity were assigned to the same group as indicated by roman numerals. Isolates for which AFLP patterns were not significantly similar to any other isolate are designated with the letter u (unique).

2.4.2 Transferrable carbapenemase genes are absent from *P. aeruginosa* isolates

The prevalence of imipenem-resistant strains of diverse clonal origins could be explained by the dissemination of transferrable resistance plasmids among different strains. To investigate whether this was the case, an isolate from each AFLP clonal group was screened for the presence of diverse carbapenem resistance genes. No previously characterized carbapenemase resistance genes including bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, bla_{GIM-1}, bla_{SIM-1}, bla_{SPM-1}, bla_{IMP}, or bla_{VIM} were identified suggesting that the imipenem resistance observed is not due to these transferrable resistance genes. In addition all isolates were negative for for metallo-β-lactamase production according to the Etest® MBL strip assay. The transfer of carbapenem resistance from resistant isolates to a susceptible recipient strain by conjugation could not be demonstrated (data not shown) suggesting that imipenem resistance in these strains is unlikely to be transferrable and could be due to chromosomal resistance mechanisms.

2.4.3 Imipenem-resistant isolates also exhibit chloramphenicol resistance implicating MexT

The LysR transcriptional regulator MexT is a central regulator of antibiotic resistance in *P. aeruginosa* which downregulates the outer membrane porin OprD leading to imipenem resistance. In addition, MexT directly activates the MexEF-OprN efflux system which confers resistance to a number of antibiotics including chloramphenicol (Figure 1) [28-29]. Mutations which lead to activation of MexT can thus cause resistance to both imipenem and chloramphenicol in clinical isolates. To investigate whether MexT played a role in mediating imipenem resistance in clinical *P. aeruginosa* isolates, chloramphenicol resistance levels were measured among the 27 clonally diverse imipenem-resistant strains. Except for a single isolate (TY5041) all strains exhibited chloramphenicol resistance, consistent with the activation of MexT in these strains.

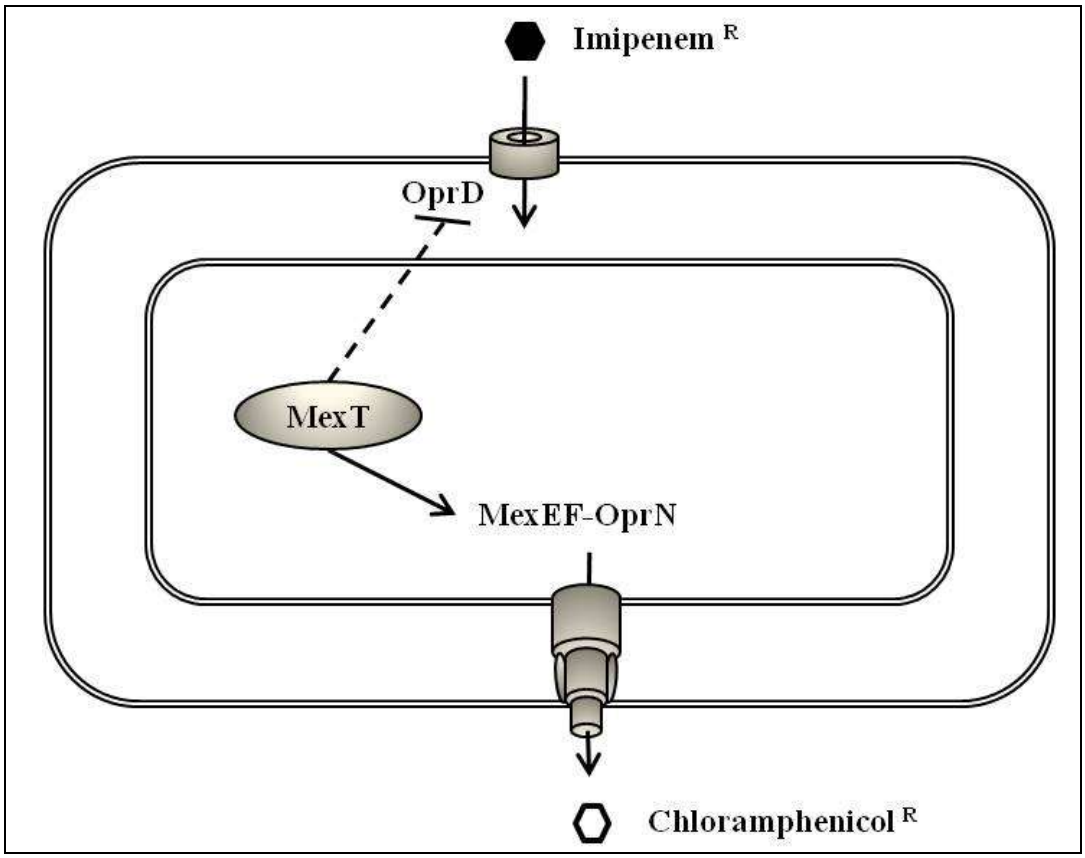

Figure 1. Mechanism of MexT-mediated imipenem and chlorampenicol resistance in *P. aeruginosa.* MexT directly induces expression of the MexEF-OprN efflux pump which extrudes chloramphenicol. MexT also represses expression of the OprD porin which is the primary route of entry to the cell for imipenem by an unknown regulatory mechanism. Thus, strains in which MexT becomes active are co-resistant to imipenem and chloramphenicol.

2.4.4 MexT is not implicated in OprD-mediated imipenem resistance

Quantitative RT-PCR was employed to determine the levels of *mexE* expression in clinical isolates in order to address whether MexT was responsible for the imipenem and chloramphenicol co-resistant phenotypes observed. The ratio of detected *mexE* transcript relative to that of *proC* in PA14*nfxC* (*mexEF-oprN* overexpressing control) was 262 ± 73 fold higher than the ratio detected in PA14 (Figure 2). In contrast, none of the isolates exhibited a *mexE:proC* expression ratio which was significantly higher than that of PA14 confirming that *mexE* is not induced in these strains. This suggests that MexT is not constitutively active in these strains and that imipenem and chloramphenicol resistance occurs independent of the MexT regulatory pathway.

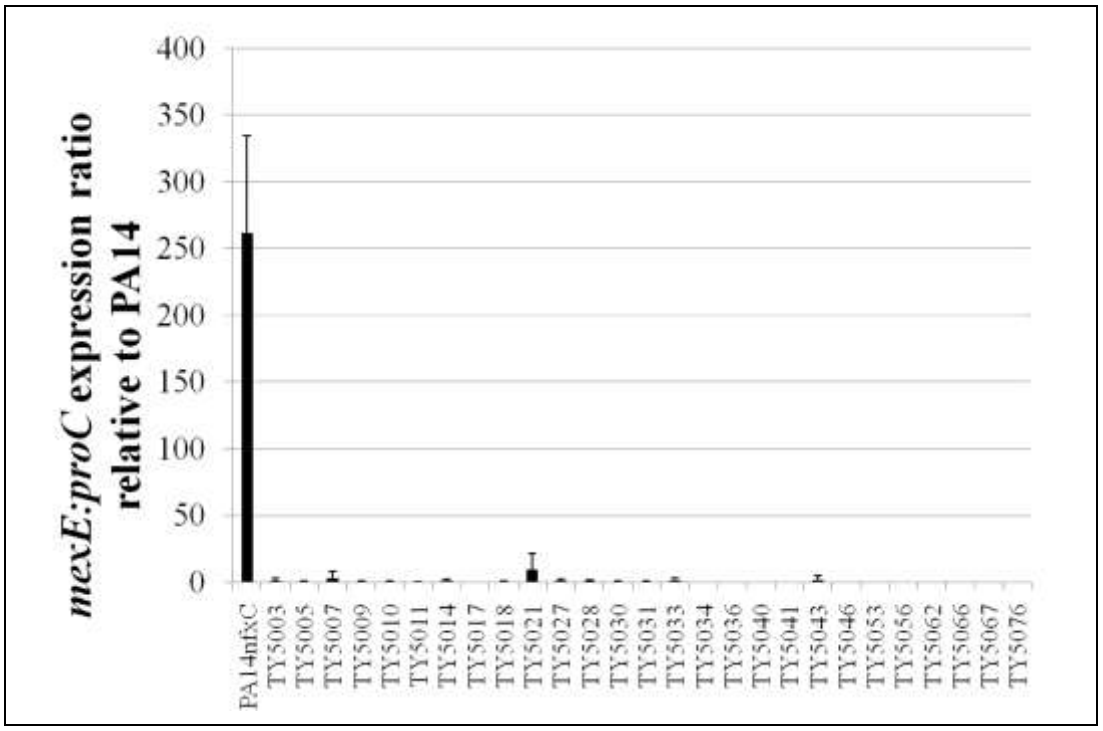

Figure 2. Expression level of *mexE* in *P. aeruginosa* clinical isolates. qRT-PCR was employed to determine the *mexE:proC* expression ratio in PA14*nfxC* and clinical isolates compared to PA14. No isolate exhibited significant *mexE* induction comparable to PA14nfxC suggesting that MexT is not involved in mediating imipenem or chloramphenicol resistance in clinical isolates.

2.4.5 Defective OprD mutations are prevalent in imipenem-resistant isolates

Mutational inactivation of *oprD* is known to occur in *P. aeruginosa* clinical isolates leading to carbapenem resistance [1]. To establish whether this could have occurred in the 27 imipenem-resistant isolates from this study, the *oprD* gene was sequenced in each isolate. This revealed that among the imipenem-resistant isolates investigated, only four harboured functional copies of the *oprD* gene. The mutations observed in *oprD* included small and large deletions, insertions and duplications causing frame shifts in the *oprD* coding sequence as well as substitutions resulting in early translational termination (Table 3).

The *oprD* sequences of the imipenem-resistant isolates were compared to those identified in the study of Pirnay *et al.* which are deposited in the Pseudomonas Genome Database [23-24]. This allowed characterization of *oprD* allelic diversity in the isolate collection compared to this broad sample of *oprD* sequences from *P. aeruginosa* isolates of diverse origins. A number of novel *oprD* sequences harbouring single nucleotide polymorphisms which are not reported in the Pseudomonas Genome Database were identified and are reported here as novel alleles (BRC1-13). Phylogenetic analysis of *oprD* alleles identified in this study as compared with those in the Pseudomonas Genome Database are illustrated in Figure 3. All *oprD* sequences were aligned using the clustalW algorithm and a phylogenetic tree was generated by UPMGA analysis. The *oprD* sequences identified in carbapenem resistant isolates exhibit diversity and are broadly distributed among three major OprD cluster groups determined by Pirnay *et al.* (Group A - 6 isolates, Group B - 11 isolates, Group C - 10 isolates). This reflects the diversity of the *oprD* genes identified in this study. Within both novel and previously described *oprD* alleles, defective OprD mutations (DOMs) were identified which are distinct from those previously characterized by Pirnay *et al.* Thus the imipenem resistance phenotypes observed in diverse *P. aeruginosa* strains isolated at this hospital can be attributed to independent mutational events leading to the inactivation of diverse *oprD* alleles. However, four imipenem-resistant isolates (TY5010, TY5017, TY5018 and TY5041) had functional *oprD* genes based on sequence analysis. This suggests that a

resistance mechanisms other that MexT-mediated OprD downregulation or DOMs were responsible for the imipenem resistance observed in these strains.

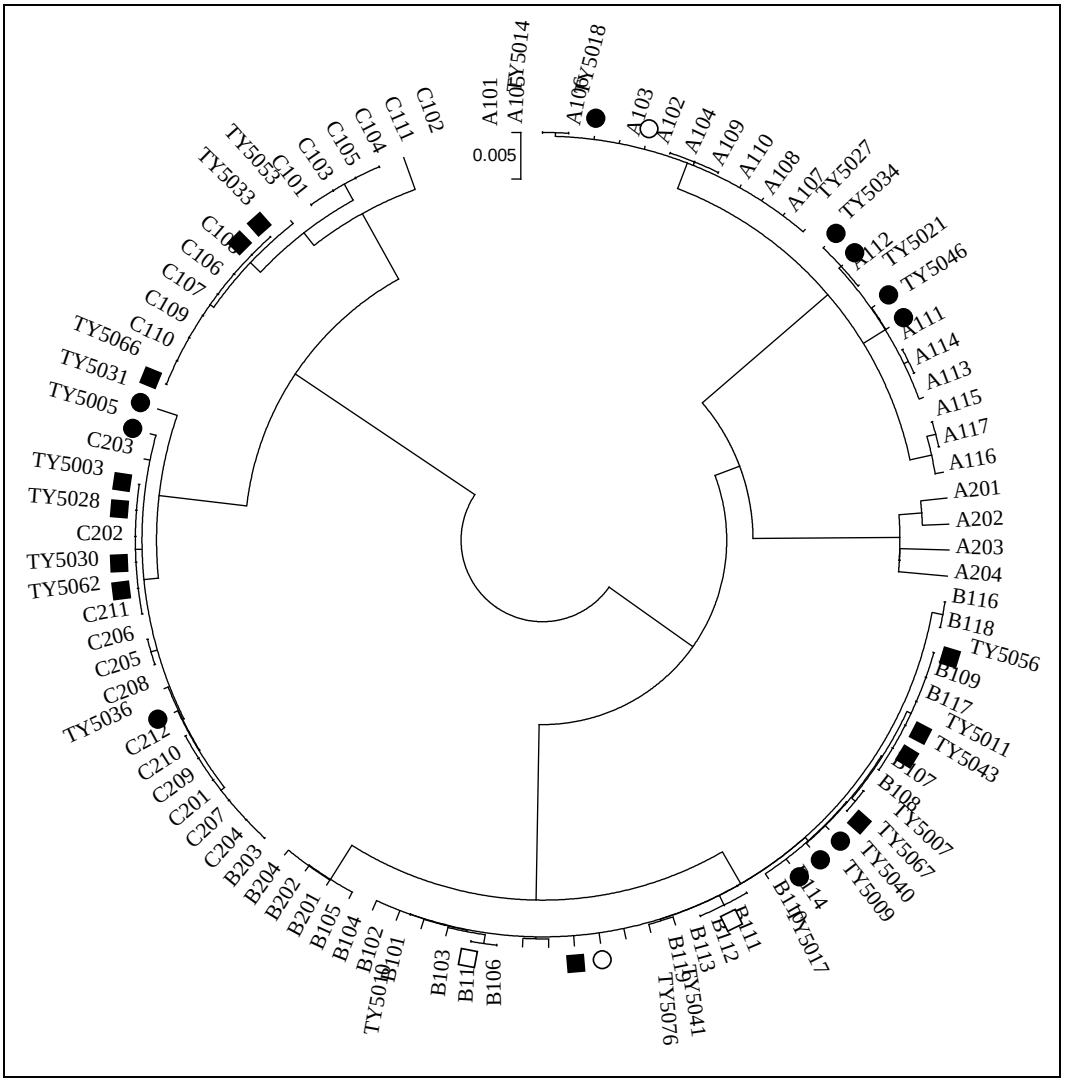

Figure 3. Sequence diversity of *oprD* in *P. aeruginosa* clinical isolates. The *oprD* alleles identified in this study were aligned with those deposited in the Pseudomonas Genome Database [23]. The previously identified *oprD* alleles are identified by the prefix A, B or C based on the identified groups into which they clustered in the study of Pirnay *et al.* and isolates identified in this study all have the prefix TY. Sequences matching previously described *oprD* alleles are indicated with square symbols and circular symbols represent newly identified *oprD* alleles. Alleles which have acquired predicted defective *oprD* mutations (DOMs) are indicated by solid fill and alleles with functional *oprD* sequences are indicated by open symbols. The Genetic distance (estimated number of nucleotide substitutions per site) is indicated at the top of the tree.

Table 3. Sequence analysis of *oprD* genes from imipenem-resistant isolates

Strain	Allele[a]	DOM[b]	Mutation
TY5003	C202	yes	large 427bp deletion from NT 47
TY5062	C202	yes	large 427bp deletion from NT 47
TY5028	C202	yes	1bp deletion of NT 903 → Frame shift → stop codon at NT 1033-5
TY5005	new allele (BRC1)	yes	1bp insertion after NT 1205 → Frame shift → stop codon at NT 1423-5
TY5007	B108	yes	large 427bp deletion from NT 47
TY5009	new allele (BRC2)	yes	C-T base substitution at NT 1279→ stop codon at NT 1279-81
TY5010	B103	no	-
TY5011	B107	yes	11bp deletion of NT 96-106 → Frame shift → stop codon at NT 147-50
TY5043	B107	yes	Duplication of CCTC at NT 69-72 → Frame shift → stop codon at NT 691-693
TY5056	B107	yes	13bp deletion of NT 63-75 → Frame shift → stop codon at NT 1021-3
TY5014	new allele (BRC3)	yes	1bp deletion of NT 38 → Frame shift → stop codon at NT 280-2
TY5018	new allele (BRC4)	no	C-G base substitution at NT 452 (Ala-Gly)
TY5021	new allele (BRC5)	yes	G-T base substitution at NT 901 → Early stop codon NT 901-3
TY5027	new allele (BRC6)	yes	Duplication of CT at NT 670-1 →Frame shift → stop codon at NT 688-90
TY5034	new allele (BRC6)	yes	4bp deletion of NT 869-872 → Frame shift → stop codon at NT 1030-2
TY5046	new allele (BRC7)	yes	C-T base substitution at NT 703 → stop codon at NT 703-5
TY5017	B110	no	-
TY5030	C211	yes	28bp deletion from of NT 662-690 → Frame shift → stop codon at NT 760-2
TY5031	new allele (BRC8)	yes	A-G base substitution at NT 1251 → early stop codon
TY5036	new allele (BRC9)	yes	1bp deletion of NT 737 → Frame shift → stop codon at NT 1033-5
TY5033	C106	yes	duplication of G at NT 455 → Frame shift → stop codon at NT 1219-21
TY5053	C106	yes	duplication of G at NT 455 → Frame shift → stop codon at NT 1219-21
TY5066	C106	yes	duplication of G at NT 455 → Frame shift → stop codon at NT 1219-21
TY5040	new allele (BRC11)	yes	C-A base substitution at NT 1197 → early stop codon
TY5041	new allele (BRC12)	no	-
TY5067	new allele (BRC13)	yes	G-A base substitution NT 195 → early stop codon NT 193-5
TY5076	B119	yes	C-T base substitution NT 1295 → early stop codon NT 1295-7

a Allele designations are those listed in the Pseudomonas Genome Database. Where novel alleles were identified a new designation is used - BRC (Biomerit Research Centre) 1-13.

b DOM - defective OprD mutation. These *oprD* sequences are predicted to encode non-functional OprD porins.

2.4.6 Investigation of alternative imipenem resistance mechanisms in isolates a with a functional copy of *oprD*

Four imipenem resistant isolates (TY5010, TY5017, TY5018 and TY5041) harboured a functional copy of *oprD* which ruled out DOMs as a mechanisms of resistance in these strains. To assess whether the downregulation of *oprD* could account for imipenem resistance in these strains, qRT-PCR was employed to assess the level of *oprD* expression in each strain. The level of *oprD* expression relative to *proC* was measured in each strain and compared to the *oprD:proC* ratio in PA14. Only TY5010 exhibited significant downregulation of *oprD* which was approximately 9-fold lower than expression levels in PA14 (Table 4). Hence other regulatory mechanisms (apart from MexT) may contribute to *oprD* down regulation in this strain (Figure 4).

Another resistance mechanisms known to play a role in imipenem resistance the chromosomal AmpC β-lactamase [18,30]. AmpC was highly in induced (>2000-fold) in TY5017 and TY5018 which could account for their increased imipenem resistance levels.

Thus, of the strains lacking DOMs, TY5010 exhibits downregulation of *oprD* and induction of *ampC* is observed in TY5017 and TY5018. No obvious mechanism which could account for imipenem resistance was observed in TY5041. However, imipenem resistance in this strain is apparently independent of OprD and AmpC.

Table 4. Relative expression levels of *oprD* and *ampC* compared to PA14 in TY5010, TY5017, TY5018 and TY5041.

	oprD[a]	*ampC*
PA14	1	1
TY5010	**0.11**	0.34
TY5017	0.53	**2690.16**
TY5018	0.54	**4340.36**
TY5041	0.53	0.53

[a] The ratio of *oprD:proC* and *ampC:proC* was measured in all strains including PA14. The ratios expressed represent the level of *oprD* and *ampC* expression measured each isolate relative to PA14.

