


A research project report submitted by

Danielle Scales

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Institute of Microbiology and Infection, University of Birmingham, UK.

Centre for Biomolecular Sciences, University of Nottingham, UK.

Under the supervision of:

Professor Steve Busby and Dr. Steve Atkinson

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Abbreviations

ABC:	ATP-Binding Cassette
AME:	Aminoglycoside Modifying Enzyme
AMR:	Anti-Microbial Resistance
ATP:	Adenosine Tri-Phosphate
BCG:	Bacillus Calmette–Guérin
BHI:	Brain Heart Infusion
CAT:	Chloramphenicol AcetylTransferases
DAP:	Diaminopimelic acid
DNA:	DeoxyriboNucleic Acid
EDTA:	Ethylenediaminetetraacetic acid
ESBL:	Extended Spectrum Beta Lactamases
ESKAPE:	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*
HIV:	Human Immunodeficiency Virus
HPV:	Human Papillomavirus
HTS:	High-Throughput Screening
KPC:	*K. pneumoniae* carbapenemases
LB:	Luria-Bertani
LPS:	Lipopolysaccharide
LR:	Log_2 likelihood ratio
NAG:	*N*-acteylglucosamine
NAM:	*N*-acetylmuramic acid
NGS:	Next Generation Sequencing
NHS:	National Health Service
MDR:	Multi-Drug Resistant
MIC:	Minimum Inhibitory Concentration
MMR:	Measles, Mumps and Rubella
mRNA:	messenger Ribosomal ribonucleic Acid
MRSA:	Methicillin-Resistant *Staphylococcus aureus*

OD_{600}:	Optical density at a wavelength of 600 nm
Omp:	Outer membrane porin
PCR:	Polymerase Chain Reaction
PBP:	Penicillin Binding Protein
PMQR:	Plasmid-Mediated Quinolone Resistance
PBS:	Phosphate Buffered Saline
PTC:	Peptidyl-Transferase Centre
pYV:	*Yersinia pseudotuberculosis* virulence plasmid
QRDR:	Quinolone Resistance Determining Region
RLU:	Relative Light Unit
RND:	Resistance/Nodulation/sell Division
RPM:	Revolutions per Minute
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
T3SS:	Type III Secretion system
TraDIS:	Transposon Directed Insertion-site Sequencing
Tn:	Transposon
tRNA:	transfer Ribosomal ribonucleic Acid
UTI:	Urinary Tract Infections
UV:	Ultra-Violet
VISA:	Vancomycin Intermediate *Staphylococcus aureus*
VRE:	Vancomycin Resistant Enterococci
VRSA:	Vancomycin Resistant *Staphylococcus aureus*
YLB:	Yersinia Luria-Bertani
Yop:	Yersinia outer protein
WHO:	World Health Organisation
WT:	Wild-Type

ABSTRACT

By 2050, it is expected that antimicrobial resistance (AMR) will overtake cancer to become the world's biggest killer. As the pharmaceutical industry spends less on antibiotic research and development, universities have an increasing role to play. As part of this degree, my two research projects approach AMR from different angles. The first takes a traditional approach to AMR, and uses transposon mutagenesis to identify novel novobiocin resistance mechanisms in *Klebsiella pneumoniae*. The second takes a genotypic approach and explores the application of anti-virulence drugs, which inhibit IscR in *Yersinia pseudotuberculosis* to disarm rather than kill the bacteria. Diversification is essential for the development of novel antimicrobial strategies.

INTRODUCTION

Introduction to Anti-Microbial Resistance (AMR)

Antibiotics form the foundation upon which modern medicine was built. Their impact is such that there has been significant demographic transition in which infectious diseases are no longer responsible for the majority of deaths, infant mortality and low life expectancy in western societies. Instead, most deaths are now attributable to lifestyle diseases including heart disease and diabetes. Such is our dependency on their use, that it is difficult to imagine a world without antibiotics. A world in which cancer patients are likely to contract infections following chemotherapy, and where even routine surgery carries a high mortality risk: particularly at the extremes of age where the risk of infection would likely outweigh the benefit to the patient. A world where HIV and other infectious diseases become death sentences once again, and where even child birth carries an increased risk of complications. Sadly, this is the future we face unless we are able to overcome antimicrobial resistance (AMR).