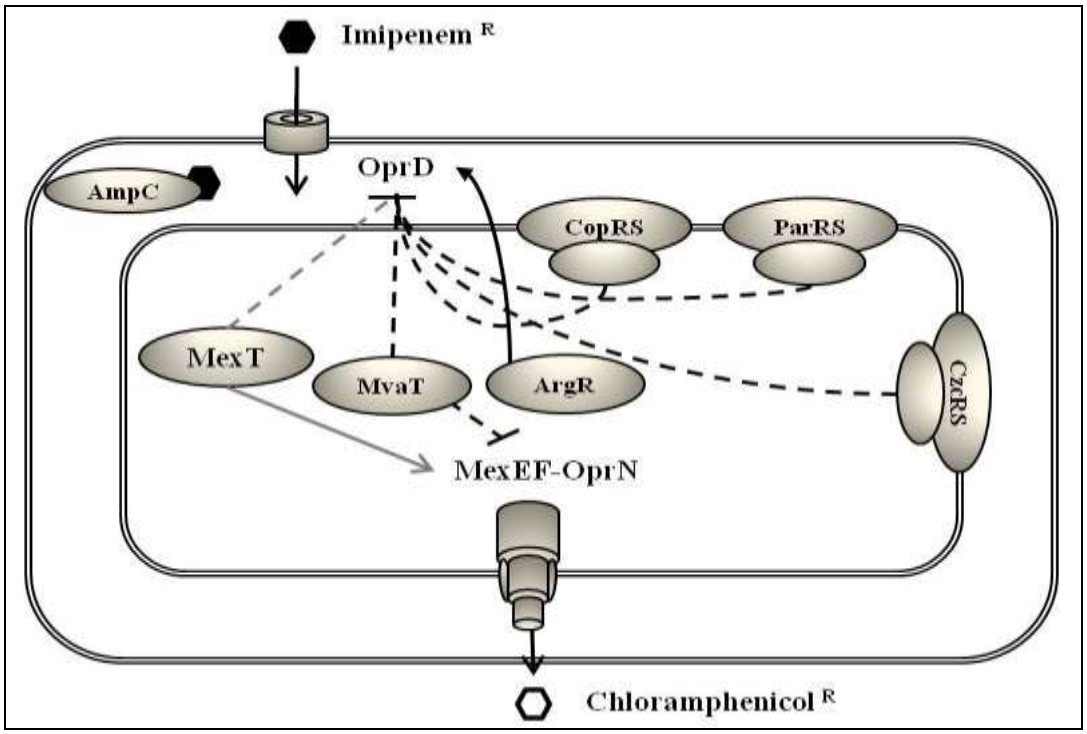

Figure 4. Alternative mechanisms to explain MexT-independent imipenem resistance in a functional OprD background. If OprD is functional at the sequence level, imipenem resistance must result either from the down regulation of *oprD* or through alternate mechanisms of resistance. In addition to MexT other regulators such as the CzcR, CopRS and ParRS two component systems can repress *oprD* expression. The HN-S-like protein MvaT is also known to negatively regulate expression of *oprD* as well as *mexEF-oprN* and ArgR directly activates expression of *oprD*. Expression of the AmpC β-lactamase is also associated with imipenem resistance and mutations in the *ampC* gene can increase its hydrolytic activity toward imipenem.

2.5 Discussion

2.5.1 Increasing carbapenem resistance in *P. aeruginosa* clinical isolates

An increase in the incidence of imipenem-resistant *P. aeruginosa* isolates was observed in at ZKUH between 2003-2006. *P. aeruginosa* carbapenem resistance rates vary globally according to geographic location and are strongly correlated with antibiotic administration patterns [31]. The Mediterranean region represents a hot-spot of endemicity for multi-drug resistant organisms and this has been associated with the preferential use of broad-spectrum antibiotics such as third-generation cephalosporins and carbapenems in this region [7,32].

In Turkey, increasing antibiotic consumption rates have been observed and this has been linked to social insurance reform policies which grant greater access to antimicrobials to a almost 70% of the Turkish population [15]. Between 2001 and 2006 the defined daily dose per 1000 inhabitant-days for cephalosporins in Turkey rose from under 2 to over 6 per 1000 inhabitant-days and was associated with increased consumption of carbapenem antibiotics [15]. Given the increases in consumption and preference for broad over narrow-spectrum antibiotics observed in Turkey, bacterial pathogens from this region including *P. aeruginosa* are under strong selection to develop resistance to broad-spectrum antibiotics [15,32]. Therefore, it is not surprising that levels of carbapenem resistance in Turkey have risen. The levels of carbapenem resistance in *P. aeruginosa* have surpassed 30% [7]. Indeed, a study in Ankara in 2004 revealed that almost 45% of nosocomial *P. aeruginosa* strains exhibited imipenem resistance [7,33]. The findings of this study are also in agreement with previous reports of increasing levels of resistance to broad-spectrum agents in *P. aeruginosa* which is likely due to the high rate of consumption these agents in this region.

2.5.2 Clonal diversity of imipenem resistant *P. aeruginosa* isolates

AFLP analysis confirmed the presence of diverse clonal lineages among imipenem resistant *P. aeruginosa* isolates. Thus increased imipenem resistance is not fully accounted for by hospital-transmission of clonal outbreak strains and genetically diverse strains have independently adapted to overcome imipenem therapy. The results reflect an increasingly endemic situation in this region regarding imipenem resistance in *P. aeruginosa*.

Although *P. aeruginosa* is not thought to be part of the normal microbiological flora of healthy individuals, increased prevalence in the isolation of *P. aeruginosa* from humans is observed in hospitalized patients [1]. *P. aeruginosa* is ubiquitous in the environment and exhibits an epidemic population structure with little evidence of clonal expansion of pathogenic strains [23]. Although some AFLP typing groups were over-represented (which could suggest hospital transmission) the overall diversity of imipenem-resistant isolates observed is in agreement with an epidemic *P. aeruginosa* population structure. A broad range of strains are capable of causing nosocomial infection and imipenem resistance arose independently in diverse genetic backgrounds as indicated by the detection of 20 diverse DOMs which occurred in 20 different *oprD* alleles identified among 27 strains of diverse AFLP typing groups.

The dissemination of carbapenem resistance can occur through horizontal gene transfer of resistance genes in association with mobile genetic elements [34]. This could explain the observation of imipenem resistance in diverse *P. aeruginosa* strains. Many transferrable carbapenem hydrolysing enzymes such as metallo-β-lactamases have been detected in *P. aeruginosa* isolates and shown to contribute to carbapenem resistance [7,35]. As none of the known carbapenemase genes screened for were detected in this isolate collection, their role in imipenem resistance is excluded. Thus, the observed imipenem resistance of these isolates may be attributable to chromosomal resistance mechanisms. This conclusion is further strengthened by the fact that repeated conjugation attempts failed to demonstrated the transferability of imipenem resistance from clinical isolates to a susceptible recipient. These

findings are in agreement with many other studies which highlight the propensity of *P. aeruginosa* isolates of diverse clonal lineages to develop resistance independent of transferrable resistance mechanisms [1].

2.5.3 Chromosomal mechanisms of imipenem resistance in *P. aeruginosa* isolates

Carbapenems are β-lactam antibiotics and as such target the penicillin binding proteins and inhibit cell-wall biosynthesis. To exert their bactericidal effect they must enter the cell which is a challenge in *P. aeruginosa* due to its inherent impermeability [36]. However, the outer membrane porin of *P. aeruginosa* contains the OprD porin which facilitates the uptake of basic amino acids but also accommodates carbapenems including imipenem. As such, OprD represents the primary portal of entry for carbapenem antibiotics [18]. Other resistance mechanisms include efflux pumps such as the MexAB-OprM system which can extrude certain carbapenem antibiotics [37]. However, in contrast to other carbapenems, imipenem is resistant to efflux due to its hydrophobic side chain. The vast majority of imipenem resistant isolates in this study harboured defective copies of the *oprD* gene reflecting its central role in the emergence of imipenem resistance. All DOMs identified in this study are distinct from those reported in other studies with the exception of TY5067 which harboured a DOM previously reported in a carbapenem resistant *P. aeruginosa* isolate from Spain (Table 3) [23,38]. In addition to mutational disruption of OprD, one of the four isolates in which an functional copy of *oprD* was identified exhibited down-regulation of this gene (Table 4). Although the mechanism of *oprD* down-regulation was not identified it was not due to activation of MexT as indicated by the lack of MexEF-OprN induction which was not observed in any imipenem resistant isolates. In line with this finding, other studies suggest MexT-mediated repression of *oprD* is not a common resistance pathway in *P. aeruginosa* clinical isolates [39-40]. This may suggest that MexT serves an important regulatory function independent from antibiotic resistance in *P. aeruginosa* and that MexT-mediated induction of MexEF-OprN is not favoured in clinical isolates because of the resultant fitness burden. This may also be true of

other efflux pumps in *P. aeruginosa*, many of which are known to extrude clinically relevant antibiotics but are rarely if ever found to be induced in clinical isolates [41-42]. Other regulators such as the CopRS, CzrRS, and ParRS two component systems, the arginine-responsive regulatory protein ArgR and the HN-S-like protein MvaT can also regulate *oprD* expression and could play a role in *oprD* downregulation in carbapenem resistant clinical isolates [1,43-46]. As the genome of *P. aeruginosa* is highly regulated, other as yet unidentified regulatory mechanisms could also impact *oprD* expression.

Upregulation of the *P. aeruginosa* chromosomal AmpC β-lactamase is also linked to carbapenem resistance. Although the affinity of AmpC for carbapenem antibiotics is low, there is a correlation between AmpC induction and imipenem resistance in *P. aeruginosa* and the interplay between these mechanisms is central to the emergence of carbapenem resistance [18]. Recently, variations in the AmpC gene have been identified which increase its affinity for carbapenems and upregulation of AmpC may thus contribute to the imipenem resistance independent of OprD [30]. The upregulation of AmpC observed in two imipenem resistant isolates which harboured functional *oprD* genes may therefore account for the observed imipenem resistance in these strains. Finally, a single imipenem resistant isolate exhibited no defective mutations in *oprD*, had levels of *oprD* expression comparable to PA14 and did non overexpress *ampC*. This suggests the involvement of yet other imipenem resistance pathways in this strain. A possible explaination could be the posttranscriptional downregulation of *oprD* which has been previously suggested [47]. Alternatively an uncharacterized resistance mechanism could contribute to imipenem resistance in this strain. However, in the majority of strains mutational inactivation of OprD appears to be the predominant pathway leading to imipenem resistance.

2.5.4 Mutational inactivation of OprD

Several DOMs were observed among diverse *oprD* alleles in imipenem-resistant isolates. It has been suggested that the sequence of OprD has a high discriminatory power in determining the lineage of *P. aeruginosa* isolates and that clonal complexes harbour identical OprD sequences [48]. The diversity of OprD alleles observed in this study supports this conclusion. As isolates in this study were chosen from diverse AFLP typing groups, the diversity of the study population was maximized. Sequencing of *oprD* revealed a corresponding level of allelic diversity. A previous study of OprD at the sequence level among a large collection of *P. aeruginosa* isolates revealed three groups into which *oprD* sequences clustered [23]. The OprD sequences observed in this study grouped almost equally to the three major OprD allele groups indicating a high level of allelic diversity. The C202 allele was identified in three strains and has previously been identified in Turkish *P. aeruginosa* isolates and could be prevalent among *P. aeruginosa* strains from this geographic location [23]. Interestingly, a large deletion event (427bp) which occurred in the C202 allele was also present in a diverse allele, B108. This suggests that this is a common deletion mechanism which can occur in diverse *oprD* alleles. Of the 20 DOMs observed in this study, only one has been previously reported reflecting the diversity of mutation types which can occur in independently in *oprD*. Hence 19/20 DOMs represent entirely novel and independent mutational events in *oprD* leading to imipenem resistance in this strain collection. This reflects the strong selective pressure on *oprD* in *P. aeruginosa* isolates which can lead to imipenem resistance independent of horizontal gene transfer or clonal dissemination.

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Chapter 3

Investigating MexT-mediated regulation of antibiotic resistance, virulence and the novel ArsR-family repressor PA4354 in *Pseudomonas aeruginosa*

Chapter 3

3.1 Abstract

In the human pathogen *Pseudomonas aeruginosa*, the LysR-family regulator MexT modulates the induction of the tripartite MexEF-OprN resistance nodulation-division (RND) multidrug efflux system resulting in increased resistance to diverse antibiotics. Induction of MexEF-OprN is also linked to repression of virulence traits, including pyocyanin, elastase, rhamnolipids and PQS and to reduced expression of type three secretion system (TTSS). Cells expressing MexEF-OprN also exhibit impaired attachment. In this study, overexpression of MexT is shown to regulate a subset of MexT-associated phenotypes including imipenem resistance, pyocyanin production, early surface attachment and the TTSS independent of MexEF-OprN. Subsequent transcriptome profiling revealed that MexT regulates novel targets in addition to MexEF-OprN including the novel transcriptional repressor PA4354. The putative role of PA4354 in mediating MexT-associated phenotypes was investigated. PA4354 did not play a direct regulatory role in mediating imipenem resistance or repression of pyocyanin production, attachment or the TTSS. PA4354 represents a novel, non-canonical member of the ArsR-family of transcriptional repressors and is part of a three gene operon *(PA4354-PA4355-xenB*). PA4354 was characterized at the molecular level revealing its function as an autorepressor and its autoregulatory binding site was determined.

Chapter 3

3.2 Introduction

Pseudomonas aeruginosa is among the top three nosocomial pathogens in terms of its prevalence and is the leading cause of mortality in cystic fibrosis sufferers of which approximately 80% become chronically colonized [1-2]. The genome of this opportunistic human pathogen encodes many virulence determinants which are highly regulated as part of intricate regulatory networks which allows *P. aeruginosa* to infect a broad range of hosts [3-5]. Multidrug resistance can arise in *P. aeruginosa* due to its inherently impermeable membrane and the acquisition of resistance through chromosomal mutation [6-7]. Many mutations which contribute to the adaptation of *P. aeruginosa* to the hospital environment are known to act by disrupting complex transcriptional networks in which virulence and antibiotic resistance determinants are embedded [6,8].

The most abundant family of prokaryotic transcriptional regulators are the LysR-type transcriptional regulators (LTTRs) of which approximately 120 are encoded in the *P. aeruginosa* genome. This large collection of transcriptional switches co-ordinate the expression of diverse target genes in response to multiple intracellular and environmental signals [9-12]. The LTTRs are characterized by an N-terminal DNA-binding helix-turn-helix domain and a C-terminal co-inducer binding domain. They are active as tetramers and on binding of a cognate inducing ligand the LTTR undergoes a global conformational change which causes relaxation of DNA bending, resulting in increased transcription through activation of the RNA polymerase. A characteristic binding motif of T-N11-A is present within the promoters of LTTR target genes which reflects the conserved structure of the LTTR family of regulators [10,13]. These regulators may act as local or global regulators influencing expression of a variable number of target genes which control diverse phenotypes including antibiotic resistance, virulence and metabolism [10].

A LTTR of clinical importance in *P. aeruginosa* is MexT which directly regulates the *mexEF-oprN* multidrug efflux pump and as such confers multidrug resistance [14]. This is in addition to other well characterized LTTRs in *P. aeruginosa* such as PqsR which is a central

regulator of 2-heptyl-3-hydroxy-4-quinolone biosynthesis (known as the Pseudomonas quinolone signal - PQS) which is a central signalling molecule in quorum sensing-regulated virulence which has been reviewed in detail [15]. MexT has homology to the characterized NodD-type transcriptional regulators of *Rhizobium* spp. which control the process of nodulation in response to plant-secreted flavonoids [16-17]. As such, the MexT transcriptional target *mexEF-oprN* contains a characteristic motif element (ATG-N9-GAT-N7-ATC-N9-GAT) termed the nod-box in its promoter region [14,18]. In contrast to other characterized LTTRs of nodulation, the natural inducing signal of MexT remains elusive. However, the MexT protein is known to be constitutively active in particular *P. aeruginosa* mutant strains termed nfxC-type phenotypic cells. These phenotypic mutants can harbour mutations in specific genes including *mexS, mvaT* and other as yet unidentified targets which influence the activity of MexT [6,19-21].

Besides their increased antibiotic resistance profile, nfxC-type phenotypic cells also exhibit reduced virulence. This is has been linked to nfxC-associated phenotypes which include reduced levels of homoserine lactone-dependent virulence traits including pyocyanin, elastase, rhamnolipids, PQS biosynthesis as well as reduced expression of type three secretion effector proteins and decreased attachment in dynamic environment [14,22-24]. Together these phenotypes lead to reduced virulence in diverse infection models [25]. In addition, nfxC-type phenotypic cells also exhibit compromised motility and attachment which are important phenotypes associated with biofilm formation which contributes to the recalcitrant nature of *P. aeruginosa* infections [23-24,26].

It has been demonstrated that induction of the MexEF-OprN pump is dependent on MexT as deletion of MexT in nfxC-type phenotypic cells restores wild type levels of antibiotic resistance [21]. By a similar approach, repression of swarming and production of elastase and rhamnolipids have been attributed to indirect effects resulting from the induction of MexEF-OprN [23]. However, given the broad-ranging physiological effects of MexT on the cell, it is

possible that MexT could regulate as yet unidentified targets which contribute to other phenotypic changes characteristic of nfxC-type phenotypic cells.

This study aims to explore whether MexT mediates the diverse phenotypes observed in nfxC-type phenotypic cells through induction of the MexEF-OprN efflux system alone, or whether MexT acts as a global regulator and regulates other target genes in *P. aeruginosa* contributing to nfxC-associated phenotypes (Figure 1). The regulation of other targets such as regulatory elements by MexT would expand the regulatory scope of the MexT regulatory network and could explain how MexT orchestrates the broad physiological changes associated with nfxC-phenotypic cells.

In this study it is demonstrated that MexT does indeed act as a global regulator and activates novel targets in addition to MexEF-OprN. This novel MexT regulon includes *PA4354* which encodes a novel transcriptional regulator. The contribution of MexT and this novel repressor to nfxC-associated phenotypes is investigated.

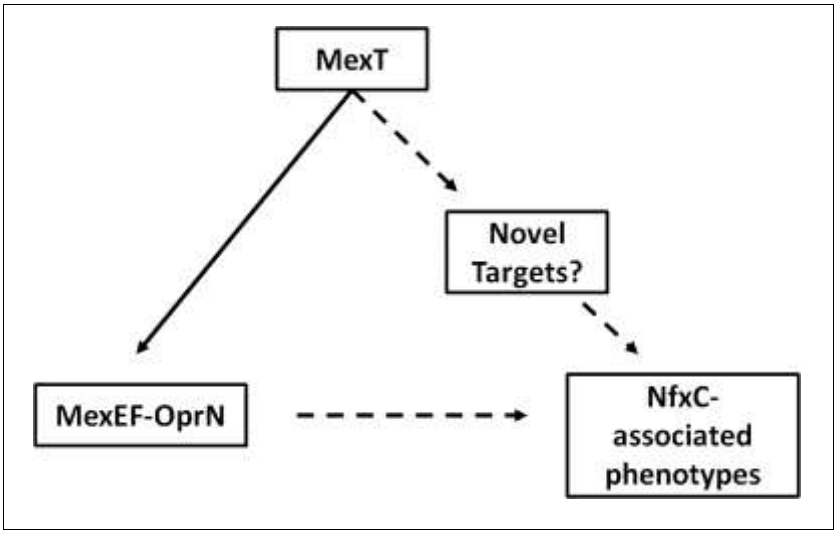

Figure 1. Conceptual framework for the study of MexT-mediated regulation of nfxC-associated phenotypes. MexT directly regulates MexEF-OprN (solid arrow) which has been attributed to indirect repression of a subset of nfxC-associated phenotypes (dashed arrow). However, in this study it is hypothesised that MexT may also contribute to nfxC-associated phenotypes through direct regulation of other regulatory target genes (dashed arrow).

3.3 Materials and Methods

3.3.1 Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. All strains were routinely cultured in LB broth at 37°C with aeration. Antibiotics were added to cultures where required at the following concentrations: *E. coli* - kanamycin 25 μg/ml, tetracycline 10 μg/ml, chloramphenicol 20 μg/ml, *P. aeruginosa* - tetracycline 20-50 μg/ml, gentamicin 20-50 μg/ml, streptomycin 100 μg/ml.