AMR is the process by which bacteria, fungi, viruses and parasites are able to survive exposure to a drug concentration that would normally result in the death of that organism. Given their large population sizes and rapid rate of reproduction, it is probable that genetic mutations spontaneously occur that give the organism a competitive advantage under these conditions. Organisms can also acquire resistance genes from other bacteria, which can

spread rapidly throughout a population. When susceptible bacteria die off in the presence of the antibiotic, resistant organisms are able to survive and thrive in the absence of competition. AMR represents a significant threat to human health as multi- and totally- resistant infections are increasingly common, therapeutic treatment options are limited, and patient prognosis is poor as a consequence.

AMR is not a new phenomenon. Sir Alexander Flemming predicted the rise of antimicrobial resistance during his Nobel Prize winning speech in 1945 for the discovery of penicillin, where he stated, "The microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and perhaps from there to others until they reach someone who gets a septicaemia or a pneumonia which penicillin cannot save" (Bartlett *et al*., 2013). In recent times, AMR has become synonymous with methicillin-resistant *Staphylococcus aureus* (MRSA), however since the NHS launched the *Clean Your Hands* campaign in 2004, basic improvements in sanitization have seen MRSA levels fall (MRSA Action UK, 2017). Presently, the emergence of multi-drug resistant (MDR) ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) is of considerable concern (Rice, 2008). The number of deaths from AMR is estimated to be in excess of 700,000 per annum, though a recent review funded by the UK government and the Wellcome Trust estimates that this number will exceed 10,000,000 by 2050 if urgent action is not taken (O'Neill,

2016). The report predicts that AMR will soon overtake cancer to become the biggest killer globally. The cost of the morbidity and mortality associated with lengthy hospital stays, complex treatment plans and lost working days is estimated to be $100 trillion. Given the economic impact, world leaders have at last resolved to tackle AMR: In 2014, the WHO quantified the spread of AMR for the first time and released their *Antimicrobial Resistance: Global Report on Surveillance* (World Health Organisation, 2014). In 2015, the White House released their own strategy for combating AMR, *The National Action Plan for Combating Antibiotic-Resistant Bacteria* (The White House, 2015), and in 2016 the United Nations General Assembly discussed a health issue for only the fourth time in its history, and committed to tackle AMR.

Such focus is long overdue. The vast majority of antibiotics in use today were developed in the 1950's and 1960's: the so-called 'golden era' of antibiotic discovery. Since then, the rate of drug discovery has slowed for various reasons. Firstly, it is increasingly difficult to find new compounds which are effective against both Gram-positive and Gram-negative bacteria. Broad-spectrum antibiotics remain the gold standard, as there is little demand for more species-specific antibiotics in the absence of rapid diagnostic tools. Traditional isolate identification and antibiotic sensitivity testing can take days, and doctors cannot afford to wait for these results before prescribing without affecting patient prognosis. This is especially true of sepsis, where the chance of mortality increases by 7.6% for every hour that antibiotic treatment is delayed (Sterling *et al*, 2015). Until the infrastructure exists for

hospitals to rapidly determine the most effective antibiotic(s) with which to treat an infection, broad-spectrum antibiotics remain first-line treatments. However, the use of broad-spectrum antibiotics also increases the risk of secondary infections such as MRSA *Clostridium difficile* and *Candida albicans*, which thrive following the disruption of the normal gut flora.

Secondly, it is estimated to cost $2.6 billion to bring a new drug to market: $1.4 billion for research and development, $1.2 billion for investment returns and $312 million for post-approval research and development. Just 11.8% of all drugs ever gain clinical approval (DiMasi *et al*, 2016). Furthermore, as antibiotics are typically taken for short durations and the average 10-day treatment cost is just $468 (Falagas *et al*, 2006), it is difficult for the pharmaceutical industry to make a return on their investment. This is compounded by the fact that when new antibiotics are licenced, their use is restricted to minimise resistance. Taken together, there has been an overall reduction in the research and development of new antibiotics, and a gradual shift towards more profitable drugs such as anti-neoplastic and immunosuppressive treatments which are prescribed for longer durations at an increased cost (Falagas *et al*, 2006). It is perhaps surprising that the pharmaceutical industry continues to invest in antibiotic discovery at all, aside from their moral obligations to improve human health, but there are numerous incentives on offer to drive innovation. Examples include the New Drugs for Bad Bugs (ND4BB) initiative which facilitates rapid drug development, and The European Commission and European Investment Bank

which offer loans specifically for antibiotic development (Renwick *et al*, 2016).

Over the years, antibiotic discovery has taken a number of distinct forms. Until the 1990's, the industry favoured a 'phenotypic' approach to drug screening. Large compound libraries were screened against whole cells and if ~80% bacterial inhibition was observed, the compound advanced to target devolution to ascertain the mode of action. However, it is curious to note that some compounds such as polymyxin B and daptomycin have been approved for clinical use with an as yet uncharacterised mechanism of action. This phenotypic approach led to the discovery of many drug classes including β-lactams, flouroquinolones and glycopeptides.

Following advancements in the field of genetics throughout the 1990's, the pharmaceutical industry adopted a 'genotypic' or 'target-based' approach. Whole genome sequencing of bacterial pathogens was used to identify essential genes which lacked a mammalian equivalent. These proteins were then expressed and purified, and High Throughput Screening (HTS) was used to identify molecules which bound to the targets. Lead compounds were then screened in whole cell models to determine whether their pharmacodynamics made them suitable antibiotics; a compound may bind to its target *in vitro* but fail to penetrate the bacterial cell envelope, or be removed by efflux pumps or induce mutational resistance *in vivo*. The genotypic approach identified many novel targets, and many compounds

which can bind to these targets, but to date no new antibiotics have been developed using this approach.

In the absence of novel antimicrobials, we have seen the re-emergence of drugs previously unlicensed or withdrawn over safety concerns. Originally licenced in 1959, colistin was discontinued in the 1980's due to nephrotoxicity. However, for Gram-negative infections which have exhausted all other treatment options, the benefits of clearing the infection outweigh any future side effects. Though resistance is rare, the mobilised colistin resistance gene *mcr-1* made headlines recently when it was found to spread via horizontal gene transfer (Liu *et al*, 2016). This phosphatidylethanolamine transferase lowers the biding affinity of colistin by adding a phosphatidylethanolamine residue to lipid A, and can be transmitted from porcine to human strains of *Escherichia coli* (Olaitan *et al,* 2015). This is particularly concerning because though the use of colistin is tightly regulated in the United Kingdom, in China polymyxins are widely used as growth promoters in veterinary medicine (Rhouma *et al*, 2016). The transmission of resistance genes from pig meat to humans could result in widespread resistance to this drug of last resort.

Mechanisms of AMR

It is a common misconception that AMR is *caused* by excessive and inappropriate agricultural, veterinary and clinical use; though it is true to say that the application of selective antimicrobial pressures has exacerbated the frequency of mutations and the spread of resistance genes (Bartlett *et al*., 2013). The ability to adapt to ever-changing environmental conditions ensures the survival of pathogen, yet this adaptability is exactly what poses such a threat to human health. In fact, most antibiotics are derived from other bacteria or fungi, which produce these compounds to gain a competitive advantage over competing organisms. Of the producer organisms, *Streptomyces* spp. are perhaps the most clinically important and have yielded a variety of antibiotics including amphotericin B, erythromycin and tetracycline. It stands to reason, however, that these producer organisms also possess mechanisms of protection to prevent self-toxicity. These self-resistance genes are early mechanisms of antimicrobial resistance.

As many resistance genes and mechanisms existed before the advent of modern antibiotics, it stands to reason that they originally served an alternative function within the cell. Take the AcrAB-TolC efflux pump in *E. coli,* which is able to actively transport a wide variety of substrates out of the cell including dyes (eg. acridine, ethidium bromide), detergents & disinfectants (eg. sodium dodecyl sulphate, cetrimide, triclosan, bile salts), in addition to antibiotics (eg. fluoroquinolones, nalidixic acid, β-lactams, tetracycline,

chloramphenicol, rifampicin) (Du *et al*, 2014). Some bacteria are able to alter their electrostatic charge to repel host defence peptides, but can also repel charged antibiotics in a similar manner. Many proteins involved in AMR are closely related to other functional proteins so the cell doesn't have to create a new resistance mechanism. Examples include the β-lactamases, which are closely related to the transpeptidases.

Mechanisms of resistance can be broadly grouped into three main categories, and examples of each will be discussed within this chapter for some common antibiotics:

1. **Innate resistance** is defined as the ability of an organism to resist an antimicrobial agent due to its structural or functional properties, and normally results in the inability of the drug to enter the cell. For example, Gram-negative bacteria are inherently resistant to vancomycin which is a large molecule unable to permeate the cell wall in sufficient quantities to achieve a therapeutic intracellular concentration (Munita & Arias, 2016).
2. **Adaptive Resistance** occurs in response to an environmental stress such as changes in nutrient bioavailability or antibiotic detection. In response to stimuli, genes and proteins are up- or down-regulated to increase the bacterial tolerance to the antibiotic. Efflux pumps are normally produced at a low level by bacteria, but can be up-regulated in response to stress. This can be permanent and over-producers demonstrate an MDR phenotype with decreased susceptibility to numerous drugs. For example, *P. aeruginosa* has

12 Resistance/Nodulation/Cell Division (RND) family efflux systems, each of which has specificity for different substrates. Constitutive expression of MexAB-OprM provides a low level broad resistance (De Kievet *et al*., 2001) whereas the expression of antibiotic specific pumps, such as MexXY-OprM, offers a higher level of resistance to fluoroquinolones and aminoglycosides (Hocquet *et al*., 2003). Efflux also underpins most other antibiotic resistance mechanisms, as other mechanisms of resistance such as enzyme production or target site change are ineffective in the absence of efflux as the cell is overwhelmed by the drug (Piddock, 2006).