Table 1. Strains and plasmids used in this study

Strain/Plasmid	Description	Reference
Strains		
PAO1	Laboratory strain	
PAO1*ΔmexEF*	*mexE-mexF* deletion mutant	This study
PAO1*ΔmexT*	*mexT* deletion mutant	This study
PAO1*ΔPA4354*	*PA4354* deletion mutant	This study
PAO1::*exoS-lacZ*	PAO1 harbouring chromosomal *exoS* transcriptional fusion	This study
E. coli DH5α	F-φ80 *lacZ*ΔM15 (Δ*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(rK- , mk-)	Invitrogen
*E.coli*S17λpir	*phoA supE44 thi-1 gyrA96 relA1*Δ- *pro thi hsdR*$^+$ Tp^r Sm^r; chromosome::RP4-2 Tc::Mu-Kan::Tn7/λpir	[27]
BL21-CodonPlus® (DE3)-RIPL	protein expression host	Merck
Plasmids		
pCR®2.1-TOPO®	Cloning vector, Ap^r , Km^r	Invitrogen
pME6032	pVS1-p15A origin, *lacI*^{q}-P*tac* expression vector, Tc^r	[28]
pME6032-mexT	pME6032-derived PAO1 *mexT* expression vector	This study
pME6032-4354	pME6032-derived PAO1 *PA4354* expression vector	This study
pET28a	T7 promoter-driven His-tag protein expression vector, Km^r	Novagen
pET28a-PA4354H6C	pET28a-derived C-terminal His6-tag PAO1 PA4354 expression vector	This study
pMP190	*IncQ* origin, low-copy-number *lacZ* fusion vector; Cm^r Str^r	[29]
pMP-PA4354p	pMP190-derived PAO1 *PA4354* promoter-*lacZ* fusion plasmid	This study
pMP-PA4354pMa	Mutated version of pMP-PA4354p harbouring mutation in PA4354 repressor binding site	This study
pEX18Tc	*oriT*+, *sacB*+, gene replacement vector Tc^r	[30]
pPS856	Gmr cassette flanked with FRT sequences Ap^r Gm^r	[30]
pFLP3	inducible Flp recombinase Tc^r	[30]

Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Str^r, streptomycin resistance; Gm^r, gentamycin resistance.

3.3.2 Generation of MexT and PA4354 overexpression plasmids

To obtain plasmid pME6032-mexT, a PCR fragment containing the *mexT* ORF and promoter region was amplified by PCR with the primers 5'-GTAGTAGACGCTGGCCTCCAC-3' and 5'-GTGAATTCGTCCCACTCGTTC-3' and cloned into pCR®2.1-TOPO® vector (Invitrogen, UK). 1.9-Kb *Eco*RI fragment was subcloned into pME6032 [28] in front of the P*tac* promoter, yielding pME6032-mexT. T4 DNA ligase and all restriction enzymes used in this study were purchased from Roche Applied Sciences (Roche, Applied Sciences, UK). The PA4354 ORF was amplified with primers 5'-GGAATTCCATGCCACTGGACATCGACG-3' and 5'-GGGGTACCTGGACTGCGAGGTGGTG-3' which incorporate *Kpn*I and *Eco*RI resistriction sites into the 5' and 3' ends of the *PA4354* ORF respectively (underlined in the primer sequences). This fragment was PCR purified the QIAquick PCR Purification Kit (Qiagen, UK) and cloned directly into pME6032 downstream of the P*tac* promoter to yield pME6032-4354.

3.3.3 Generation of deletion mutants

To introduce gene deletions in PAO1, deletion plasmids were constructed using the gene replacement vector pEX18Tc. Regions flanking the *PA4354* were amplified and cloned into pEX18Tc. Chimeric PCR was employed to link *PA4354*-flanking regions generating a 1.8kb HindIII-BamHI fragment which was ligated into pEX18Tc. This fragment was engineered harbour a restriction site between the target gene flanking regions so as to allow the insertion of the FRT-containing gentamicin resistance cassette excised from plasmid pPS856 [30]. This allowed facile screening during subsequent selection of second cross-over mutants. The pEX18Tc deletion constructs were transformed into *E. coli* S17λpir and subsequently transferred to PAO1 by conjugation following routine cloning procedures [31]. Following specific integration of pEX18Tc into the PAO1 chromosome and subsequent selection for second crossover PA4354 deletion mutants, the gentamicin resistance cassette was excised

from the PAO1 chromosome using the pFLP3 plasmid which harbours the Flp recombinase gene as previously described [30]. The PAO1*ΔmexEF* and PAO1*ΔmexT* deletion mutants were generated using the same strategy. The successful disruption of target genes in deletion mutants was verified in each strain by PCR amplification and DNA sequencing.

3.3.4 Generation of a *PA4354* reporter fusion

A 0.4kb *xba*I-*kpn*I fragment containing the promoter region of *PA4354* was amplified using primers 5'-GCTCTAGAACACGTCCAGCAGGACG-3' and 5'-GGGGTACCGTCCGTTGGCCGTCCATATC-3'. This fragment was purified and ligated into the pMP190 *lacZ* reporter construct upstream of the *lacZ* gene to yield the pMP190-PA4354p transcriptional reporter fusion [29].

3.3.5 Semi-quantitative RT-PCR

Semi-quantitative reverse transcriptase PCR (RT-PCR) was employed to assess whether MexT overexpression induced the *mexEF-oprN* efflux system. RNA was extracted using the RNeasy® minikit (Qiagen, UK) from log phase cultures of PAO1(pME6032) and PAO1(pME6032-mexT) (OD600 0.4-0.7) grown in LB broth with appropriate selection. RNAprotect™ Bacteria Reagent (Qiagen, UK) was added to stabilize bacterial RNA. Contaminating DNA was successfully removed by RNase-free DNase I (Promega, UK) as verified by the failure to detect PAO1 genomic DNA in RNA samples by PCR. Using DNA-free RNA samples as a template, cDNA was synthesised using reverse transcriptase and random primers (Promega, UK) in accordance with manufacturer's instructions. Synthesised cDNA was used in the specific PCR amplification of the *rpsL* and *oprN* genes using specific primers - *rpsL* (5'-GTACATCGGTGGTGAAGGT-3' and 5'-TACTTCGAACGACCCTGC-3'), *oprN* (5'-GCGCGAGAAGATTGCCCTGAG-3', 5'-GCGGCGAAAGGTCCACTGTCA-3'). PCR amplification of *rpsL* and *oprN* was performed using GoTaq® Master mix (Promega, UK). Cycling conditions were as follows:

an initial denaturation step of 95°C for 2 mins, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 sec and a final elongation step at 72°C for 10 mins, the abundance of PCR-amplified *rpsL* and *oprN* products were separated by electophoesis on a 2% agrose gel. PCR products were compared visually following staining with EtBr and UV transillumination.

3.3.6 Determination of antibiotic and metal MIC

The minimum inhibitory concentration (MIC) of diverse antibiotics was determined by microbroth dilution in different strains. Overnight cultures of each strain were diluted 1:100 in fresh Mueller-Hinton broth containing 50 μg/ml tetracycline and grown to mid-log phase (OD600 of 0.4-0.6), harvested and washed in PBS. An inoculums of 10^4-10^5 cfu/ml was added to wells containing a dilution series of each antibiotic in Mueller-Hinton broth. The MIC was defined as the concentration at which bacterial growth was completely inhibited after static incubation for 20 h at 37 °C. In the case of metal MIC determination, Mueller-hinton was substituted for M9 minimal media (0.2% glucose, 10 mM ammonium sulfate as carbon and nitrogen source respectively).

3.3.7 Pyocyanin quantification

Overnight cultures were grown in LB, washed in PBS and diluted to an OD of 0.01 in 20 ml glycerol-alanine media (112 mM DL-alanine, 2% glycerol, 0.8 mM K_2HPO_4, 0.4 mM ferric citrate 0.02 M MgCl2 and 0.1 M Na2SO4) in 100 ml conical flasks. After 24 h incubation at 37°C, pyocyanin was quantified in cell-free stationary phase culture supernatants by HCL extraction as previously described [32].

3.3.8 Attachment assay

Strains were grown overnight in M9 minimal medium. Cells were diluted 1:100 into fresh M9 medium. These bacteria were allowed to grow to mid-log phase (OD600 of 0.4-0.7) before dilution to an OD600 of 0.1 in 20 ml of M9 medium. One milliliter cultures were incubated at 37 °C in 24-well polystyrene plates for 2 h. Unattached cells were removed and attached cells were stained with 0.25% crystal violet for 30 min, washed twice with PBS and air dried. Two millilitres of ethanol was added to the wells to extract crystal violet and levels of attachment were quantified at OD595 nm.

3.3.9 Quantification of *exoS* induction

The putative regulatory effect of MexT and PA4354 on the expression of the TTSS effector gene *exoS* was investigated by β-galactosidase assay. Into an isogenic PAO1 strain chromosomally tagged with an *exoS-lacZ* reporter fusion the pME6032-mexT and pME6032-4354 overexpresison constructs were introduced. Expression of *exoS* was induced by growing strains in TTSS inducing media (LB, 10 mM nitrilotriacetic acid [NTA], supplemented with 5 mM, $MgCl_2$). Overnight cultures were were inoculated into 20ml TTSS-inducing media with appropriate antibiotic selection at a starting OD of 0.02 and incubated at 37°C with shaking (150 r.p.m). Cells were harvested at an OD 600 of ~1.5 and the expression of *exoS* was measured by β-galactosidase assay as previously described [33].

3.3.10 Transcriptome analysis

For transcriptomic analysis of PAO1(pME6032), PAO1(pME6032-mexT) and PAO1*ΔmexEF*(pME6032-mexT), RNA was extracted from bacterial cultures which were grown at 37°C with shaking (150 r.p.m.) in 20ml of LB medium supplemented with tetracycline in 100-ml culture flasks. At an OD600 of 0.5, cell cultures were mixed with Qiagen RNAprotect™ Bacteria Reagent (Ratio of 1:2) to stabilize RNA (Qiagen, UK). Total RNA was prepared with an RNeasy® mini kit (Qiagen. UK) and contaminating DNA was

removed by RNase-free DNase (Ambion, UK). For each strain, RNA was prepared from three independent cultures. cDNA synthesis and hybridization to Affymetrix GeneChip ® *P. aeruginosa* genome arrays were carried out by a commercial Affymetrix Genechip service supplier (Conway Institute of Biomolecular & Biomedical Research, UCD, Ireland). Array data were normalized using the GC-RMA algorithm, and the data were analyzed using the microarray software package Genespring ® GX 10.0.1 (Agilent, UK). Genes whose levels of expression were significantly influenced (Fold change >2, P<0.05) were identified with Genespring® GX, using a Benjamini - Hochberg multiple-testing correction and a false detection rate of 5%.

3.3.11 Identification of conserved motif in MexT-regulated targets

The promoter regions of the ten putative MexT target genes identified by transcriptome analysis (*PA1744*, *PA1970*, *PA2486*, *mexE*, *PA2759*, *PA2813*, *PA3229*, *PA4354*, *PA4623* and *PA4881*) were analysed for the presence of a conserved MexT binding site using the Multiple Em for Motif Elicitation (MEME) algorithm [34]. The following parameters were used: motif occurrence - one per sequence, optimum motif width - >=6, <=50. A sequence logo was generated to visually represent the position-specific scoring matrix which describes the conservation of nucleotides within the putative MexT-binding motif.

3.3.12 Sequence alignment of PA4354 and other ArsR-family repressors

PA4354 was aligned with other representative ArsR-family repressors in the Prints database [35]. The sequences of ArsR-family proteins in the multiple sequence alignment which define the HTHARSR-family fingerprint were retrieved and aligned with PA4354 using the T-coffee algorithm [36]. The predicted secondary structure of ArsR-repressors in the alignment is based on the previous multiple sequence alignment of Harvie *et al* [37].

3.3.13 Induction of *PA4354* expression in the presence of diverse metals

Induction of *PA4354* in the presence of diverse metals was investigated in a plate-based assay using the *PA4354-lacZ* promoter fusion pMP-PA4354p as a reporter in PAO1. Overnight cultures of PAO1 harbouring the pMP-PA4354p were diluted to an OD 600 of 0.125 and a bacterial lawn was inoculated onto M9-agar plates containing 0.4 % glucose, 40 µg/ml Xgal and 100µg/ml streptomycin. Filter paper disk containing metal salts including $ZnSO_4$,V_2SO_4, $MnCl_2$, MO_4Na_2, $NaAsO_2$, CdCl, $CuSO_4$, K_2TeO_3, $CoCl_2$, $NiCl_2$, $FeCl_3$, $CrCl_3$, $C_8H_4K_2O_{12}Sb_2$, $AgNO_3$, $Pb(NO_3)_2$, $AuCl_3$, $BiNO_3$, $SeNa_3$ and $HgCl_2$, were placed on the centre of each inoculated plate which was incubated at 37 °C for 16-24 h and examined for induction of expression from the *PA4354-lacZ* reporter fusion as indicated by an increase in blue pigmentation in the agar resulting from induction of the *lacZ* gene and the breakdown of Xgal. The ability of inducing metals to activate expression from the pMP-PA4354p was also investigate in PAO1*ΔPA4354* and PAO1*ΔmexT* deletion backgrounds.

3.3.14 Quantification *PA4354* promoter activity

To validate the induction of *PA4354* by MexT as observed in the transcriptome analysis, the MexT overexpressor pME6032-mexT and the pME6032 vector control were introduced to PAO1 harbouring the pMP-PA4354p reporter fusion. Overnight cultures were inoculated into 20ml of fresh LB broth with appropriate antibiotic selection at a starting OD600 of 0.02. Cultures were grown to mid-log phase (OD600 of 0.4-0.7) and harvested for determination of β-galactosidase activity as previously described [33]. To investigate the autoregulatory effect of PA4354, the β-galactosidase activity was measured from the pMP-PA4354p reporter in PAO1 in the presence of the pME6032-4354 overexpression construct from which PA4354 expression was induced in the presence IPTG concentrations ranging from 1-100 µM. Expression from pMP-PA4354p promoter fusion was also assayed in PAO1*ΔPA4354* and PAO1*ΔmexT*.

3.3.15 Identification of a putative PA4354 autoregulatory motif

To determine whether PA4354 and homologous repressors exhibited conserved autoregulatory binding sites in their promoters, the upstream regions of 57 putative PA4354 orthologues were retrieved from the Database of Prokayotic Operons (DOOR) and analysed for the presence of conserved autoregulatory motifs [38]. Upstream sequences of approximately 200bp retrieved from each identified PA4354-like regulator were searched using the Multiple Em for Motif Elicitation (MEME) algorithm for the presence of conserved motifs using the following parameters: motif occurrence - one per sequence, optimum motif width - >=6, <=50 [34]. A position-specific scoring matrix was generated to describe the observed conservation of nucleotides upstream of PA4354 orthologues which constitute a putative PA4354 binding site. To determine the distribution of this putative PA4354 binding motif in the PAO1 genome the Motif Alignment and Search Tool (MAST) was employed to search upstream promoter regions in the PAO1 genome for the presence of a putative PA4354 autoregulatory sites [39].

3.3.16 Site directed mutagenesis of the putative PA4354 autoregulatory motif

To generate a mutated promoter fusion construct the *xba*I-*kpn*I PA4354 promoter fragment described in section 3.2.4 was cloned into the pCR®2.1-TOPO® cloning vector (Invitrogen, UK). Primers 5'-GTCGCAATCGATCCCCATCGCGTCCCACACCGATAT-3' and 5'-ATATCGGTGTGGGACGCGATGGGGATCGATTGCGAC-3' were designed to amplify the entire pCR2.1-TOPO plasmid and introduce site specific mutation within the putative PA4354 autoregulatory motif as previously described [40]. The amplified plasmid was transformed into *E. coli* DH5α and the presence of the introduced mutations was verified by sequencing. Once verified, this mutated promoter region was excised from pCR®2.1-TOPO® and ligated into pMP190 to yield the mutated promoter fusion pMP-PA4354pMa. The ability of PA4354 (as expressed from the pME6032-4354 overexpression construct) to repress activity from the mutated promoter region of pMP-PA4354pMa was assessed in

PAO1 by β-galactosidase assay as described in section 3.2.13. In this case a concentration of 1μM IPTG was added to induce expression of *PA4354* from pME6032-4354.

3.3.17 Investigation of *PA4354-PA4355-xenB* operon structure

RNA extracted from PAO1(pME6032-mexT) as was used as a template in the synthesis of cDNA as described in section 3.2.5 with the exception that a specific primer complementary to the 3' region of *xenB* (5'-TCGGATAGTCCAGGTAGCCT-3') was used in order to target cDNA synthesis from the putative *PA4354-PA4355-xenB* transcript. The co-transcription of the *PA4354-PA4355-xenB* transcript was investigated by PCR using primers which flanked the *PA4354-PA4355* (5'-AACGAGGAGACCATCCAGGC-3', 5'-ACGATGAAGATGCCCATCA-3') and *PA4355-xenB* (5'-CGGCAGCGTCATCGACCA-3', 5'-AGAGTTGCAGGAAGATGCGC-3') intergenic regions and specifically synthesized cDNA as a template.

3.3.18 Phylogenetic distribution of *PA4354*, *PA4355*, *xenB* and *mexT*

To assess the distribution and putative interaction of PA4345, PA4355, xenB and MexT in diverse organisms the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online analysis tool was employed [41]. This allowed visual representation of the distribution and synteny of *PA4354*, *PA4355*, *xenB*, and *mexT* homologues in diverse species. The genomes of species in which strong MexT homologues were identified were searched for the presence of homologous PAO1 MexT-regulated targets identified in the transcriptome analysis (*PA1744*, *PA1970*, *PA2486*, *mexE*, *PA2759*, *PA2813*, *PA3229*, *PA4354*, *PA4623* and *PA4881*). This was achieved using the BLAST search tool on the Pseudomonas genome database (version 2) [42]. To investigate whether MexT might regulate a similar complement of gene targets in diverse Pseudomonads, the MAST algorithim was employed to search the genomes of *P. mendocina* YMP, *P. stuzeri* A1501 *P. syringe* 1448A, *P. entomophila* L48,

P. fluorescens Pf-01, and *P. putida* KT2440 for the occurrence of the identified MexT binding motif of identified in PAO1[39].

3.3.19 His-tag Purification of PA4354

In order to purify the PA4354 repressor, a His-tagged overexpression construct was generated. The PA4354 ORF was cloned into the *not*I and and *xho*I sites of the pET28a vector to yield pET28a4354H6C which expressed a C-terminal His-tagged PA4354 protein from its IPTG-inducible T7 promoter. This construct was transformed into the *E. coli* expression host strain BL21-CodonPlus®(DE3)-RIPL (Merck, UK). and grown at 37°C with vigorous shaking in 200-ml LB medium containing kanamycin (50 mg/ml) to an OD 600 of 1. A this point 1mM of IPTG was added to the culture to induce expression of the His-tagged PA4354 protein. After 4 hours cells were harvested and by centrifugation at 5000 x g at 4°C and stored overnight a -70°C. Cell pellets were subsequently thawed and re-suspended in CelLytic™ B II buffer (Sigma, UK) (10 ml per gram of cell paste) with 5 mg/ml DNase and 200 µl per gram cell paste of Protease Inhibitor Cocktail (Sigma, UK). Soluble protein was extracted in accordance with the manufacturer's instructions. The protein extract were applied to a Poly-Prep® Chromatography Column (Bio-Rad, UK) containing 1ml of HIS-Select™ Nickel Affinity Gel (Sigma, UK). The gel was washed with 2ml of sterile de-ionized water and equilibrated with 5ml of wash buffer (HEPES 100mM, pH 7.5, 10mM imidazole). Following washing and equilibration steps, the crude protein extract (10ml) was passed through the column by gravity flow. Protein which bound to the resin was washed twice with 10ml wash buffer and purified His-tagged PA4354 protein was eluted in wash buffer containing 250mM imidazole. The homogeneity of the purified PA4354 protein was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Purified protein aliquots were either frozen in 20% glycerol at -70°C or used promptly for electromobility shift assays (EMSAs). As a control, the protein purification procedure was also performed with *E. coli* BL21-CodonPlus®(DE3)-RIPL cells harbouring

the pET28a empty vector. Protein concentrations were determined by the Bio-Rad protein assay.

3.3.20 Electromobility Shift Assay

The direct binding of PA4354 to its own promoter region was demonstrated by EMSA. Purified PA4354 protein was incubated with an infrared-labelled PA4354 promoter fragment generated with DY-682 infrared-labelled primers 5'-GATCATCGTCATCAGCCCT-3' and 5'-AGTGGCATGTGGTCCGTT-3' (Eurofins MWG Operon, UK). EMSA was set up with 20µl reaction volumes containing varying concentrations of purified PA4354 protein (10-2000nM) in the presence of 10fmol of labelled promoter DNA in EMSA binding buffer (20mM HEPES pH 7.6 containing 30mM KCl, 5mM $(NH_4)_2SO_4$, 1mM EDTA and 1mM DTT 0.2% w/v Tween 20, 5µg/ml Poly[d(I-C)]). The reaction was incubated at room temperature for 45 mins and samples were then separated by electrophoresis on 6% native polyacrylamide gel and were visualized on an Odyssey® Infrared Imaging System (Li-COR Biosciences, UK). Control binding reactions were performed with extracts from *E. coli* BL21-CodonPlus®(DE3)-RIPL cells harbouring the pET28a vector control and with a non-specific labelled DNA fragment amplified from the PA4881 promoter region which did not contain the putative conserved motif from the PA4354 promoter region using DY-682 infrared-labelled primers 5'-AGCAAGACCCGTCCGAGAG-3' and 5'-GATTTCGAAGATTTCGTAAAGGA-3'.