3. **Acquired resistance** includes the mutation of existing genes, and the acquisition of extrachromosomal elements such as plasmids, transposons and integrons via horizontal gene transfer (Alekshun & Levy, 2007). This allows the rapid spread of genetic material throughout a population of related or mixed species. Such resistance genes are normally proceeded by promoters so that the genes are only induced in the presence of the substrate. This limits the fitness cost of carrying these genes to the organisms.

Cell Wall Biosynthesis

Introduction

Though the composition of the bacterial cell envelope differs between Gram-positive and Gram-negative bacteria (Fig. 1), it serves to provide strength and support for the cell and an effective barrier against antimicrobial agents (Silhavy *et al*., 2010). Lipophilic antibiotics such as the macrolides and fluoroquinolones are limited in the ways they can cross cell membranes, but the cell envelope is not impenetrable and low molecular weight hydrophilic antibiotics with a molecular weight less than 1000 Da that can pass through non-specific aqueous diffusion channels known as outer membrane porins (Omp's). These β-barrel structures allow the passage of nutrients into the cell, so although Omp's are often down-regulated in response to antibiotic stress, their repression only provides low level resistance as they are required for normal cell homeostasis.

Peptidoglycan is comprised of repeating units of the disaccharide pentapeptides *N*-acteylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), which are joined by β-1, 4 linkages (Sobhanifar *et al*, 2013). It is required for binary fission during cell growth and division, and also to maintain the turgor pressure within the cell (Silhavy *et al*., 2010). Defects to the Mur enzyme peptidoglycan biosynthesis pathway, or to the polymer itself, will result in osmolysis and cell death (Kouidmi *et al*., 2014). This makes peptidoglycan an attractive target for several different classes of antibiotics.

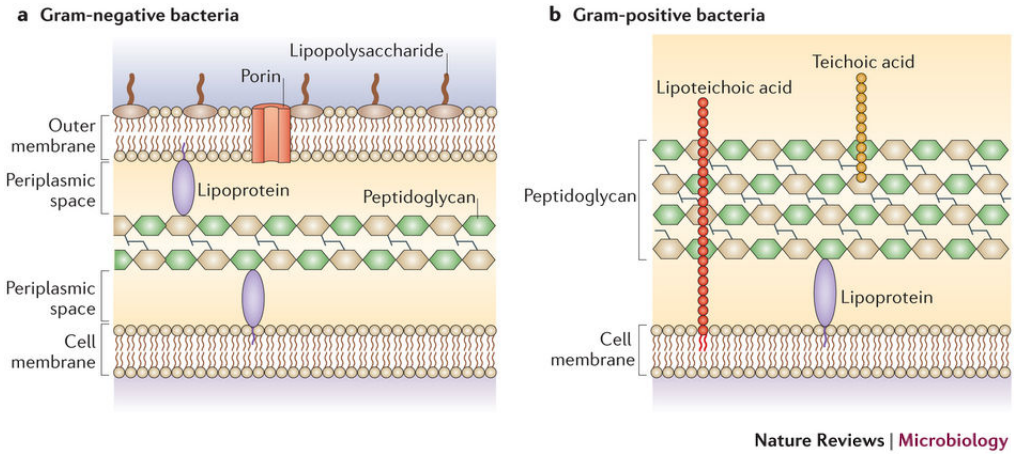


Figure 1: A comparison between the Gram-negative and Gram-positive cell envelopes

(A) Gram-negative bacteria possess a thin layer of peptidoglycan (7 - 8 nm), sandwiched between the asymmetric cytoplasmic and outer membranes. The cytoplasmic membrane is a phospholipid bilayer embedded with protein and ion channels, whilst the outer membrane is comprised of two distinct layers: a phospholipid inner leaflet interdigitated with lipoproteins, and a lipopolysaccharide (LPS) outer leaflet. The outer membrane is coupled to a thin layer of peptidoglycan located in the aqueous periplasmic space by the Braun lipoprotein. Here, complex machinery such as efflux pumps, secretion systems and fimbriae are assembled in response to stimuli from individual protein monomers located in the periplasm. (B) The Gram-positive bacterial cell envelope is comprised of a thick layer of peptidoglycan (20 - 80 nm), coupled to the cytoplasmic membrane. Polymers such as teichoic acids and lipoteichoic acids provide rigidity to the structure. Figure taken from Brown *et al*. (2015).