3.4 Results

3.4.1 MexT is a global regulator of antibiotic resistance and virulence

Overexpression of LTTRs including MexT has been previously employed to investigate the regulation of target genes by LTTRs in a substrate-independent manner [14]. In order to investigate the global phenotypic effects of MexT on the physiology of *P. aeruginosa*, a MexT overexpression construct, pME6032-mexT, was introduced into the model strain PAO1. This resulted in increased expression of *oprN* as measured by semi-quantitative RT-PCR, indicating that the *mexEF-oprN* transcript had been induced (Figure 1). This was accompanied by increased resistance to chloramphenicol, trimethoprim, norfloxacin and imipenem as well as hypersusceptibility to carbenicillin and kanamycin compared to PAO1 harbouring the pME6032 vector control (Table 1). This resistance profile is typical of nfxC-type phenotypic mutants. In addition, overexpression of MexT lead to reduced pyocyanin production, impaired attachment to polystyrene and abrogated induction of the type three secretion system (TTSS) (Figure 2). Hence, overexpression of MexT induced phenotypic changes consistent with those observed in nfxC-type phenotypic cells confirming its role in antibiotic resistance and virulence.

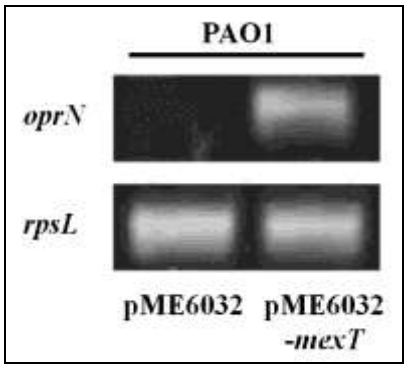

Figure 1. Induction of the *oprN* transcript by overexpression of MexT in PAO1. Increased transcript levels of *oprN* were detected in PAO1 harbouring a MexT overexpression construct (pME6032-mexT) compared to PAO1 harbouring the vector control (pME6032) relative to the stabily expressed *rpsL* control.

Table 1. Increased antibiotic resistance induced by overexpression of MexT in PAO1.

	Antibiotic MIC (μg/ml) [a]					
Strain	CB	CM	TMP	NFX	KM	IMP
PAO1 (pME6032)	64	32	64	0.25	32	0.25
PAO1 (pME6032-mexT)	32	2048	1024	4	16	2

[a] MIC - minimum inhibitory concentration. CB - carbenicillin CM - chloramphenicol TMP - trimethoprim NFX - norfloxacin KM - kanamycin IMP - imipenem

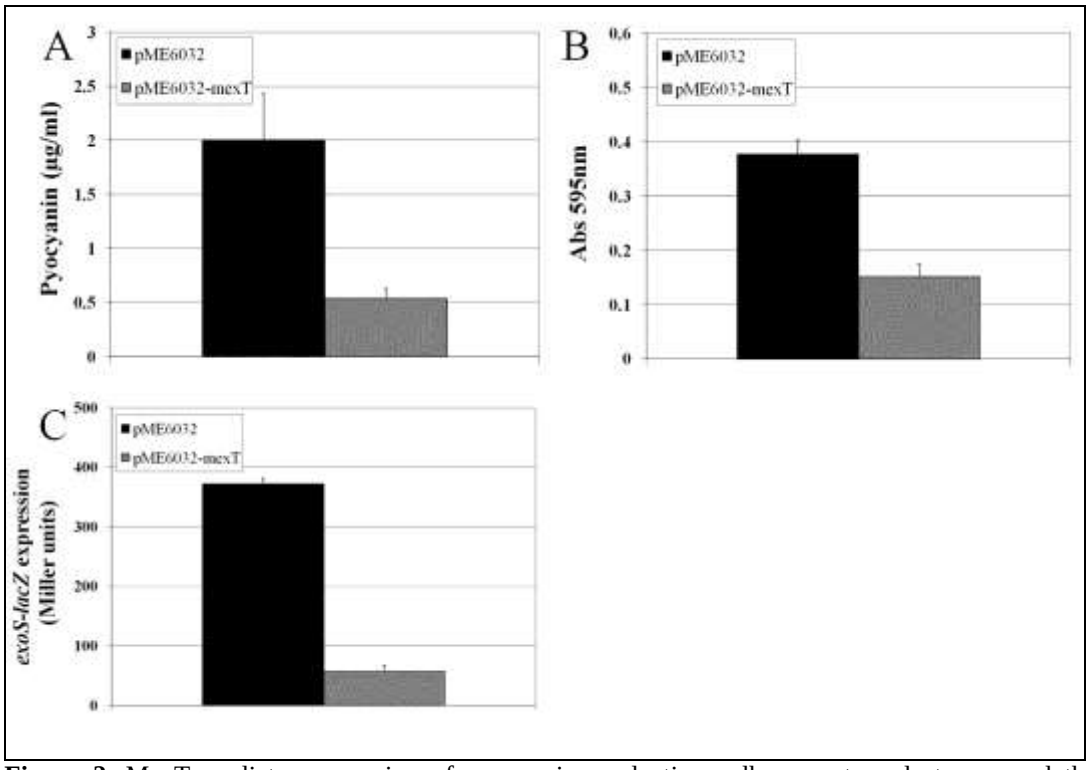

Figure 2. MexT mediates repression of pyocyanin production, adherence to polystyrene and the TTSS in PAO1. **A -** The presence of the MexT overexpression construct pME6032-mexT in PAO1 reduced the level of pyocyanin detected in stationary phase supernatants of cultures grown in DL-alanine media. **B -** Overexpression of MexT also negatively affected adherence to polystyrene after 2 hours in M9-glucose minimal media as measured by crystal violet staining of attached cells. **C -** MexT overexpression in PAO1 inhibits induction the TTSS effector-encoding *exoS* gene under TTSS-inducing conditions. Expression of *exoS* is given in Miller units measured by β-galactosidase assay from a chromosomally inserted *exoS-lacZ* promoter fusion in PAO1.

3.4.2 MexT effects phenotypic changes independent of the MexEF-OprN efflux system

To assess the contribution of the MexEF-OprN efflux system to nfxC-associated phenotypes, a *mexEF* deletion mutant was generated (PAO1Δ*mexEF*) into which the MexT overexpression construct pME6032-mexT was introduced. The effect of MexT overexpression on nfxC-associated phenotypes including antibiotic resistance, pyocyanin production, early attachment and repression of the TTSS effector-encoding gene *exoS* were assessed in the PAO1Δ*mexEF* deletion mutant.

In a *mexEF* deletion background, overexpression MexT failed to induce resistance to the known antibiotic substrates of MexEF-OprN chloramphenicol, trimethoprim and norfloxacin to the same level as in the wild type PAO1 background demonstrating the contribution of MexEF-OprN to resistance against these antibiotics. In contrast, imipenem resistance was still observed in the PAO1Δ*mexEF* on overexpression of MexT, in line with previous data reporting the MexEF-OprN-independent effect of MexT on resistance to this carbapenem antibiotic (Table 2) [14].

In addition to increasing imipenem resistance, overexpression of MexT also mediated repression of pyocyanin production, adherence to polystyrene and induction of the TTSS effector-encoding gene *exoS* in a *mexEF* deletion background verifying that these phenotypes are mediated by MexT independent of the MexEF-OprN efflux system (Figure 3). Hence the induction of *mexEF-oprN* by MexT is not sufficient to explain the global changes in PAO1 physiology which accompany MexT activation. This suggested the influence of MexT on alternative regulatory pathways.

Table 2. Effect of MexT overexpression on antibiotic resistance in PAO1Δ*mexEF*.

	Antibiotic MIC (μg/ml) [a]					
Strain	CB	CM	TMP	NFX	KM	IMP
PAO1Δ*mexEF* (pME6032)	64	32	64	0.25	32	0.25
PAO1Δ*mexEF* (pME6032-mexT)	128	64	256	0.25	32	4

[a] MIC - minimum inhibitory concentration. CB - carbenicillin CM - chloramphenicol TMP - trimethoprim NFX - norfloxacin KM - kanamycin IMP - imipenem

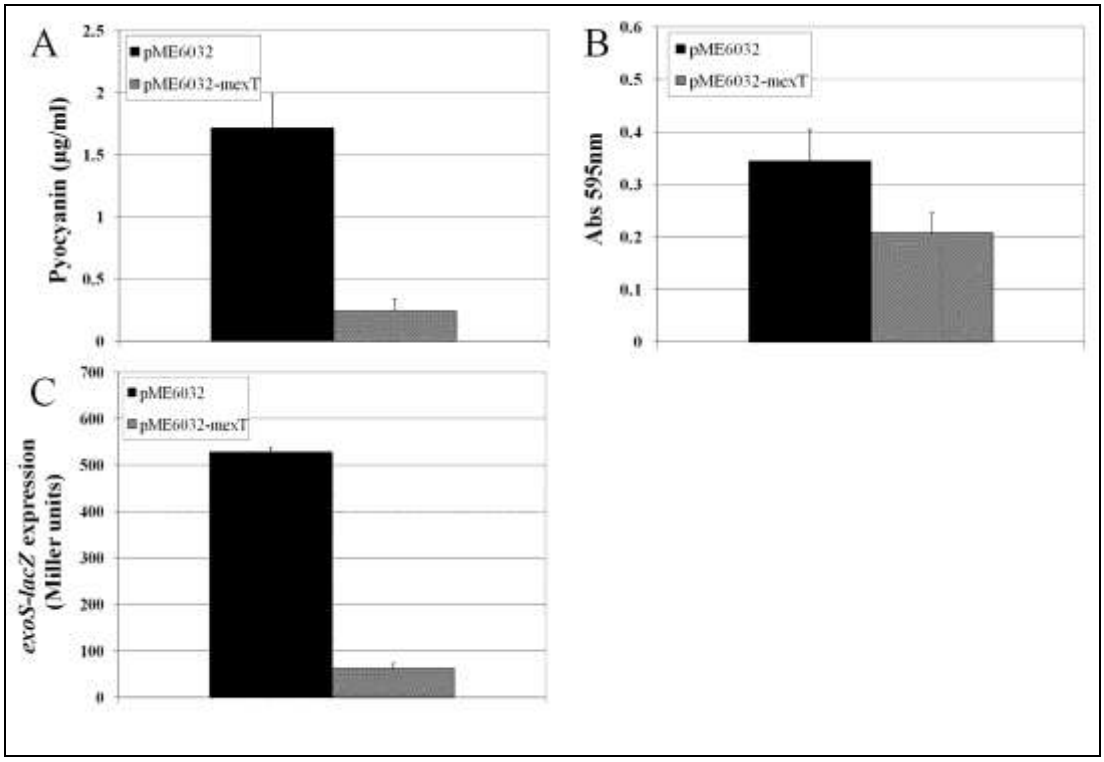

Figure 3. MexT-mediate repression of pyocyanin production, adherence to polystyrene and the TTSS is independent on the MexEF-OprN efflux system. **A -** Deletion of *mexEF* did not affect MexT-mediated repression of pyocyanin production as measured in stationary phase culture supernatants. **B -** Overexpression of MexT impaired adherence to polystyrene in PAO1Δ*mexEF*. **C -** The presence of pME6032-mexT in PAO1 inhibited induction of expression of *exoS* under type three-inducing conditions in PAO1Δ*mexEF*. Expression of *exoS* is given in Miller units measured by β-galactosidase assay from a chromosomally inserted *exoS-lacZ* promoter fusion in PAO1.

3.4.3 Identification of a novel MexT regulon

The identification of phenotypes regulated by MexT independent of MexEF-OprN suggested that MexT might directly regulate targets in addition to *mexEF-oprN*. To investigate whether MexT could mediate direct activation of other target genes in PAO1, microarray analysis was carried out comparing global gene expression profiles of PAO1 harbouring the pME6032 vector control - PAO1(pME6032), PAO1 harbouring the MexT overexpression construct - PAO1(pME6032-*mexT*) and the isogenic PAO1 *mexEF* deletion mutant harbouring the MexT overexpression construct - PAO1Δ*mexEF*(pME6032-*mexT*).

This allowed the identification of genes which were induced by MexT overexpression independent of the MexEF-OprN efflux system. Genes which were highly induced (>10 fold) in the global transcriptional comparison of PAO1(pME6032) *vs* PAO1(pME6032-*mexT*) and exhibited comparable expression in the comparison of PAO1(pME6032-*mexT*) *vs*

PAO1Δ*mexEF*(pME6032-*mexT*) are shown in Table 3. Comparison PAO1(pME6032-mexT) and PAO1Δ*mexEF*(pME6032-*mexT*) global expression profiles revealed that 14 genes were induced by MexT in PAO1 (in addition to *mexEF-oprN*) which all showed comparable expression in a PAO1Δ*mexEF* background. These genes were therefore induced to comparable levels in both PAO1 and PAO1Δ*mexEF* by MexT and hence represent putative direct regulatory targets of MexT (Table 3).

The MEME (Multiple Em for Motif Elicitation) motif finding algorithm was employed to identify conserved sequences in the upstream regions of MexT induced genes [34]. Of the 14 genes induced by MexT independent of MexEF-OprN, nine were found to harbour a putative MexT consensus binding motif in their upstream regions. These putative MexT binding sites shared sequence similarity with the proposed MexT binding site previously identified upstream of *mexE* (Figure 4) [14]. The MexT-induced genes harbouring the putative MexT consensus motif are indicated in Table 3 by bold typeset. The significance of this motif (E value = 3.5e-022) and the high level of induction of these genes by MexT in the in both PAO1 and PAO1Δ*mexEF* transcriptome datasets is consistent with the direct regulation of these genes by MexT. The identification of novel genes regulated by MexT independent of MexEF-OprN represent a possible mechanism by which MexT mediates MexEF-OprN independent phenotypes such as imipenem resistance, reduced adherence to polystyrene, and repression of the type three secretion system (Figure 5).

One of these novel target identified was *PA4354* which encodes a novel ArsR-family transcriptional repressor. Members of the ArsR-family of transcriptional repressors are known to repress their targets at the transcriptional level by binding to their upstream regions causing repression. As MexT directly induces expression of *PA4354*, increased expression of this negative transcriptional regulator may contribute to the downregulation of genes which contribute to MexT-associated phenotypes.

Table 3. Genes differentially regulated (>10 fold) in response to MexT overexpression independent of *mexEF-oprN*.

Gene No. [a]	Gene name	PAO1 mexT++ vs PAO1 [b]	PAO1ΔmexEF mexT++ vs PAO1 mexT++ [c]	Protein description
PA1744 [d]		**48**	**<2**	**Hypothetical protein**
PA1970		**237**	**<2**	**Hypothetical protein**
PA2486		**338**	**<2**	**Hypothetical protein**
PA2491	*mexS*	75	<2	Probable oxidoreductase
PA2492	*mexT*	23.3	<2	Transcriptional regulator MexT
PA2493	***mexE***	**2033**	**−1714**	**RND multidrug efflux membrane fusion protein MexE**
PA2494	*mexF*	1382	−9.5	RND multidrug efflux transporter MexF
PA2495	*oprN*	340	−10	Multidrug efflux outer membrane protein OprN precursor
PA2759		**72**	**<2**	**Hypothetical protein**
PA2811		34	<2	Probable permease of ABC-2 transporter
PA2812		12	<2	Probable ATP-binding component of ABC transporter
PA2813		**20**	**<2**	**Probable glutathione *S*-transferase**
PA3205		11	<2	Hypothetical protein
PA3229		**1789**	**<2**	**Hypothetical protein**
PA4354		**11**	**<2**	**Conserved hypothetical protein**
PA4623		**74**	**<2**	**Hypothetical protein**
PA4881		**751**	**<2**	**Hypothetical protein**

a Gene number from the Pseudomonas Genome Project [42].

b Fold change in gene expression of PAO1 (pME6032-mexT) compared to PAO1 (pME6032); positive value means expression increased and negative

c Fold change in gene expression of PAO1Δ*mexEF*(pME6032-mexT) compared to PAO1(pME6032-mexT).

d Genes in bold type represent MexEF-OprN independent induced targets which harbour a putative MexT binding motif.

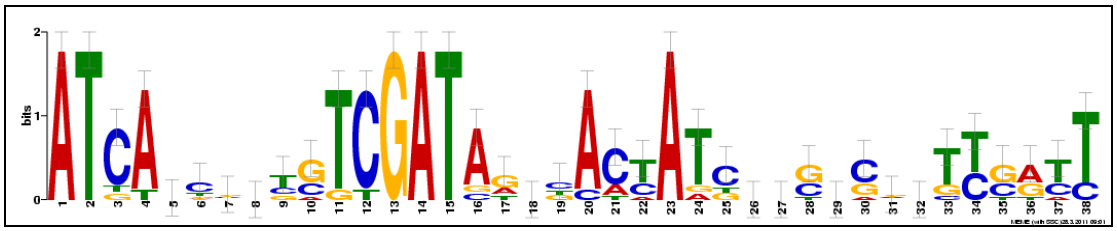

Figure 4. Identification of a conserved motif upstream of highly MexT-induced target genes. The sequence logo illustrates the position-specific scoring matrix which describes the putative MexT binding motif present upstream of *mexE*, *PA1744*, *PA1970*, *PA2486*, *PA2759*, *PA2913*, *PA3229*, *PA4354*, *PA4623*, *PA4881*.

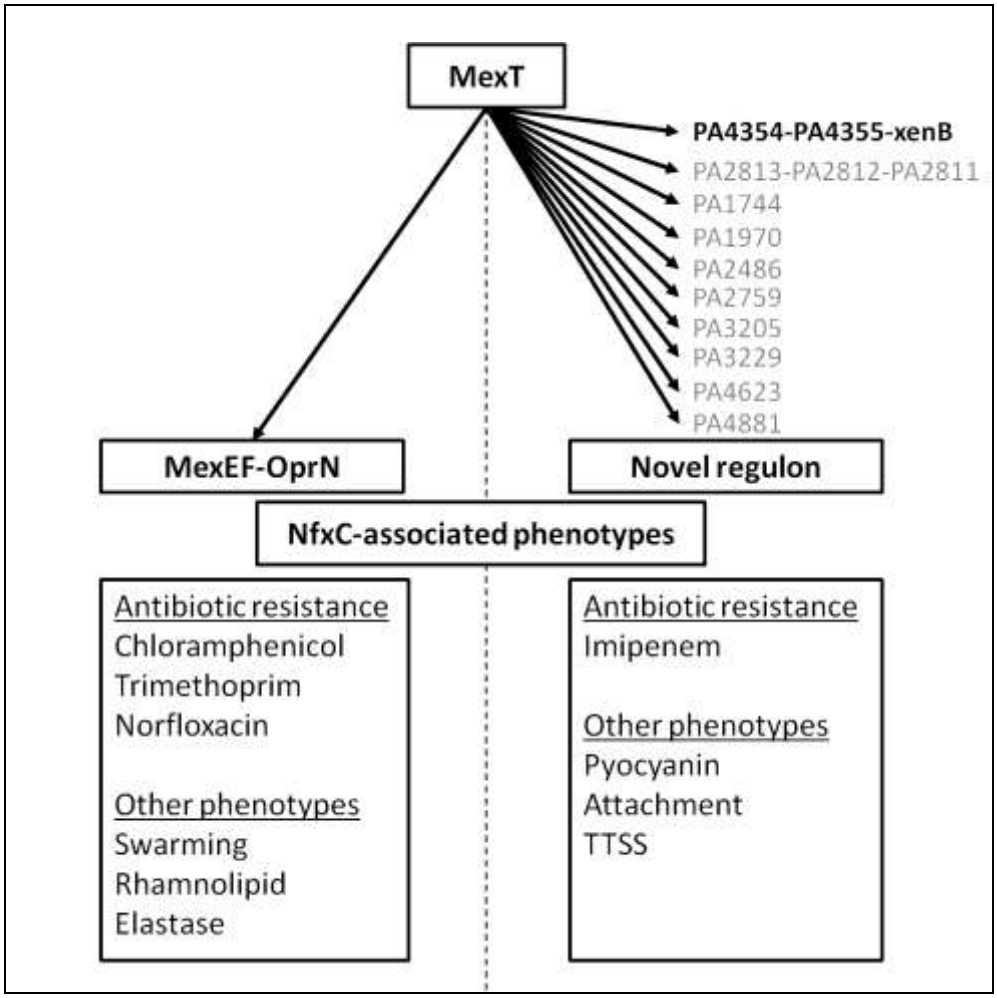

Figure 5. The novel MexT-regulon may contribute to antibiotic resistance and virulence phenotypes via MexEF-OprN-dependent pathways in nfxC-type phenotypic cells. MexT-mediated phenotypes such as resistance to chloramphenicol, trimethoprim and norfloxacin as well as repression of swarming, and production of rhamnolipids and elastase are dependent on the MexEF-OprN system. However, other phenotypes including imipenem resisitance, pyocyanin production, attachment to polystyrene and repression of the TTSS are mediated by MexT independent of MexEF-OprN. The novel MexT provides a possible mechanism by which MexT mediates MexEF-OprN-independent phenotypes. Regulation of the novel transcriptional repressor PA4354 could explain how MexT influences virulence and antibiotic resistance genes independent of MexEF-OprN via direct transcriptional repression.

3.4.4 MexT regulates a novel ArsR-family transcriptional repressor

Among the newly identified targets of MexT is *PA4354* which encodes a novel transcriptional repressor of the ArsR-family (Figure 5). To validate the direct induction of *PA4354* by MexT, a *PA4354-lacZ* promoter fusion (pMP-PA4354p) was constructed and introduced into PAO1. Overexpression of MexT caused induction of expression from the *PA4354* promoter in agreement with the results of the transcriptome analysis confirming that MexT directly induces *PA4354*. Trancriptome analysis also revealed that MexT induced

expression of *PA4355* (9-fold) and *xenB* (8-fold) which are present immediately downstream of PA4354 suggesting that these three genes are present in an operon. RT-PCR verified the operon structure of *PA4354-PA4355-xenB* in PAO1 (Figure 7). Complementary cDNA was synthesised from RNA isolated from PAO1(pME6032-mexT), in which expression from the *PA4354* promoter is induced (Figure 6). Primers flanking the intergenic regions between *PA4354* and *PA4355* as well as *PA4355* and xenB were designed to verify that the tri-cistronic transcript was expressed (Figure 7). The amplification of the PA4354-PA4355 and PA4355-xenB intergenic regions from cDNA specifically reverse transcribed from isolated RNA confirmed the co-transcription of *PA4354, PA4355* and *xenB.*

To assess whether the *PA4354-PA4355-xenB* operon might be regulated by MexT in divergent bacterial genera, the conservation of these four genes among diverse organisms was assessed using the online Search Tool for the Retrieval of Interacting Genes/Proteins (STRING v8.3) [41]. Genes homologous to *PA4354, PA4355* and *xenB* are found in the same genetic neighbourhood in divergent species suggesting the conservation of operon structure among *PA4354, PA4355* and *xenB* orthologues in diverse bacteria (Figure 8). MexT is not found in the vicinity of homologous *PA4354-PA4355-xenB* operons and is not strongly conserved outside the *Pseudomonas* genus which indicates that the integration of *PA4354-PA4355-xenB* into the MexT regulatory network is specific to this genus (Figure 8).

In addition, among diverse *Pseudomonas* species in which MexT is conserved, the MexT consensus binding site may be present or absent from the upstream region of *PA4354* orthologues suggesting that the *PA4354-PA4355-xenB* operon is regulated by MexT in some species of *Pseudomonas* but not in others. This suggests that the MexT regulon has undergone regulatory "re-wiring" in different *Pseudomonas* species. In *P. aeruginosa* PAO1, *P. mendocina* YMP, *P. stuzeri* A1501 and *P. syringe* 1448A, *PA4354* harbours a MexT binding site in its upstream region but in *P. entomophila* L48, *P. fluorescens* Pf-01, and *P. putida* KT2440 this regulatory motif is absent upstream of *PA4354* suggesting that it is not regulated my MexT in these species (Table 4). That MexT regulates a diverse complement

of target gene outside *P. aeruginosa* is also evidenced by the fact that homologues of other genes of in the MexT regulon identified in PAO1 are absent from the genomes of other species of *Pseudomonas* (Table 4). Thus MexT may activate expression of a diverse complement of target genes among the *Pseudomonas* genus and may therefore elicit divergent physiological functions across different species of *Pseudomonas*.

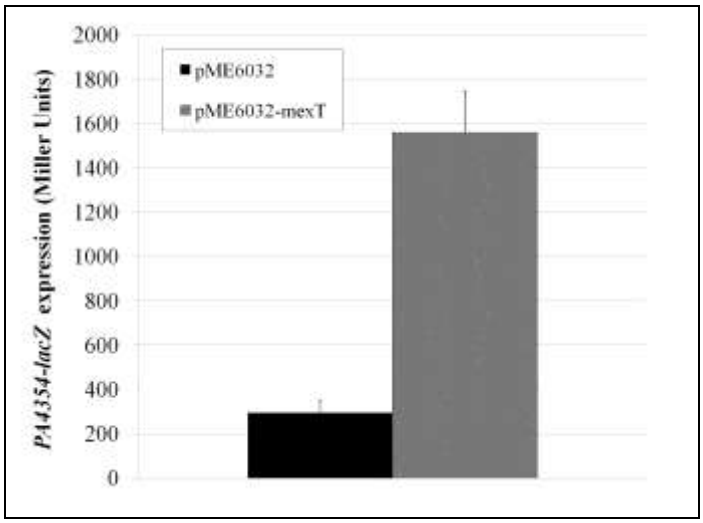

Figure 6. MexT-mediated induction of *PA4354*. Overexpression of MexT resulted in increased expression from the *PA4354-lacZ* promoter fusion (pMP-PA4354p) compared to the vector control as measured by β-galactosidase assay. Expression from pMP-PA4354p in the presence of pME6032-mexT or the pME6032 vector control is given in Miller Units.

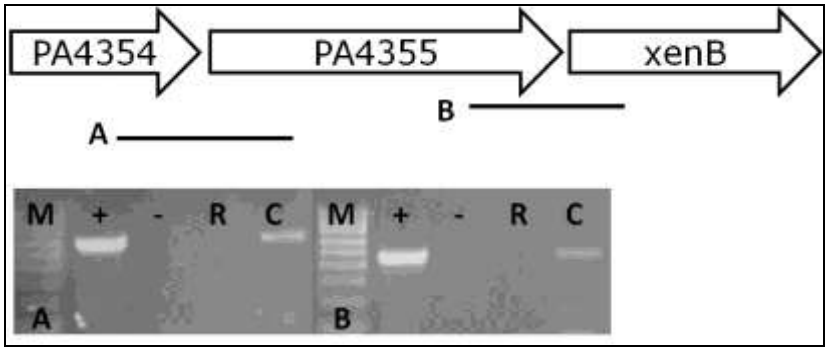

Figure 7. The *PA4354-PA4355-xenB* operon structure was confirmed by RT-PCR in PAO1. **M**:DNA ladder, **+**: positive control (PAO1 gDNA), **-**:negative control (cDNA synthesis reaction mixture to which no template added), **R**: RNA isolated from PAO1(pME6032-mexT), **C**: cDNA synthesised from RNA isolated from PAO1(pME6032-mexT) using a specific primer complementary to the 3' end of the xenB transcript. The overlapping regions amplified in images **A** and **B** are indicated by solid black lines in the diagram of the *PA4354-PA4355-xenB* operon structure above the gel image.

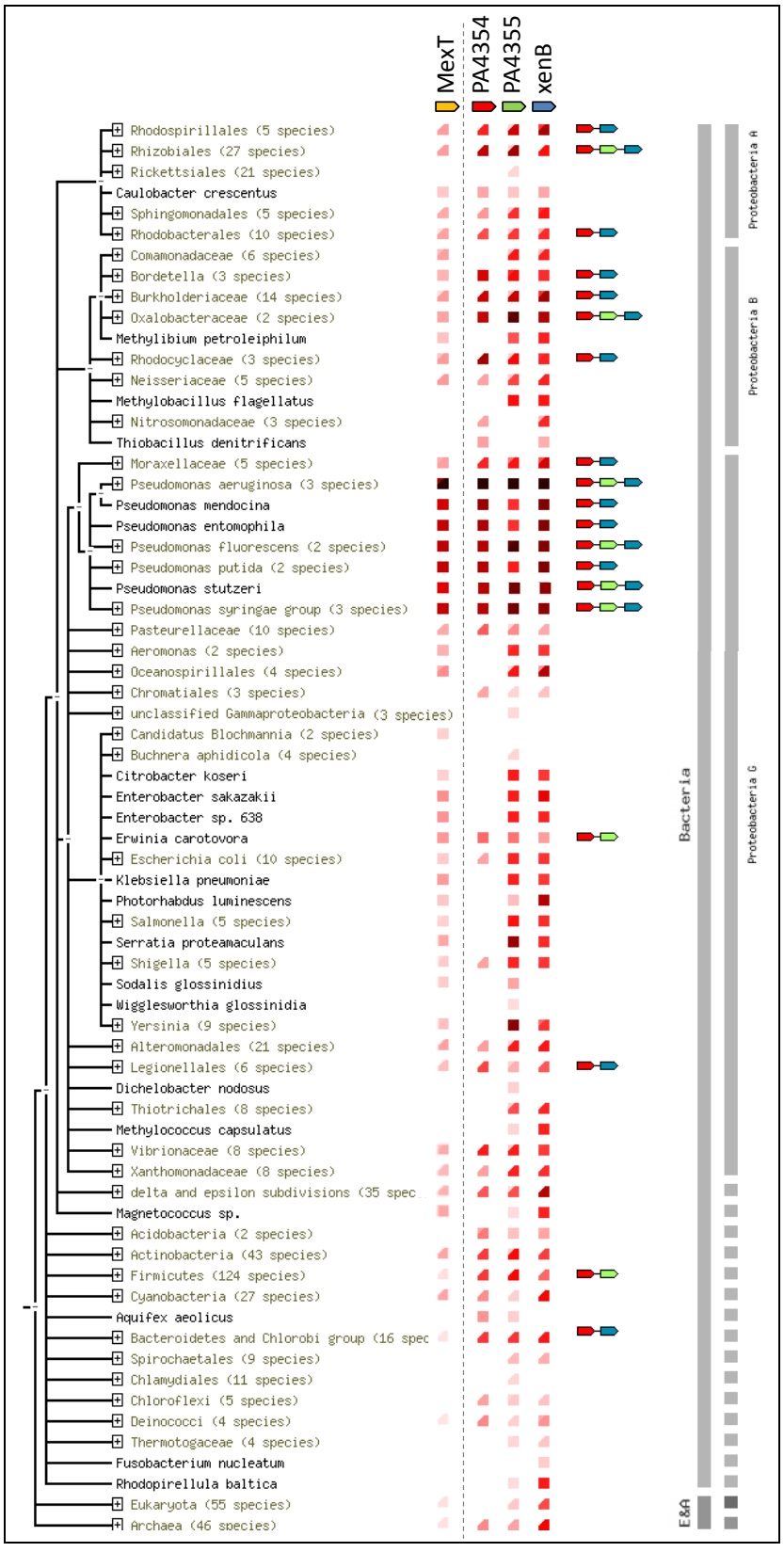

Figure 8. Occurrence of genes homologous to *PA4354 PA4355* and *xenB* in diverse organisms. Boxes are shaded from pink to black based on homology. In diverse species, *PA4354*, *PA4355* or *xenB* are co-located in the same genetic neighbourhood suggesting that they present in an operon. This is indicated by linked arrows to the right of the homology boxes. The occurrence of MexT in the gene neighbourhood of *PA4354*, *PA4355* or *xenB* orthalogous sequences was not detected, hence MexT is separated by a broken grey line above.

Table 4. Conservation of the MexT regulon among diverse species of *Pseudomonas*.

P. aeruginosa **PAO1**	***P. mendocina*** **YMP**	***P. stuzerei*** **A1501**	***P. entomophila*** **L48**	***P. syringe*** **1448A**	***P. fluorescens*** **Pf-01**	***P. putida*** **KT2440**
PA2493[a,b] **(*mexE*)**	Pmen2181	PST2166	PSEEN2312	PSPPH2273	Pfl012659	PP3425
PA4354	Pmen1121	PST1565	PSEEN1128	PSPPH4056	Pfl011284	PP0921
PA1970	-	-	PSEEN3318	PSPPH3154	Pfl013914	PP3519
PA2813	-	-	PSEEN1595	PSPPH1622	Pfl014182	PP1894
PA2759	-	-	-	-	Pfl013288	PP3014
PA4623	-	-	PSEEN4907	-	Pfl013748	-
PA1744	-	-	-	-	-	-
PA2486	Pmen2588	-	-	-	-	-
PA3229	Pmen1533	-	-	-	-	-
PA4881	-	-	-	-	-	PP4858

a Gene number from the Pseudomonas Genome Project [42].

b Grey highlighting indicates conservation of the MexT binding motif upstream of PAO1 MexT regulon and orthologous genes in diverse *Pseudomonas* species.

3.4.5 MexT is not the sole regulator of expression from the PA4354 promoter

The PA4354 repressor is itself an independent regulatory element within the MexT regulon. ArsR-family regulators typically repress expression from their own promoter regions from which they dissociate in the presence of inducing metals causing upregulation of the repressor as well as downstream, co-transcribed genes [43-45]. Hence both MexT and PA4354 could both act upstream of *PA4354* controlling expression from the *PA4354* promoter.

As many previously characterized ArsR-family regulators exhibit autorepression, the ability of PA4354 to repress expression from its own promoter was investigated. An IPTG inducible PA4354 overexpression construct, pME6032-4354, was introduced to PAO1 harbouring the pMP-PA4354p transcriptional reporter. Overexpression of PA4354 resulted in repression of expression from the *PA4354* promoter region suggesting that PA4354 is an autorepressor (Figure 11).

ArsR-family repressors are known to dissociate from their targets in the presence of inducing di- and multivalent heavy metal ions. The binding of diverse metals to ArsR-family members has been demonstrated and metal-sensory motifs which define their metal specificity have been characterized [37]. PA4354 exhibits significant sequence divergence from previously characterized ArsR repressors most notably at its N-terminal region where a sequence region which does not align with any characteristic ArsR-family representative sequences is present (Figure 9). This region precedes the only cysteine residue in PA4354

which are known to act as critical sensory residues in ArsR-family repressors [44,46]. This suggests that this region defines the specific sensory interaction of PA4354 with its cognate ligand. However, PA4354 lacks any of these previously characterized metal-specific binding sites which precludes bioinformatic prediction of putative cognate binding metals. Nonetheless, induction of expression from the *PA4354-lacZ* promoter fusion pMP-PA4354p in PAO1, in the presence of diverse metals was measured by a plate-based assay to investigate whether PA4354 was induced by metals. Results indicated that lead and tellurium induced expression from the *PA4354-lacZ* promoter fusion (Figure 10) culture. However, similar induction of *PA4354* expression in the presence of lead and tellurium was observed both in a *MexT* and *PA4354* deletion backgrounds suggesting that induction of *PA4354* is caused by yet other transcriptional regulators in addition to PA4354 and MexT. Overexpression of MexT in PAO1 had no effect on the MIC of tellurium which was 0.06mM for both PAO1(pME6032) and PAO1(pME6032-mexT) suggesting that MexT and by extension the *PA4354-PA4355-xenB* operon do not play a role in resistance to this metal. Determination of resistance to lead could not be determined due to the precipitation of lead salts in both M9 and LB media.

In support of the autoregulatory function of PA4354, expression from the *PA4354* promoter was higher in PAO1*ΔPA4354* and deletion of PA4354 also caused increased MexT-mediated induction of *PA4354* (Table 5). In addition, it was noted that expression from the *PA4354* promoter declines in stationary phase cultures and that this drop in expression exhibited independence on the PA4354 repressor (Table 5). Stationary phase repression of *PA4354* expression also exhibits independence on MexT as a decline in *PA4354* promoter activity is still observed even when MexT is overexpressed. In agreement with this, a separate experiment demonstrated that the basal level of *PA4354* expression observed in PAO1 (287 ± 15 Miller units) was comparable to that observed in PAO1*ΔmexT* (256 ± 30.46 Miller units) further suggesting that expression of the *PA4354-PA4355-xenB* operon is regulated by other transcriptional regulators in addition to PA4354 and MexT.

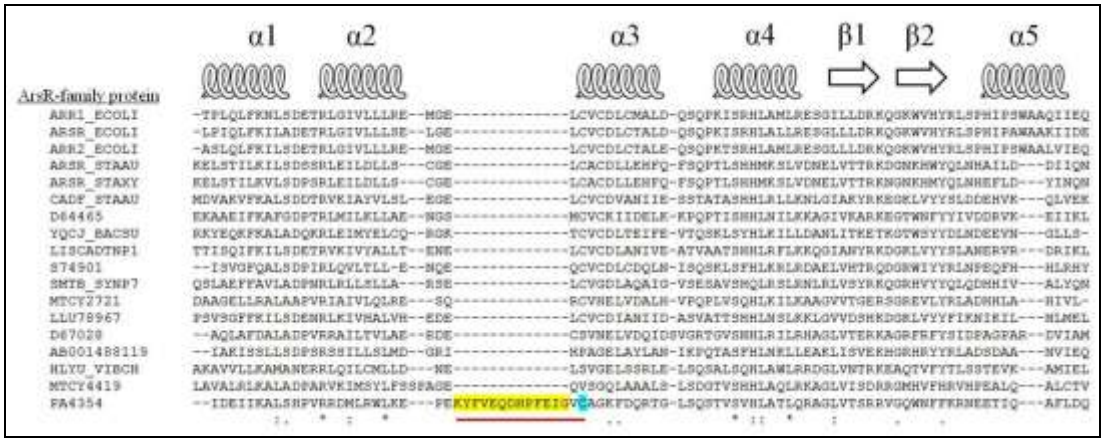

Figure 9. Alignment of PA4354 to the multiple sequence alignment of seed sequences which characterizes the ArsR-family (HTHARSR - Prints database). PA4354 exhibits sequence divergence at the highlighted region (yellow). This region does not align with other representative ArsR-family repressors. The predicted secondary structure of the ArsR-family repressors in the alignment (α1-α2-α3-α4-β1-β2-α5) is indicated above the alignment - springs indicate predicted α helical regions and arrows indicate predicted extended β-sheet regions. The region of sequence divergence in PA4354 precedes the only cysteine residue (blue) in PA4354 an may represent a unique sensory site (underlined in red).

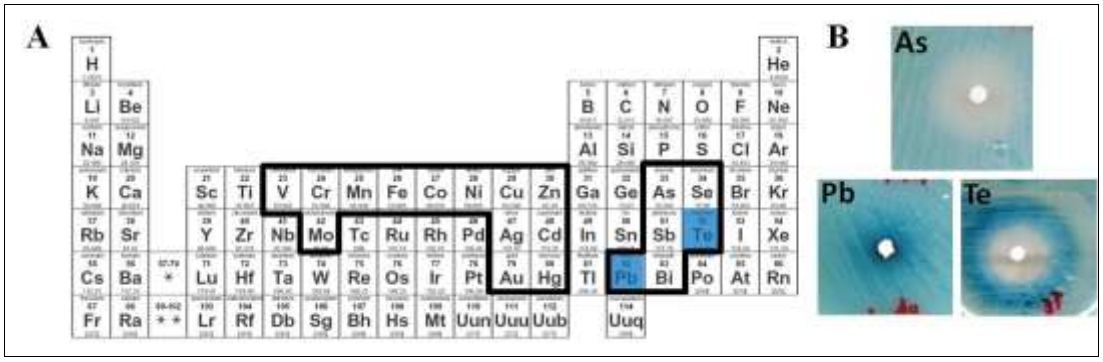

Figure 10. Induction of expression from the *PA4354* promoter region in the presence of diverse metals. The ability of diverse metals to induce expression from the pMP-PA4354p *lacZ*-promoter fusion was tested by plating PAO1 harbouring pMP-PA4354p on minimal media containing Xgal impregnated with filter paper disks containing diverse metals. **A** - metals tested in the screen are enclosed within a black outline in the periodic table. Metals which induced expression from the *PA4354* promoter are indicated in blue (Te and Pb). **B** - The induction of *PA4354* expression as indicated by blue ring around metal containing disks resulting from the breakdown of Xgal is illustrated for metal salts of Arsenic (As) which inhibits growth but does not induce expression of PA4354, tellurium (Te) which inhibits growth and induces expression of *PA4354*, lead (Pb) which does not inhibit growth but induces expression of *PA4354*.