β-lactams

Penicillin's, cephalosporin's, carbapenem's and all other members of the β-lactam family target a class of enzymes known as transpeptidases (also known as Penicillin Binding Proteins or PBP's). Transpeptidases remove the terminal D-alanine of the NAM pentapeptide side chain, allowing peptidoglycan cross-links to form between the position 4 D-alanine and the position 3 of diaminopimelic acid (DAP) (Sobhanifar *et al*, 2013). The formation of these cross-links results in a rigid structure that maintains a turgor pressure 2 - 6 times that of atmospheric pressure within the cell. β-lactams make such effective antibacterial agents because they are analogues of the terminal D-alanine-D-alanine of peptidoglycan, and possess the characteristic 4-membered lactam ring that acylates the S403 residue in the transpeptidase active site to form an acyl-enzyme complex. With the active site of the enzyme permanently blocked, cross-linking and peptidoglycan biosynthesis are inhibited and cell lysis occurs. As the transpeptidases are highly conserved between bacteria, most β-lactams have broad spectrum activity against both Gram-positive and Gram-negative bacteria.

Since penicillin was discovered in 1928, the parent compound has been modified to create numerous natural and synthetic derivatives that share the characteristic β-lactam ring. The addition of various side chains alters properties of the drug such as side effects, bioavailability and membrane penetration (Hamilton-Miller, 1999). The cephalosporin's are a subclass of β-lactams which were originally isolated from *Acremonium* spp. Compared to

penicillin's, they have a lower affinity to β-lactamases so are less susceptible to hydrolysis (Phelps *et al*, 1986). Carbapenems are another subclass of β-lactams with a broader spectrum of antibacterial activity than penicillin's and cephalosporin's, and are used sparingly for the treatment of MDR infections. The spread of carbapenem-resistant infections is concerning to clinicians given the lack of alternative treatment options. In the United Kingdom, carbapenem-resistant Enterococci are reportable to the Antimicrobial resistance and healthcare associated infections (AMRHAI) reference unit, part of Public Health England's bacteria reference department (BRD), in order to monitor outbreaks and identify transmission pathways (AMRHAI, 2017).

Penicillin resistance is mediated by β-lactamases that hydrolyse the β-lactam ring so it is unable to react with the transpeptidase. β-lactamases are grouped into classes A – D based on their amino acid sequence. Classes A, C and D utilise a serine residue to hydrolyse the β-lactam ring, whereas Class B β-lactamases requires a Zinc co-factor (Bush & Jacoby, 2010). Extended Spectrum Beta Lactamases (ESBL's) are β-lactamases that can also hydrolyse cephalosporin's, and carbapenemases are able to hydrolyse carbapenems in addition to penicillin's and cephalosporin's. Of particular concern are the KPC's (*K. pneumoniae* carbapenemases) which have spread beyond *K. pneumoniae* to a wide range of Gram-negative bacteria. The bla_{KPC} gene encodes a class A enzyme which is able to hydrolyse all β-lactams and β-lactamase inhibitors (Arnold *et al*, 2011). Many β-lactamases are located on plasmids which facilitates their rapid spread throughout a population. As

plasmids carrying β-lactamases also tend to carry resistance genes for aminoglycosides, fluoroquinolones and trimethoprim, treatment options are limited to a handful of drugs of last resort such as colistin and polymyxin B.

β-lactamase inhibitors including clavulanic acid and tazobactam have a β-Lactam structure but weak antimicrobial properties. Instead, their β-lactam ring binds strongly to the β-lactamase, blocking the active site of the enzyme so that it is unable to bind to the antibiotic. β-lactamase inhibitors are co-prescribed with β-lactams for the treatment of β-lactamase producing infections which would otherwise be rendered resistant, though clavulanic acid is only able to inhibit Group 2 β-lactamases. Some β-lactam/β-lactamase inhibitor preparations are commercially available including Tazocin (piperacillin/tazobactam) and co-amoxiclav (amoxicillin/clavulanic acid).

Glycopeptides

The glycopeptides are another class of antibiotics which inhibit peptidoglycan biosynthesis. Vancomycin was the first drug of this class to be isolated from *Amycolatopsis orientalis* in 1958. This large hydrophilic molecule (1448 Da) is too large to permeate the Gram-negative outer membrane, so it is used to treat β-lactam resistant Gram-positive infections. Vancomycin forms a five-point hydrogen bond with the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides to prevent the synthesis of new cell wall polymers and cross-linking of any backbone polymers, resulting in cell lysis.

Very little vancomycin resistance was observed during the drug development process, and it was more than 20 years later when vancomycin resistant Enterococci (VRE) were first reported in 1988 (Uttley *et al*, 1988). This is likely due to the fact that vancomycin use was limited during this time due to the availability of drugs with fewer side effects such as the β-lactams. However, the increase in pseudomembranous colitis and the widespread appearance of MDR organisms such as MRSA saw the clinical use of vancomycin increase throughout the 1980's.

Vancomycin resistance can occur by several different mechanisms. Vancomycin-intermediate *S. aureus* (VISA) are defined as requiring 8–16 μg/mL vancomycin for inhibition (CDC, 2002), and characterised by a thicker cell wall containing non-cross linked side chains which bind vancomycin outside of the cell. This results in a titration effect whereby less vancomycin is available to bind to its intracellular target. Vancomycin-resistant *S. aureus* (VRSA) strains are defined as requiring ⩾32 μg/mL vancomycin for inhibition and were first reported in 2002 (CDC, 2002). This is concerning given that vancomycin is the first line treatment for MRSA. Eleven plasmid-mediated gene clusters have been described which confer vancomycin resistance, including the recently described *vanI* (Kruse *et al*, 2014). The most prevalent of which is encoded by the *vanA* gene cluster, and is believed to have originated from glycopeptide producing bacteria (Marshall *et al*, 1998).

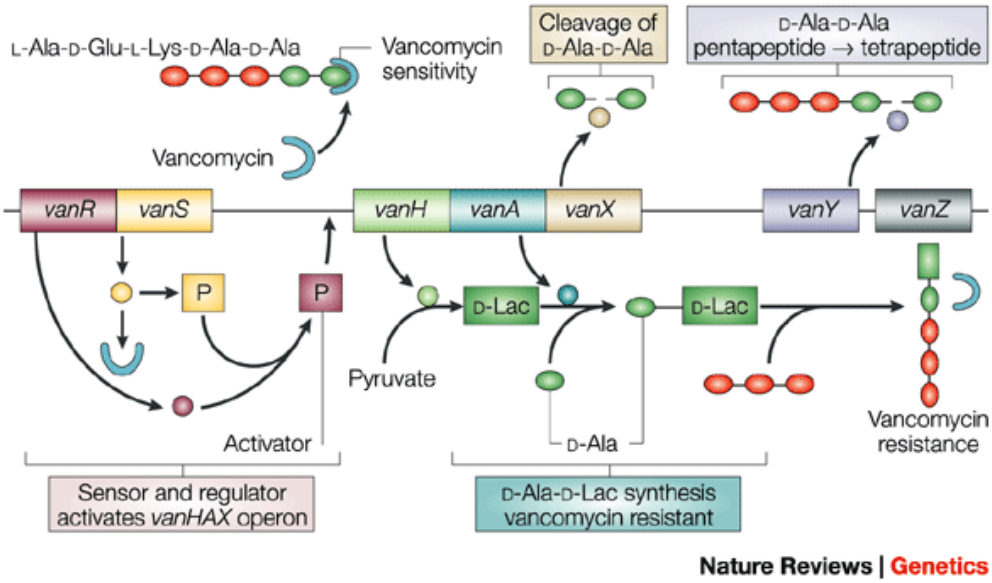


Figure 2: The *vanA* gene cluster

The *vanA* gene cluster confers resistance in vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). Expression of the *vanA* resistance operon is regulated by a two-component regulatory system VanRS, which is induced when vancomycin is detected in the cell by the membrane-associated sensor kinase (VanS), and switches off the cytoplasmic response regulator (VanR). Genes *vanH*, *vanA* and *vanX* encode three biosynthetic enzymes: VanH is a dehydrogenase which reduces the cellular metabolite pyruvate to D-lactate. VanA is a ligase which catalyzes the formation of an ester bond between D-alanine and D-lactate to produce the depsipeptide D-alanine-D-lactate. As MurF is able to use both D-alanine-D-alanine and D-alanine-D-lactate as a substrate, no additional enzymes are required to complete peptidoglycan synthesis. VanX is a D,D-dipeptidase which hydrolyses any D-alanine-D-alanine synthesised through the normal pathway in error. Accessory gene VanY is a D,D-carboxypeptidase which cleaves the terminal D-alanine of any membrane-bound peptidoglycan which may occur through incomplete hydrolysis by VanX. The role of accessory genes VanW and VanZ are not known. Taken from Hughes (2003).