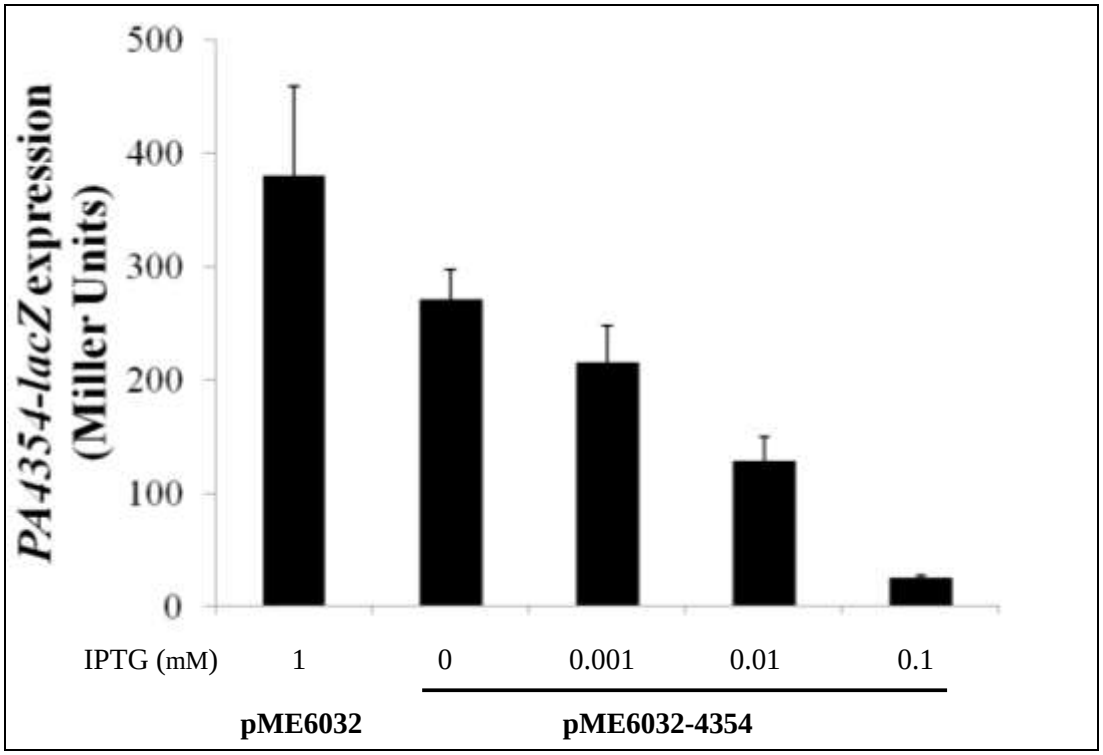

Figure 11. PA4354 exhibits autorepression. Into PAO1 harbouring a *PA4354* promoter-*lacZ* fusion, an inducible PA4354 overexpression construct (pME6032-4354) was introduced. Wild-type *PA4354* promoter activity was repressed on induction of PA4354 expression from pME6032-4354 in the presence of IPTG compared to the pME6032 vector control indicating that PA4354 is an autorepressor.

Table 5. Effect of *PA4354* deletion on *PA4354* promoter activity.

	Exponential[a]		Stationary[b]	
	PAO1	PAO1*ΔPA4354*	WT	PAO1*ΔPA4354*
pME6032	295 ± 54	468 ± 70	116 ± 9	154 ± 27
pME6032-mexT	1560 ± 188	2572 ± 33	560 ± 26	881 ± 75

a Exponential phase cultures (OD 600 = 0.4-0.7)

b Stationary phase cultures (OD 600 >2.5). Samples take at 24h timepoint

3.4.6 Characterization of the PA4354 autoregulatory site

As PA4354 was demonstrated to exhibit autorepression, this would suggest that the upstream region of PA4354 is likely to contain a conserved PA4354 regulatory site. To identify a putative autoregulatory site, PA4354 orthologues were retrieved from the database of prokaryotic operons (DOOR) [38]. These sequences were aligned and searched for the presence of a commonly conserved motif using the MEME algorithm [34]. Figure 12 illustrates the conservation of a putative PA4354 auto-

regulatory site which was highly conserved among 57 retrieved upstream regions from PA4354 orthologues.

To confirm the role of the identified PA4354 binding motif in PA4354 autorepression, site-directed mutagenesis of this region was performed. Mutation of conserved residues of the putative PA4354 autoregulatory site (Figure 12) present in the *PA4354-lacZ* promoter fusion pMP-PA4354p abrogated the ability of the PA4354 overexpressor (pME6032-4354) to down regulate expression from its own promoter (Figure 13). This demonstrates the importance of the identified cis-acting regulatory site in mediating repression from its own promoter by PA4354.

Direct binding of the PA4354 to its own promoter was confirmed by EMSA. A His-tagged PA4354 expression construct was generated (pET28a-PA4354H6C) and the PA4354 repressor was purified by nickel affinity column chromatography. The purified PA4354 protein caused a shift in the presence of labelled DNA amplified from the PA4354 upstream region. In contrast, no shift was observed in the presence of non-specific DNA probe containing no PA4354 motif amplified from the PA4881 upstream region (Figure 14). This demonstrated the direct and specific binding of PA4354 to its own upstream promoter region consistent with the autoregulatory function of the PA4354 autorepressor.

PA4354 exhibits characteristics typical of ArsR-family proteins including conserved primary sequence features, an autoregulatory function and presence in an operon. However, PA4354 also exhibits atypical sequence features, does not contain any characterized metal binding sites and does not appear to directly sense the presence of metals. PA4354 may therefore belong to a novel sub-family of ArsR regulators which are not responsive to metal ions.

As MexT is a positive transcriptional regulator, is not immediately clear how it downregulates nfxC-associated phenotypes. It was hypothesized that PA4354 may act globally, negatively regulating diverse transcriptional targets which contribute to MexT-associated phenotypes.

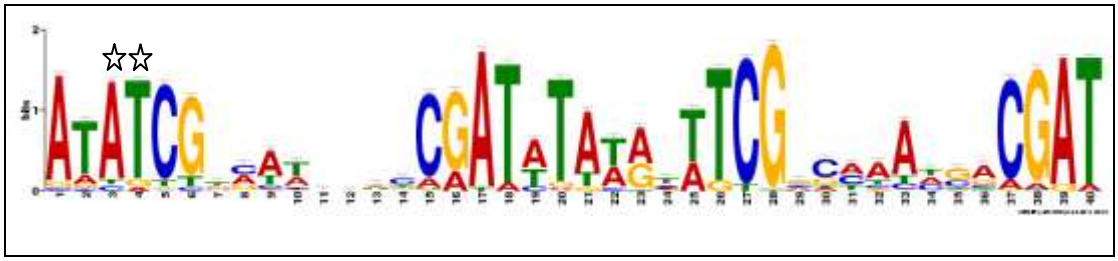

Figure 12. Position-specific consensus logo of the identified PA4354 autoregulatory binding motif conserved in the upstream regions of 57 *PA4354* orthologues from diverse bacterial species/genera. Conserved nucleotides targeted by site directed mutagenesis are indicated by stars.

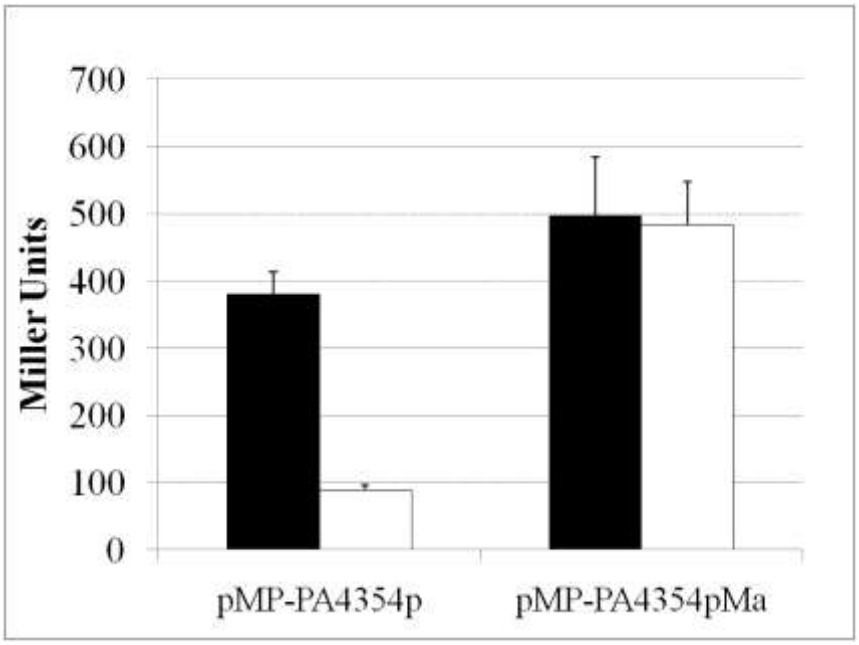

Figure 13. Site directed mutagenesis of the PA4354 binding site. An AT-GC change was introduced into the second and third conserved nucleotides of the PA4354 binding motif (Figure 12) of the PA4354 promoter-*lacZ* fusion pMP-PA4354p to yield the mutated promoter fusion pMP-PA4354pMa. Overexpression the PA4354 repressor (white bars) repressed promoter activity from pMP-PA4354p compared to the empty vector control (black bars) but not from the mutated promoter in pMP-PA4354pMa.

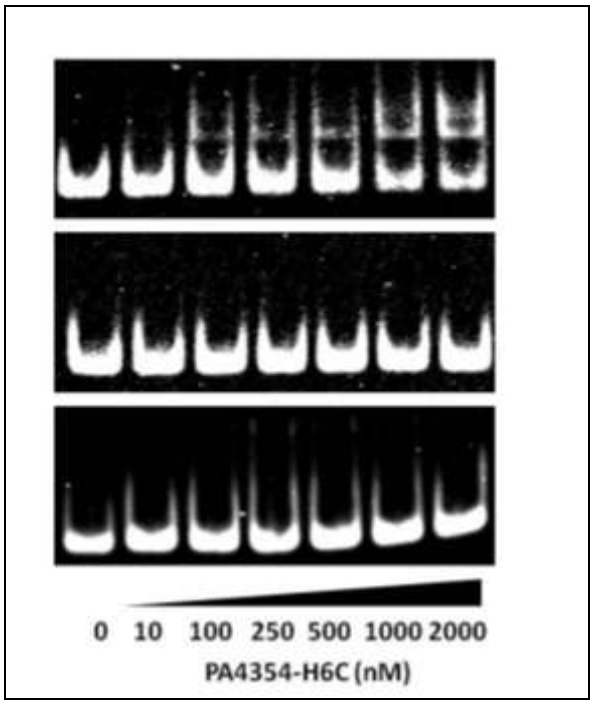

Figure 14: Demonstration of direct binding of PA4354 to its own promoter by EMSA. **Top lane**: His-tag-purified PA4354 causes a shift in the presence of the PA4354 promoter region. **Middle lane**: Control - protein isolation performed on E. coli harbouring pET28a empty vector- no shift. **Bottom lane**: no shift was observed with a non-specific DNA target amplified form the PA4881 upstream region in the presence of the purified PA4354 repressor protein.

3.4.7 PA4354 does not play a direct role in the downregulation of MexT-associated phenotypes

As PA4354 is the only regulatory protein identified in the MexT regulon it could explain how MexT modulates diverse phenotypes independent of MexEF-OprN such as imipenem resistance, pyocyanin production, adherence to polystyrene and the TTSS. If PA4354 has a global role in regulating diverse target genes then the identified PA4354 regulatory motif identified in the PA4354 promoter should also be present in the upstream regions of other PA4354 regulated targets. To investigate if the identified PA4354 binding site is present upstream of other genes in *P. aeruginosa*, a genome wide screen of the upstream regions of genes in the PAO1 genome was undertaken using the MEME-generated position specific scoring matrix which describes the PA4354 binding site (Figure 12) using the MAST motif alignment and search tool [39]. Candidates which were identified below a cut off E-value of 1 are illustrated in Table 6. The E-value represents the probability of obtaining an equally good match to the defined PA4354 position-specific scoring matrix as that observed, in a

database of random sequences equal in size to that searched (all PAO1 upstream regions). Hence the E-value reflects the likelyhood that a region matched to the position-specific scoring matrix is a true regulatory site. The specificity of the matches obtained for PA4354 and PA4353 were far in excess of other genes identified. The genes identified (PA4353, flgF, PA3236, PA3237) are not altered in the MexT transcriptome datasets which suggests that they are not part of the MexT or PA4354 regulatory cascades. The highly significant matches identified upstream of *PA4354* and *PA4353* represent the same sequence and occur due to the fact that *PA4353* is divergently transcribed relative to PA4354 and that the PA4354 binding site is palindromic. This is also true of the *PA3326* and *PA3237*. The matching motifs upstream of *flgF, PA3236* and *PA3237* have significantly higher E-values compared to *PA4354* and *PA4353* which is reflective of the weakness of these matches and the lower probability that *PA4354* specifically binds these regions (Table 6).

Table 6. Identified hits to the PA4354 binding motif identified in the upstream regions of genes in the PAO1 genome using the MAST algorithm.

Gene	E-value
PA4354	1.50E-08
PA4353	1.50E-08
flgF	0.47
PA3236	0.5
PA3237	0.5

Although no putative PA4354 regulatory targets which could explain the MexEF-OprN independent effects of MexT were identified bioinformatically, the role of PA4354 in mediating MexT-repressed phenotypes was investigated in order to rule out its direct regulation of MexT-repressed phenotypes.

Imipenem resistance has been demonstrated to occur in nfxC-type phenotypic mutants due to the negative regulatory effect on MexT on the outermembrane porin OprD which acts as a portal of entry for this carbapenem antibiotic [6]. To investigate whether MexT caused this negative regulation via PA4354, the effect of MexT overexpression on Imipenem resistance was assessed in a *PA4354* deletion mutant PAO1Δ*PA4354* compared to PAO1 wild type.

Deletion of *PA4354* did not abrogate MexT-mediated imipenem resistance suggesting that MexT mediates imipenem resistance independent of both MexEF-OprN and the PA4354 repressor. In support of this, overexpression of PA4354 had no effect on imipenem resistance in PAO1 (Table 7).

Table 7: PA4354 does not play a role in MexT-mediated imipenem resistance

Strain	Imipenem MIC (μg/ml)
PAO1 pME6032	0.25
PAO1 pME6032-mexT	4
PAO1ΔPA4354 pME6032	0.25
PAO1ΔPA4354 pME-mexT	4
PAO1 pME6032-4354	0.25

By a similar approach PA4354 was shown not to play a role in adherence to polystyrene which was identified as a MexEF-OprN independent MexT-mediated phenotype (Figure 15). MexT repressed pyocyanin production independent of the MexEF-OprN efflux system hence it is possible that PA4354 could mediate direct repression of this phenotype. However, overexpression of PA4354 had no effect on pyocyanin production suggesting that it is not involved in directly regulating pyocyanin production (Figure 16).

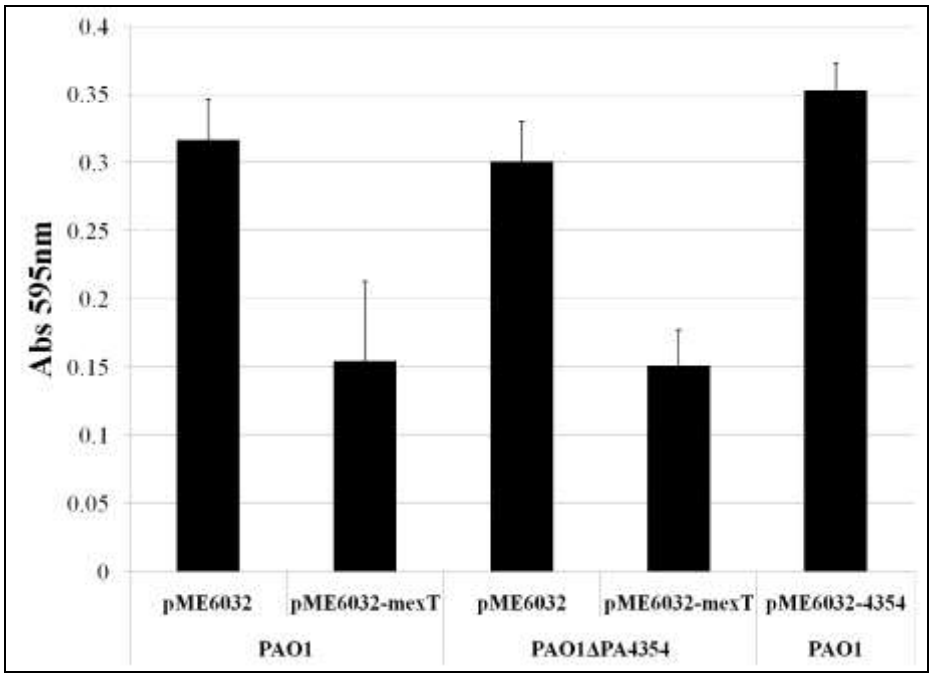

Figure 15. Deletion *PA4354* did not affect MexT-mediated impairment of adherence to polystyrene. Impaired attachment was observed in both PAO1 and PAO1*ΔPA4354* on overexpression of MexT from pME6032-mexT. In addition, overexpression of PA4354 did not impair attachment in PAO1.

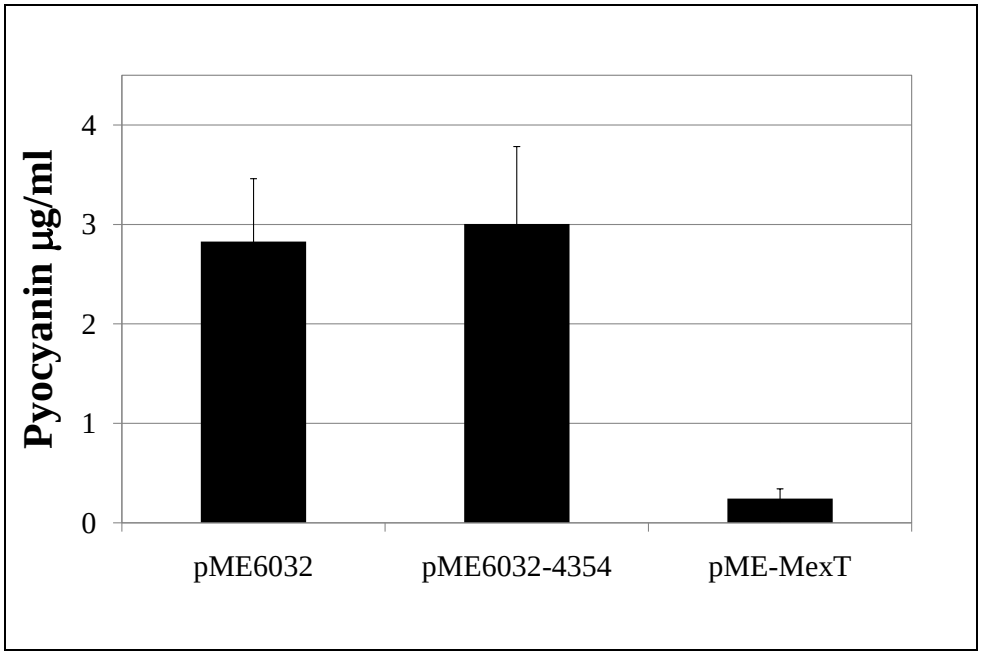

Figure 16. PA4354 does not represses pyocyanin production in PAO1. The level of pyocyanin in stationary phase culture supernatants on overexpression of the PA4354 repressor (pME-4354) was comparable to PAO1 harbouring the pME6032 vector control in contrast to the marked reduction in pyocyanin production observed on overexpression of MexT (pME6032-mexT).

Finally, to investigate whether PA4354 was responsible for MexT-mediated repression of the TTSS, the PA4354 overexpression construct was introduced into PAO1 chromosomally tagged with a *exoS-lacZ* promoter fusion construct. Although overexpression of MexT repressed induction of *exoS* expression under TTSS-inducing conditions, PA4354 overexpression had no observable effect on the type three secretion system (Figure 17). Hence it is concluded that although MexT mediates several nfxC-associated phenotypes independent of MexEF-OprN, the PA4354 repressor does not have a direct regulatory role in mediating these phenotypes.

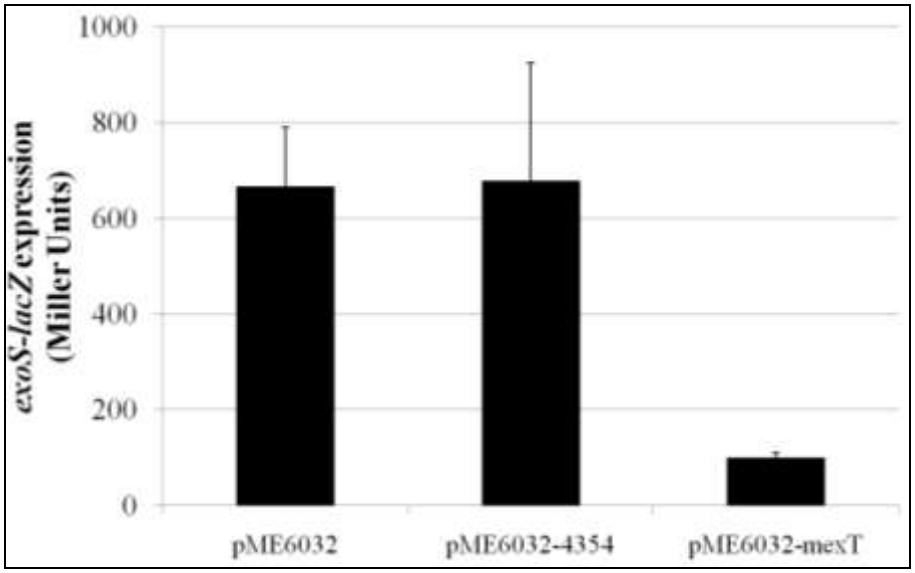

Figure 17. PA4354 does not inhibit induction of the TTSS in PAO1. The level of *exoS* expression in TTSS-inducing conditions on overexpression of the PA4354 repressor (pME6032-4354) was comparable to PAO1 harbouring the pME6032 vector control in contrast to the marked reduction in *exoS* induction observed on overexpression of MexT (pME6032-mexT).Expression of *exoS* is given in Miller units measured by β-galactosidase assay from a chromosomally inserted *exoS-lacZ* promoter fusion in PAO1.

3.5 Discussion

Many transcriptional regulators modulate the expression of antibiotic resistance genes. Mutations affecting such regulators are selected during antibiotic therapy resulting in the induction of resistance genes in clinical isolates. Global transcriptional regulators of antibiotic resistance can have pleiotropic effects on the cell resulting from their regulatory effect on multiple gene targets. Regulators which originally came to light as important mediators of antibiotic resistance such as AmpR and MarA have subsequently been demonstrated to have global regulatory functions [47-48]. In *P. aeruginosa*, MexT directly activates expression of the MexEF-OprN RND efflux system which is associated with antibiotic resistance and reduced virulence as demonstrated in this and other studies [14,22-23,25]. RND efflux pumps have been linked to virulence due to their ability to extrude host-secreted toxins such as bile salts, hormones, fatty acids, antimicrobial peptides and plant isoflavinoids [49]. This suggests RND pumps play a role in virulence as part of broader regulatory programs which co-ordinate infection [8,49]. The hypothesis that MexT acts as a global regulator in co-ordinating the expression of MexEF-OprN as part of a broader regulatory network involved in pathogenesis was investigated.

3.5.1 MexT is a global transcriptional regulator

Transcriptome analysis revealed that MexT directly activates expression of nine novel targets in addition to MexEF-OprN demonstrating for the first time the precise global regulatory scope of MexT. The novel MexT targets identified provide a possible mechanism by which MexT downregulates virulence determinants. Previous studies have implicated MexEF-OprN as the primary contributor to reduced virulence in nfxC-type phenotypic mutants due to its impact on the quorum sensing network which results in the reduced expression of virulence genes [14,22-23,25]. In it has been demonstrated that a subset of MexT-associated phenotypes are modulated by MexT independent of the MexEF-OprN efflux system and hence might be influenced by the newly identified genes of the MexT regulon.

Seven of these novel MexT targets identified are small hypothetical proteins of unknown function, six of which harbour type I or type II export signal sequences suggesting that they are secreted or cell envelope-associated proteins [50-51]. MexT also directly regulates two putative operons *PA2813-PA2812-PA2811* and *PA4354-PA4355-xenB*. Both of these operons have putative detoxification and transport functions. PA2811 encodes a protein belonging to the glutathione *s*-transferase family known to play a role in cellular detoxification. The *xenB* gene encodes an NADPH-dependent flavoprotein oxidoreductase of the old yellow enzyme (OYE)-family which has been characterized for its role in the transformation of toxic nitro-aromatic compounds including 2,4,6-trinitrotoluene in *Pseudomonas fluorescens* [52]. *PA2812-PA2813* and *PA4355* encode transporters of the ATP-binding cassette (ABC), and Major facilitator superfamily (MFS) transport families respectively which, in addition to RND-family transporters, have been implicated in virulence due to their protective roles against host toxins [8,49]. The *PA2813-PA2812-PA2811* and *PA4354-PA4355-xenB* operons could be co-regulated by MexT in concert with MexEF-OprN to protect the cell from host-secreted toxins during infection.

The presence of the PA4354 transcriptional repressor within the *PA4354-PA4355-xenB* operon adds another level of complexity to the MexT regulatory cascade and provides a possible regulatory mechanisms by which MexT mediates repression of its associated phenotypes independent of the MexEF-OprN efflux system. However, PA4354 does not play a direct role in regulating antibiotic resistance, repression of pyocyanin production, attachment or the TTSS. This suggests that other genes of the regulon mediate these MexT-associated phenotypes independent of both MexEF-OprN and the PA4354 repressor. In line with this hypothesis, recent work has demonstrated that the novel MexT target PA2486 (*ptrC*) identified in this study can mediate downregulation of the TTSS [53]. Although PA4354 was found not to be involved in MexT-associated antibiotic resistance or virulence phenotypes, the role of this novel regulatory component of the MexT regulon was further characterized.

3.5.1 PA4354 is a non-canonical ArsR-family regulator

The ArsR-family of metallo-regulatory transcriptional repressors are implicated in resistance to metals and are often co-transcribed with genes involved in metal resistance. They dissociate from their promoters on binding of their cognate di- and multi-valent metal ligands [44]. The metal-specific binding sites of diverse ArsR-family repressors have been characterized in detail and conserved metal binding motifs have been identified which allows prediction of the cognate metal ligands of ArsR repressors [37]. In line with the ArsR paradigm, PA4354 exhibits autoregulation and is transcribed as part of an operon. However, in contrast to characteristic ArsR metallo-repressors, PA4354 exhibits atypical sequence features, lacks a conserved metal binding site and appears not to play a direct role in metal perception or resistance. Several more recently described ArsR regulators exhibit divergent functions unrelated to metal resistance [46,54-56]. For example, the SdpR repressor of *Bacillus subtilis* which regulates cannibalistic behaviour, dissociates from its promoter via sequestration at the cell membrane by a cell membrane immunity protein [56]. The HlyU repressor of *Vibrio vulnificus* activates expression of its target by acting as an antirepressor of a H-NS protein which negatively regulates expression of the *rtxA1* toxin-encoding gene [57]. Based on bioinformatic and experimental data, PA4354 is likely to group with ArsR-family repressors which exhibit atypical functional characteristics from archetypal metal-sensing ArsR repressors.

Recently, the ArsR-family repressor CyeR of *Corynebacterium glutamicum* has been shown to be induced by oxidative and thiol-specific stress rather than the presence of metals and also regulates the OYE-family enzyme Cye1 which has homology to *xenB* [46]. This is interesting as both metals which were found to induce expression of *PA4354-PA4355-xenB* are implicated in oxidiative stress. Tellurium has been shown induce an thiol-specific stress response in *Pseudomonas pseudoalcaligenes* and the toxicity of lead to eukaryotic organisms is attributed to its elicitation of oxidative stress [58-59]. As induction of expression from the *PA4354* promoter was observed in both *mexT* and *PA4354* deletion backgrounds, it

is likely that the *PA4354-PA4355-xenB* operon is regulated by another unidentified regulator in response to metal-induced changes in the redox state independent of both MexT and PA4354. Although the redox-sensing function of CyeR of *Corynebacterium glutamicum* was demonstrated *in vitro*, in the *in vivo* study of CyeR induction the involvement of another regulator as observed here for *P. aeruginosa* was not ruled out. Hence induction *cyeR* and *cye1* might also involved another regulator in *C. glutamicum*. The *PA4354-PA4355-xenB* may have diverse functions within divergent regulatory pathways. The high level of conservation of *PA4354*, *PA4355* and *xenB* in divergent organisms suggests that it may function in detoxification or biotransformation processes which are widespread across diverse organisms.

3.5.2 Conservation of the MexT-PA4354 regulatory interaction

The induction of PA4354 by MexT is the first report of a LysR and ArsR-family co-regulating a common target operon. It has been noted that LysR regulators are overrepresented in Gram-negative lineages in contrast to ArsR-family regulators which are more abundant in the genomes of Gram-positive species [60]. This is certainly reflected in the genome of *P. aeruginosa* which harbours approximately 120 LysR regulators and only three proteins of the ArsR-family. Interaction between these families of transcriptional regulators is therefore less likely to occur as a result of their separate ancestries. MexT is conserved across diverse *Pseudomonas* species but is not broadly conserved outside this genus. This is in contrast to the genes of the *PA4354-PA4355-xenB* operon which are conserved in diverse bacterial genera as well as among eukaryotic and archaeal species. *PA4354*, *PA4355* and *xenB* are therefore likely to be regulated by other regulatory elements outside the *Pseduomonas* genus where MexT is not conserved. This suggests that MexT is ancestral to *Pseudomonas* species such as *P. aeruginosa* and that *PA4354-PA4355-xenB* has subsequently become incorporated into the MexT regulon. Even within the *Pseudomonas* genus, the regulatory interaction between MexT and PA4354 does not appear to be

conserved as PA4354 orthologues in *P. entomophila* L48, *P. fluorescens* Pf-01 and *P. putida* KT2440 do not harbour conserved MexT binding sites in there promoter regions. This suggests that the function *PA4354-PA4355-xenB* as part of the MexT regulon is specific to certain *Pseudomonas* species. Thus, *PA4354, PA4355* and *xenB* may be integrated into diverse regulatory pathways in diverse species where they may serve divergent physiological functions.

3.5.3 The regulatory scope of the PA4354

Upregulation of the PA4354 transcriptional repressor by MexT suggests that it could serve global regulatory functions beyond its local regulation of the *PA4354-PA4355-xenB* operon. However, the identified PA4354 binding site is not well conserved beyond the *PA4354* promoter region in PAO1. Furthermore, global transcriptome studies of other ArsR-family repressors KmtR and CyeR have demonstrated they that they are local repressors [46,61]. It is not immediately apparent why MexT should upregulate expression of a local repressor, however, the co-induction of local repressors and their associated operons is not uncommon. Many ArsR-family repressors are present in operon structures and hence are induced at the transcriptional level with their downstream metal resistance genes [43-45,62]. The MarR repressor is also induced as part of the MarRAB (multiple-antibiotic-resistance) operon in *E. coli* and both AcrR and MexR repressor are induced along with the AcrAB-TolC and MexAB-OprM in *E. coli* and *P. aeruginosa* respectively [63-64]. A possible explanation for the induction of these apparently local transcriptional regulators is that they may function as part of negative feedback pathways. Such a modulating role has been suggested for AcrR in its local regulation of *AcrAB* operon. AcrR is proposed to act as a secondary modulator which prevents excessive expression of the *AcrAB* operon which is induced by other global transcriptional activators [64-65]. A similar situation appears to have evolved in the case of MexT and the *PA4354-PA4355-xenB* operon, the expression of which appears to be primarily controlled by MexT and other unidentified transcriptional regulators as opposed to

PA4354. In the case of the *PA4354-PA4355-xenB* operon, the end product of the biosynthetic/degradative pathway catalysed by XenB might be toxic to the cell. The level of this hypothetical molecule might be sensed by PA4354 which could repress expression of *PA4354-PA4355-xenB* through a PA4354-mediated negative feedback loop.

3.5.4 Physiological function of the *PA4354-PA4355-xenB* operon

The function of XenB and the majority of described OYE-family proteins remains elusive [52,66]. Although XenB is known to catalyse the reductive removal of nitro-groups from electrophillic pollutants including 2,4,6-trinitrotoluene and nitro-glycerine, these are unlikely to reflect the natural substrates of XenB. The only OYE enzymes for which a precise physiological role as been delineated are those that belong to the 12-oxophytodienoate reductase (OPR) family which reduced 9S,13S-12-oxophytodienoate (9S,13S-OPDA) as part of the jasmonic acid biosynthesis pathway in diverse plant species [67-68]. It is possible that XenB also catalyses a specific biotransformation within a synthetic or biodegradative pathway rather than functioning as a general detoxifying enzyme. This is suggested for members of the OYE family due to their highly conserved structure which contradicts a general detoxifying function [66]. Although OYE enzymes have been know for over 70 years, a precise physiological function has been described only for the OPR family. Hence the delineation of a natural XenB substrate and function may prove challenging. However, the elucidation of macromolecular regulatory networks within which OYEs are embedded such as the MexT regulon and the *PA4354-PA4355-xenB* operon may provide clues as to the natural physiological roles of the members of the OYE family. The natural XenB substrate is likely to interact with PA4354 and PA4355 which may function in the perception and transport of the substrates or products of XenB.

Recently, MexT has been shown to induce the *PA4354-PA4355-xenB* operon in the presence of the nitrosative stress elicitors *s*-nitrosoglutathione **(**GNSO) and diethylaminetriamine NONOate (DETA). The experiments of Fetar and co-workers demonstrate that *xenB*

induction is dependent on the functional status of the MexEF-OprN system and suggest the XenB substrate or inducing compound is extruded by MexEF-OprN under conditions of nitrosative stress. Only when MexEF-OprN is disrupted does the putative XenB substrate/inducer accumulate to sufficient levels to induce the *PA4354-PA4355-xenB* operon under nitrosative stress. This suggests that other regulators must regulate the expression of *PA4354-PA4355-xenB* because, although nitrosative stress induces MexT activity (increasing expression of MexT targets *mexEF-oprN*, *PA2813-PA2812-PA2811*, *PA3229* and *PA4881*), expression of *PA4354-PA4355-xenB* is not induced [69]. This is in line with the data presented in this thesis which demonstrate that an unidentified regulator influences expression from the PA4354 promoter independent of both MexT and PA4354. The observation that MexT is activated in response to nitrosative stress further implicates the MexT regulon in infection as nitric oxide is produced by airway epithelial cells in the presence of bacteria [69-70]. Previous transcriptome analysis suggests that MexT is activated in response to human airway epithelial cells which suggests that MexT is a important in bacterial-host interactions [71]. In this context, MexT may induce expression of MexEF-OprN and the novel regulon to protect against natural host defences in tandem with the regulation of virulence in the co-ordination infection.

3.6 Conclusion

In addition to mediating resistance to anthropogenic antibiotics, efflux systems have been demonstrated to protect bacteria from host-secreted toxins [8,49]. This implicates RND efflux systems such as MexEF-OprN in bacterial-host interactions and would explain their integration into regulatory networks which co-ordinate infection. MexT has immediate clinical relevance as a global regulator of multidrug resistance but may also have an important role in the infection process [14,25]. In addition to MexEF-OprN, MexT also directly induces a novel regulon including the *PA4354-PA4355-xenB* operon which encodes a novel ArsR-family transciprional repressor which may also function in bacterial host interaction. The inverse regulation of MexEF-OprN and virulence is suggestive of a defensive bacterial response whereby efflux systems including MexEF-OprN, PA2813-PA2812 and PA4355 may be induced by MexT to protect from host toxins whilst immunostimulatory virulence factors are repressed in an attempt to avoid detection by the host. However, the precise role of the *PA4354-PA4355-xenB* operon within the MexT regulon remains to be elucidated. Given its conservation outside the *Pseudomonas* genus, the *PA4354-PA4355-xenB* operon may function as part of a broadly conserved physiological pathway which is related to bacterial-host interactions in divergent bacterial species.

3.7 References

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General Discussion

4.1 General Discussion

The increasing incidence of antibiotic resistance observed among Gram-negative bacteria at both the Mercy University Hospital (MUH) and Zonguldak Karaelmas University Hospital (ZKUH) reflects the emerging global problem of increasing antibiotic resistance. An increasing number of Gram-negative pathogens exhibit multidrug-resistance phenotypes globally including isolates which exhibit pan-drug resistance and as such are resistant to all known classes of antibiotics [1]. For antibiotic therapy to remain a viable therapeutic option, we must curtail the spread of resistance to currently efficacious antibiotics and develop novel antibiotic therapies as resistance to 'older' antibiotics gradually arises in clinical pathogens. Molecular investigation of emerging resistance mechanisms in nosocomial pathogens provides insights into the dissemination of resistance and the key strategies adopted by bacterial pathogens to circumvent antibiotic therapy. Such insights can inform infection control and disease management policies and can contribute to the development of innovative treatment strategies. This thesis has focused on characterizing the resistance mechanisms which have recently emerged in clinical isolates of important Gram-negative pathogens.

Multidrug-resistant (MDR) Enterobacteriaceae are becoming an increasing problem in Ireland as well as throughout Europe and carbapenem resistant *P. aeruginosa* isolates have become highly prevalent in the Mediterranean region particularly in Turkey and represent major health concerns [2-8]. Characterization of the resistance mechanisms in MDR Enterobacteriaceae and carbapenem-resistance *P. aeruginosa* from these geographic locations has revealed how antibiotic resistance is emerging at the molecular level in these pathogens. Further investigation of the regulatory mechanisms governing expression of multidrug efflux in *P. aeruginosa* has shed new light on the global regulatory role of MexT, providing further insight to its natural physiological function which is likely to expand beyond antibiotic detoxification [9-10].

4.1.1 The emergence of multidrug resistance among the Enterobacteriaceae in Ireland

MDR Enterobacteriaceae isolated from patients admitted to MUH harboured diverse Extended spectrum β-lactamase (ESBL) resistance genes. Given that the key selective force behind the emergence of resistance is antibiotic administration, this observation is not surprising. Ireland has a high rate of antibiotic usage compared to other European countries such as the UK where ESBL producers are already prevalent [8,11-12]. The increasing levels of resistance to broad-spectrum agents such as third generation cephalosporins, fluoroquinolones and aminoglycosides signifies that Ireland is approaching a critical point where many broad-spectrum agents will no longer suffice for the empiric treatment of common nosocomial infections. As the prevalence of multidrug resistant Enterobacteriaceae increases, so too will the burden they impose on the healthcare system in Ireland as well as globally.

4.1.2 Dissemination of transferrable resistance genes

The importance of horizontal gene transfer in the emergence of multidrug resistance is evident in MDR Enterobacteriaceae isolated at MUH where diverse horizontally acquired resistance genes were detected. The pace of microevolution allows nosocomial pathogens to adapt quickly to the challenge of antimicrobial therapy [13]. This is greatly accelerated by the phenomenon of horizontal gene transfer through which pathogens can acquire resistance genes of diverse chromosomal origin [13]. Diverse β-lactamase genes such as bla_{SHV-12} and $bla_{CTX-M-15}$ as well as the plasmid-mediated fluoroquinolone resistance genes *qnrA* , *qnrB* and *aac(6')IB-cr* which have not previously been reported in Irish clinical isolates were detected in MDR isolates at MUH. Resistance plasmids were identified which harboured both $bla_{CTX-M-15}$ and *aac(6')IB-cr* and conferred resistance to multiple antibiotics including third generation cephalosporins, aminoglycosides and fluoroquinolones illustrating their potential to transfer multidrug resistance to diverse strains. Recently, the sequence of diverse plasmids harbouring bla_{CTX-M} genes revealed the presence of *aac(6')Ib-cr* among other resistance

genes conferring resistance to up to eight antibiotic classes [14]. Thus, multidrug resistance in the majority of isolates identified at MUH can be attributed largely to the acquisition of multidrug resistance plasmids [5]. Under antibiotic selection, such multidrug resistance plasmids and the pathogenic strains to which they have been transferred are maintained and may accumulate resulting in their increases prevalence [13,15]. Hence the presence of multidrug resistance plasmids and their associated resistance genes contribute significantly to multidrug resistance in Irish isolates and are likely to be further disseminated resulting in their increased endemicity.

4.1.3 The contribution of mobile genetic element in the emergence of resistance

The integration of diverse resistance genes into transferrable plasmids is crucial to the rapid emergence of resistance [15]. Many globally disseminated resistance genes have been mobilized from the chromosomes of diverse species through their association with mobile genetic elements [15-17]. In two *E. coli* strains isolated at MUH, $bla_{CTX\text{-}M\text{-}9}$ was identified on a plasmid where it was associated with an ISCR mobile element which is likely to have had a key role in the global dissemination of this ESBL-encoding gene [18]. It is noteworthy that many resistance genes have their origin in non-pathogenic species reflecting the wide gene pool which can be accessed by nosocomial pathogens in the development of resistance [15,17]. The $bla_{CTX\text{-}M\text{-}9}$ gene is almost certain to have been mobilized from the chromosome of *Kluyvera* species prior to its global dissemination in pathogenic *E. coli* [18]. It has been demonstrated that the mobile genetic element IS*Ecp1B* can facilitate the transfer of the chromosomal $bla_{CTX\text{-}M\text{-}2}$ gene of *Kluyvera ascorbata* to *E. coli* via its integration into a transferable plasmid [19]. This demonstrates three crucial genetic events in the emergence of resistance via horizontal gene transfer: (i) the association of mobile genetic elements with resistance genes which facilitates their mobilization, (ii) the integration of such resistance genes into transferrable resistance plasmids and (iii) the transfer of resistance-conferring plasmids to clinical pathogens. Once efficient resistance genes become mobile via their

integration into transferrable genetic entities, their dissemination into prevalent pathogenic strains can facilitate the progression toward endemic or even pandemic resistance to an antibiotic class [15,17,20].