The *vanA* gene cluster encodes a number of enzymes and accessory genes that replace the terminal D-alanine-D-alanine of the peptidoglycan peptide stem with D-alanine-D-lactate in a complex mechanism which alters the binding target of vancomycin (Fig. 2). Alanine and lactate are almost identical amino acids except the carbonyl-NH peptide bond at positions 4 and 5 between the alanine and vancomycin is replaced with a carbonyl-O. This difference in atomic structure alters the hydrogen bonding so vancomycin binds with 1000x less affinity to lactate than alanine (Reynolds & Courvalin, 2005). The *vanB* and *vanI* resistance operons encode related enzymes to confer resistance by the same mechanism, whilst the *vanC*, *vanE* and *vanG* resistance cassettes synthesise D-alanine-D-serine to sterically blocks vancomycin binding (Hughes, 2003). This is a weaker form of resistance and only results in 6x less susceptibility to Vancomycin (Reynolds & Courvalin, 2005).

Recently, vancomycin analogues have been synthesised which inhibit cell wall biosynthesis in a three pronged attack: alongside D-alanine-D-alanine binding, the addition of a quaternary ammonium salt increases cell wall permeability, and the addition of (4-chlorobiphenyl)methyl (CBP) inhibits bacterial transglycosylase. These analogues are effective against VRE and 1,000-fold more potent than traditional vancomycin, so less drug is required for treatment (Okano *et al*, 2017).

Daptomycin

Daptomycin is a non-ribosomally synthesised cyclic peptide that is used a drug of last-resort to treat MDR Gram-positive infections except *Streptococcus pneumoniae*. Though the compound was isolated from *Streptomyces roseosporus* by Eli Lily in the 1980's, it wasn't approved for clinical until 2003. This is because development was abandoned due to myopathic side effects observed during Phase II clinical trials. The exact mechanism of action is not known, but Daptomycin is believed to depolarise the cytoplasmic membrane and increase permeability causing ions to leak out of the cell. This mechanism is calcium-dependent (Taylor & Palmer, 2016). Although resistance is still relatively uncommon, organisms with reduced levels of phosphatidylglycerol in the membrane or increased conversion to lysyl-phosphatidylglycerol show reduced susceptibility to the drug (Taylor & Palmer, 2016). This is believed to be the reason why *S. pneumoniae* shows resistance.

DNA replication

Fluoroquinolones

In 1962, while researchers were attempting to synthesise the anti-malarial drug quinine, they accidently synthesised Nalidixic Acid: a quinolone with antibacterial properties (Lesher *et al*., 1962). Subsequent research revealed that the addition of a fluorine atom to the central C6 or C7 position broadened the spectrum of activity, and thus the fluoroquinolones were formed. These antibiotics are effective against both Gram-positive and Gram-negative infections, primarily genitourinary infections such as bacterial prostatitis and catheter associated urinary tract infections (UTI's). Four generations of fluoroquinolones have been approved for clinical use: The first generation is comprised of all non-fluorinated drugs, the second generation includes most of the fluoroquinolones in clinical use, while the third generation has an increased spectrum of activity and is effective against Streptococci. Fourth generation fluoroquinolones have a duel mechanism that targets both DNA gyrase and topoisomerase IV, and show reduced resistance. The most frequently prescribed fluoroquinolones include ciprofloxacin, levofloxacin, and moxifloxacin (Redgrave *et al*, 2014).

The fluoroquinolones target DNA replication by inhibiting two essential type II topoisomerases: DNA gyrase and topoisomerase IV. Whilst type I topoisomerases are responsible for reducing the level of supercoiled DNA within a cell, type II topoisomerases introduce supercoiling and prevent

double strand breaks during cell division. In response to external stimuli such as stress, DNA gyrase relaxes replication forks and introduces negative supercoiling to enable more DNA to be packed into the cell, whilst topoisomerase IV decatenates the daughter chromosomes. Both enzymes are heterotetrameric: DNA gyrase is comprised of two subunits each of GyrA and GyrB, whilst topoisomerase IV is comprised of two ParC and two ParE subunits. Each fluoroquinolone binds preferentially to one of the two enzymes, with varied affinity between bacterial species (Redgrave *et al*, 2014). The amine group of the fluoroquinolone binds irreversibly to an acidic residue on helix-4 of GyrA or ParC to form a drug-enzyme-DNA (ternary) complex which prevents the two DNA strands from rejoining. Crucially, this binding is mediated by a magnesium ion, which is required for the formation of a water-metal ion bridge. For this reason, flouroquinolones are unable to bind in the absence of magnesium (Wohlkonig *et al*, 2010). The resultant drug-enzyme-DNA cleaved complexes result in the death of the cell by fragmenting chromosomal DNA and inducing oxidative stress.