4.1.4 Emergence of resistance in prevalent strain types

A key event in the emergence resistance is the acquisition of multidrug resistance plasmids by 'successful' pathogenic strains which serve as vehicles for the dissemination of resistance [21]. As multidrug resistance plasmids are acquired by diverse pathogenic strains including both nosocomial and community-acquired isolates, resistance becomes more widespread. In addition, diverse plasmids can accumulate resistance genes and hence resistance mechanisms are disseminated from chromosome to plasmid by association with mobile genetic elements and from strain to strains by the transfer of plasmids. Eventually resistance becomes endemic and unrelated MDR strains of diverse environmental origin become prevalent in community-acquired as well as nosocomial infections. Among Irish Enterobacteriaceae isolated at MUH, ESBL genes (including $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}12}$ which were detected in clonally unrelated strains) appear to be endemic. The twelve $bla_{CTX\text{-}M\text{-}15}$ producing *E. coli* isolates which exhibited highly similar typing profiles could represent the a pathogenic *E. coli* strain prevalent to this region which has acquired $bla_{CTX\text{-}M\text{-}15}$. At a national level, an endemic situation regarding $bla_{CTX\text{-}M}$ producing *E. coli* appears to have emerged in Ireland since they were first reported in 2000 [2]. Hence it has taken approximately ten years from initial detection to widespread dissemination of this ESBL in Irish *E. coli* isolates where the current rate of cephalosporin resistance is now 6.5% [7].

4.1.5 Carbapenem resistance in Irish Enterobacteriaceae

Most ESBL-producing organisms are treatable with other broad-spectrum agents such as the carbapenems which are not susceptible to hydrolysis by ESBLs. However, the recent emergence of the $bla_{NDM\text{-}1}$ carbapenemase among members of the Enterobacteriaceae may

signal the next pandemic wave of antibiotic resistance [22]. Unfortunately the bla_{NDM-1} gene has recently been detected in clinical isolates in Northern Ireland [23]. By analogy with the emergence of CTX-M-family ESBLs in Ireland, in approximately ten years we may predict significant increases in carbapenem resistance among Irish Enterobacteriaceae. In addition, other carbapenemase resistance genes including bla_{KPC-2} and bla_{VIM-1} have already been reported in Irish *Klebsiella pneumoniae* isolates which may accelerate the emergence of carbapenem resistance should strains producing these carbapenemases become established [24-25]. Although carbapenem-resistant strains can be treated with alternative antibiotic therapies, their increased prevalence can cause treatment failure due to inappropriate empirical therapy [26-27]. An increase in carbapenem resistance among the Enterobacteriaceae would have negative clinical implications and efforts should be made to maximize infection control to limit the dissemination of carbapenemase producing organisms and to curtail the use of carbapenem antibiotics. However, given that ESBL-producing organisms have become prevalent in Ireland, one of the consequences will be increased administration of carbapenem antibiotics for their treatment. This will further encourage the selection of strains harbouring bla_{NDM-1}, bla_{KPC-2}, bla_{VIM-1} or other carbapenem resistance mechanisms.

4.1.6 Emerging carbapenem resistance in *P. aeruginosa*

In contrast to MDR Enterobacteriaceae in Ireland and globally, non-fermentative Gram-negative pathogens such as *P. aeruginosa* are not reliant on transferable resistance mechanisms in the development of broad-spectrum antibiotic resistance [28-32]. This was reflected in the study of imipenem-resistant *P. aeruginosa* isolates from ZKUH where no known transferrable carbapenem resistance genes were identified. The emergence of transferrable carbapenemase genes in *P. aeruginosa* has been a concern since their first identification but they have not disseminated widely in this species by comparison with the emergence of ESBL-mediated cephalosporin resistance among the Enterobacteriaceae [33]. Rather than acquiring resistance via horizontal gene transfer, carbapenem resistance in *P.*

aeruginosa isolates from ZKUH resulted from native chromosomally resistance mechanisms. *P. aeruginosa* can develop broad resistance profiles through chromosomally encoded resistance mechanisms alone [34]. Nonetheless *P. aeruginosa* strains harbouring transferrable carbapenemase resistance genes have been responsible for major outbreaks and their emergence remains a threat [35-36].

There is a strong correlation between antibiotic consumption and the emergence of resistance, and the level of antibiotic resistance in *P. aeruginosa* is largely dependent geographic location [33]. Geographic locations which have a high rate of broad-spectrum antibiotic administration such as the Mediterranean region are hotspots for multidrug resistant infections [3]. The primary mechanism of carbapenem resistance identified in *P. aeruginosa* in our investigation was mutational inactivation of the OprD porin which compromises entry of carbapenems to the cell. Globally, this appears to be a common mechanism of carbapenem resistance in *P. aeruginosa* and given the clonal diversity of the imipenem resistant *P. aeruginosa* isolates, mutational deactivation of *oprD* has occurred independently in diverse strains. The observation of *oprD* disruption in diverse strains suggests that *oprD* deficient strains are becoming increasingly endemic in Turkey in response to high rates of broad-spectrum antibiotic consumption. Although rates of carbapenem resistance in *P. aeruginosa* isolates from Ireland are lower than those observed in Turkey, increased consumption of broad-spectrum agents due to the emergence of ESBL producing bacteria may promote a similar increase among *P. aeruginosa* isolates as observed in Turkey.

Other structural analogues of imipenem such as meropenem and doripenem have been developed which are less affected by mutational loss of *oprD* in *P. aeruginosa*. However, in contrast to imipenem these agents have an associated disadvantage in that they can be extruded by the diverse multidrug efflux systems of *P. aeruginosa* [37-38]. Thus diverse chromosomal resistance mechanisms present in *P. aeruginosa* as well as other Gram-negative pathogens limit the efficacy of antibiotic therapy. The upregulation of RND efflux

systems in non-fermentative species such as *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia* spp. is frequently observed in MDR clinical isolates [30,39-42].

4.1.7 The diverse roles of RND efflux systems in *P. aeruginosa*

Among MDR *P. aeruginosa* isolates, induction of diverse chromosomal RND efflux systems is a commonly observed resistance mechanisms [34]. Although the majority of described efflux systems in *P .aeruginosa* have the ability to extrude diverse antibiotics, some pumps appear to be more frequently induced compared to others in response to antibiotic selection which may reflect their physiological roles independent of antibiotic resistance. For example, the MexAB-OprM and MexXY efflux systems are commonly found to be induced in resistant isolates whereas induction of the MexEF-OprN and MexCD-OpmJ systems is less frequently observed [43-47]. None of the imipenem-resistant *P. aeruginosa* isolates from our study expressed the MexEF-OprN efflux system even though MexT-mediated induction of this pump would confer multidrug resistance including increased resistance to imipenem [38]. The frequent induction of MexAB-OprM and MexXY suggest they serve general detoxification functions suited to the efflux of exogenous compounds whereas other pumps such as MexEF-OprN may be embedded in native physiological processes and hence impart a greater fitness burden if induced by mutation. In line with this, the MexEF-OprN and MexCD-OprJ efflux systems cause reduced expression of virulence determinants when induced in *P. aeruginosa* [48-50]. This could explain their infrequent upregulation in clinical isolates due to their negative impact on pathogenicity. It has been suggested that RND efflux systems are linked to virulence due to their putative roles in protecting against host secreted toxins and their impact on the level of virulence-related signalling molecules [51-57]. This would explain their regulation by global transcriptional regulators which integrate them into broader physiological processes such as infection. Efflux pumps have been targeted as a therapeutic strategy to counteract multidrug resistance [57]. Given that efflux systems may also have a central role in virulence, their attractiveness as a therapeutic targets is increased as

inhibition of efflux could not only counteract efflux-mediated resistance, but also sensitize pathogens to host defence molecules and disrupt the co-ordination of infection.

The elucidation of regulatory networks associated with RND efflux systems thus provides insight to their natural physiological function which can broaden the scope for novel therapeutic strategies. A number of resistance mechanisms, particularly RND efflux systems, have been demonstrated to be under complex transcriptional regulation including by global transcriptional regulators [9,57-63].

4.1.8 The function of MexT as a global regulator

MexT induces the MexEF-OprN RND efflux system causing antibiotic resistance but also the downregulation of multiple virulence-associated phenotypes [10]. This pleiotropic shift in physiology could play a significant role in pathogen-host interactions. The role of MexT in infection is supported by the induction of MexEF-OprN in response to human airway epithelial cells as well as nitrosative stress (which is encountered during infection) and the interfacing of other global regulators of virulence with MexT [64-67]. The role of MexT in the context of infection could be interpreted as a stealth function whereby MexT coordinates downregulation of virulence to avoid detection by the immune system and increases efflux to offer protection from host secreted toxins. Alternatively, MexT may coordinate an exit strategy as the pathogen no longer seeks to cause damage to the host (downregulation of virulence factors) and adopts a more planktonic mode of growth in an attempt to disseminate from the infection site (reduced attachment phenotype). The identification of a novel MexT regulon provides insight into the putative physiological processes coordinated by MexT. Many of the direct regulatory targets of MexT are uncharacterized proteins of unknown function which may be involved in novel aspects of pathogen-host interactions. The MexT regulon is an important example of the integration of efflux and virulence via a global transcriptional network which may represent a common theme among bacterial pathogens.

Investigating the function of the newly identified targets of MexT may reveal novel aspects of pathogenesis relevant to the treatment of *P. aeruginosa* infections.

4.1.9 MexT-mediated regulation of the novel ArsR-family regulator PA4354

One of the novel targets of the MexT regulon characterized in this thesis is the PA4354 transcriptional repressor. This is an interesting regulatory interface as these regulators appear to have divergent ancestries: ArsR-family regulators are overrepresented in Gram-positive bacteria whereas LysR regulators exhibit increased prevalence among Gram-negative bacteria [68]. Hence, the transcriptional interaction between these regulatory families is likely to be rare and is, to our knowledge, the first report of a LysR-ArsR interaction.

The ArsR-family of transcriptional repressors is a large family and many members may have been demonstrated to bind and function in detoxification of diverse metals [69-70]. The archetypical founding members of the ArsR-family include the ArsR repressor of *E. coli,* ZiaR of *Synechocystis* spp., CadC and CzrA of *Stapylococcus aureus* and NmtR of *Mycobacterium tuberculosis* (Figure 1) [69]. All are autoregulatory proteins which dissociate from their promoters in the presence of their cognate metals [69,71]. More recently, variations on the ArsR-paradigm have become evident among ArsR-family repressors which do not exhibit typical ArsR-family characteristics. Such ArsR-family repressors including PA4354 may represent subfamilies within the ArsR-family which have associated functions divergent from metal resistance or homeostasis (Figure 1).

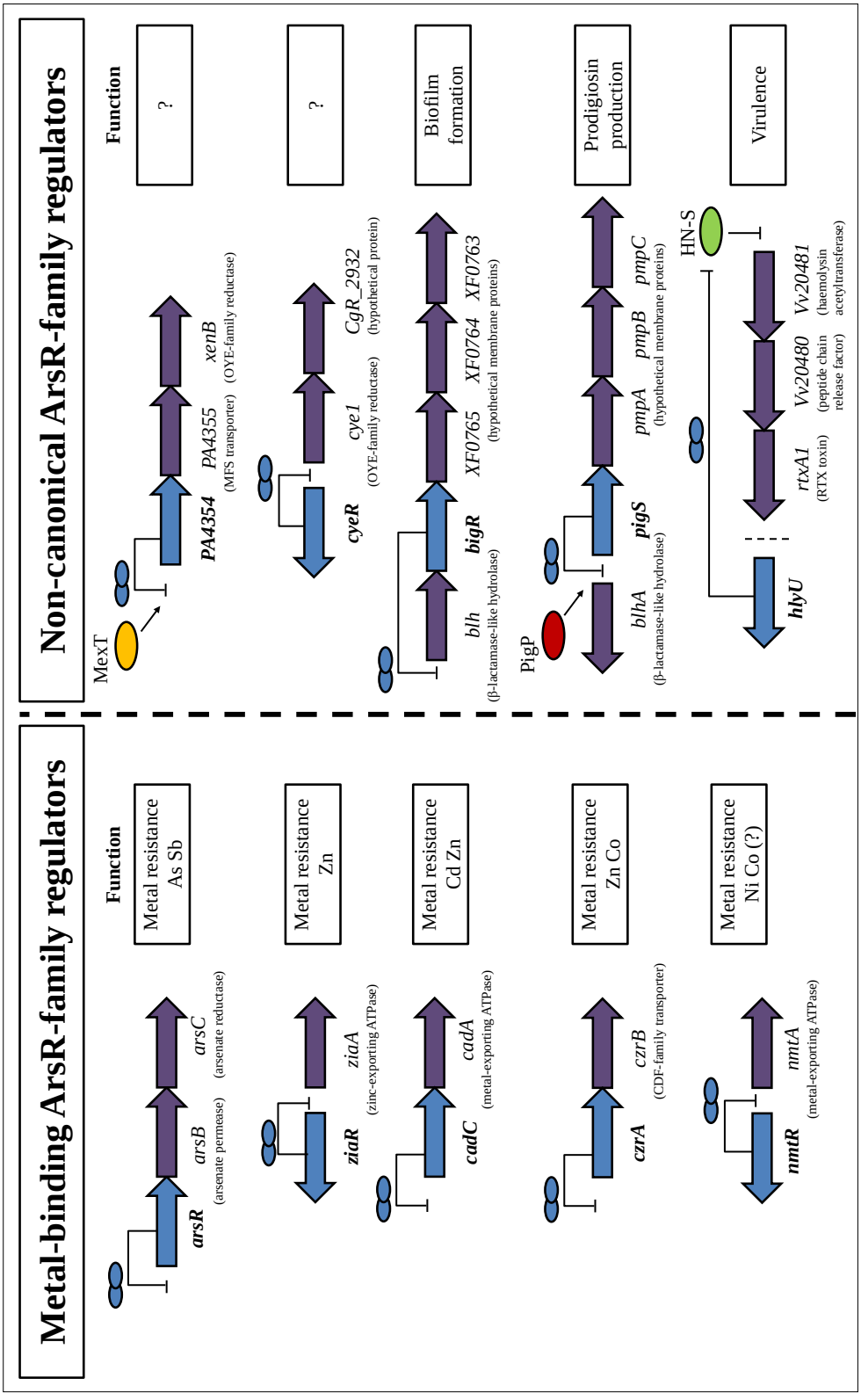

Figure 1. Regulation and function of ArsR-family regulators. Blue arrows represent genes encoding ArsR-family regulators. Blue ellipses represent the encoded repressors and their regulatory sites are indicated by black lines. Genes regulated by ArsR repressors are indicated in purple. Other regulatory elements controlling non-canonical ArsR-regulated operons are indicated by coloured ellipses. The metal resistance function of *nmtA* is inferred by homology rather than experimental evidence.

4.1.10 Emerging functions associated with non-canonical ArsR-family regulators

In contrast to canonical metal-responsive ArsR-family repressors, PA4354 regulates an operon which does not contain metal reductases, metallothionins, or metal efflux pumps and does not appear to function in metal detoxification. This is a characteristic shared by other non-canonical ArsR-family repressors including CyeR of *Corynebacterium glutamicum*, BigR of *Xylella fastidiosa*, PigS of *Serratia* spp., and HlyU of *Vibrio vulnificus* which are not associated with metal resistance operons and function in processes divergent from metal resistance (Figure 1) [72-75]. Although tellurium and lead induce expression from the *PA4354* promoter, this occurs independent of the PA4354 repressor and is possibly an indirect effect due to oxidative stress elicited by these metalloids/metals [76-77]. The CyeR repressor of *Corynebacterium glutamicum* has been implicated in oxidative stress and also controls expression an old-yellow-enzyme (OYE) family reductase Cye1 with homology to XenB. OYE-family enzymes have been implicated in oxidative stress and hence redox signals rather than metals may be sensed by a subset of ArsR-repressors including CyeR and PA4354. Although it has been demonstrated that CyeR can sense changes in the redox state, no specific function or phenotype has been attributed to this repressor or its regulatory targets. In contrast, other non-canonical ArsR regulators have been demonstrated to play a role in diverse physiological phenomena including biofilm formation, pigment production and virulence (Figure 1). Less is known however about the cognate inducing ligands of these non-canonical repressors compared with canonical ArsR-family repressors for which direct binding to diverse metals has been demonstrated [69,73-75,78-79].

4.1.11 Non-canonical ArsR-family repressors may recognise non-metal ligands

The characterization of metallo-regulatory ArsR-family repressors has allowed identification of conserved metal-binding motifs which allows prediction of cognate metal ligands of diverse ArsR repressors [71,80]. Similarly, more in depth characterization of non-canonical ArsR repressors may identify conserved non-metal sensory binding sites which would allow

prediction of ligand specificity of non-canonical repressors within the ArsR-family. PA4354 is highly conserved in diverse bacterial genera and commonly associated with XenB and PA4355 orthologues. As metals are the substrates of the metal-resistance machinery regulated by metallo-regulatory ArsR repressors, it seems logical that the substrates of XenB and PA4355 could represent the ligand sensed by the PA4354 repressor. If this is the case then the ligand-specific binding sites of non-metal binding ArsR-family repressors may also be ellucidated through characterization of representative repressors. Identification of specific ligand binding sites is important as sequence identity does not represent a robust predictor of ligand specificity among the ArsR-family where diverse metal-specific binding motifs are dispersed among repressors with low sequence similarity [71,80]. Metal-binding motifs have shown better predictive power compared to sequence similarity in determining the cognate metal ligands of ArsR-repressors [71,80]. The identification of ligand-predictive motifs among non-metal binding ArsR-repressors would greatly contribute to the functional characterization of ArsR-family repressors. Thus, a key challenge in the characterization of this large family of transcriptional repressors is to uncover the identity of non-metal ligands of non-canonical ArsR-repressors. This can be guided by analysis of the associated operons which are regulated by non-canonical repressors as well as global regulators which integrate ArsR-repressors into coordinated gene networks which provides insight to their physiological function.

4.1.12 Integration of ArsR repressors into global regulatory networks.

Some of the archetypical founding members of the ArsR-family such as ArsR of *E. coli* are plasmid located and have been studied in isolation rather than as integrated components of transcriptional regulatory networks. However, work presented in this thesis and in other recent studies have highlighted the integration of ArsR-family regulators into broader regulatory networks where their function is likely to expand beyond metal resistance and homeostasis. Analogous to PA4354, the ArsR-family regulator PigS of *Serratia* spp. is an

integrated component of the PigP regulon which regulates production of the pigmented antibiotic prodigiosin. Hence, ArsR-family repressors do not necessarily function in isolation, responding to the presence or absence of metals, and can be into complex regulatory pathways. The ArsR-family repressor HlyU of *Vibro vulnificus* controls expression of the RtxA1 toxin by acting as an antirepressor of the histone-like nucleoid structuring protein H-NS. H-NS is a global regulator which mediates transcriptional silencing of virulence genes including *rtxA1* [75,81]. Thus HlyU interfaces with the global H-NS network and is implicated in virulence, further adding to the number of physiological phenomena influenced by ArsR-family repressors (Figure 1) [74-75]. The characterization of PA4354 presented in this thesis contributes to knowledge on the emerging non-canonical repressors within the ArsR family of transcriptional regulators. The PA4354 repressor does not have a direct regulatory role in any of the characterized MexEF-OprN independent antibiotic resistance or virulence phenotypes regulated by MexT and is a local regulator of the *PA4354-PA4355-xenB* operon. XenB encodes an enzyme of the OYE-family of reductases which are known to be involved in oxidative stress pathways [82]. Hence the *PA4354-PA4355-xenB* operon may be involved in ancillary biotransformations involved in redox balance under the global physiological changes mediated by MexT.

4.2 Concluding remarks

Multidrug resistance is an emerging problem in bacterial pathogens which encourages investigation of the detailed molecular interactions which define antibiotic resistance. This can shed light on the regulatory pathways within which resistance mechanisms are embedded which ultimately leads to a more concise understanding of the natural physiological roles of antibiotic resistance mechanisms. This provides scope for the development of innovative therapeutic strategies to circumvent the resistance mechanisms which continue to evolve in bacterial pathogens.

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