Fluoroquinolone resistance typically arises due to alteration of the binding site: mutations occur in one of the genes located on a short sequence of DNA known as the Quinolone Resistance Determining Region (QRDR). Common amino acid substitutions include S80L and E84K in topoisomerase IV, and S83L and D87N in DNA gyrase (Johnning *et al*, 2015). However, if a mutation occurs within the primary target of the fluoroquinolone then it will still bind to its secondary target site, but with weaker affinity. Plasmid-mediated

quinolone resistance (PMQR) genes including *qnr* can also decrease susceptibility to fluoroquinolones. The qnr protein family are typified by their pentapeptide tandem repeat. The biological function of these proteins is not known, but they dimerise and fold to form a right-handed quadrilateral β-helix that mimics the structure and charge properties of B-DNA (Hegde *et al*, 2011). The protein then binds to the enzyme to prevent drug inhibition. The *qnr* genes normally confer mild fluoroquinolone resistance, however susceptibility is determined by plasmid copy number and subsequent gene expression levels.

Another PMQR is the aminoglycoside *N*-acetyltransferase AAC(6')-1b, which normally acetylates the NH_2 group of kanamycin B to prevent ribosomal binding. This Aminoglycoside Modifying Enzyme (AME) is highly mobile and as the fluoroquinolones also contain an NH_2 group, mutations D179Y & W102R in AAC(6')-1b give rise to fluoroquinolone resistance too (Robicsek *et al*, 2006). The plasmid-mediated *qepA* gene also encodes an efflux pump to prevent antibiotic accumulation within the cell (Yamane *et al*, 2007).

Quinolone resistance has been linked to the use of the common household disinfectant triclosan, which is used in everything from cleaning products to soap and shampoo. Webber *et al*, (2017) showed that quinolone-resistance mutations S83F and D87G in GyrA also conferred resistance to triclosan. Furthermore, exposure to triclosan increased the frequency of GyrA mutations in *E. coli*. The use of triclosan was recently banned in antibacterial

soap by the FDA, due to concerns over its efficacy and long term health implications (FDA, 2016).

Aminocoumarins

The Aminocoumarins are a class of antibiotics that inhibit DNA gyrase in a non-quinolone fashion. They are derived from *Streptomyces antibioticus*, and contain a tetraene group that binds next to the magnesium ion, and a polyketide group that binds in a separate pocket. Examples within this group include coumermycin, clorobiocin and novobiocin: the latter of which is discussed in depth in Research Project 2.

Protein Synthesis

The bacterial ribosome is the organelle responsible for the majority of protein biosynthesis within the cell. The genetic code is carried to the ribosome as an mRNA message comprised of three-nucleotide sequences known as codons, each of which corresponds to one amino acid. The ribosome is comprised of two subunits: the smaller 30S subunit is responsible for correctly proof-reading and decoding, whilst the larger 50S subunit brings in amino acid loaded tRNA from an elongation factor and makes Crick-Watson base pairing to form the anti-codon. By joining the amino acids together in this way, a polypeptide chain is formed. As the ribosome is highly conserved between bacteria and protein synthesis is such a fundamental process within the cell, it is unsurprising that the ribosome is the target for many diverse antibiotics:

30S subunit

The 30S ribosomal subunit is comprised of ~1500 nucleotides and 21 proteins (Yonath, 2005). This decoding centre contains three active sites for binding tRNA: all but the first aminoacyl tRNA's enter the 30S subunit at the *A site*. From here, the aminoacyl tRNA passes from the *A site* to the *P site* where peptidyl tRNA is constructed. The *P site* is also where the first aminoacyl tRNA enters the ribosome. Once the charged tRNA has donated its amino acid to the growing polypeptide chain, deacylated tRNA leaves the ribosome at the *E site*. This motion is powered by guanosine triphosphate (GTP). If the

codon matches with the newly synthesised anti-codon, then bases A1492 and A1493 of the 16S rRNA ribosome undergo a conformational change and flip up from helix 44 to signal to the ribosome that catalysis should occur. Antibiotics that target the 30S ribosome subunit increase the error rate of translation so that the bacteria encode for the wrong proteins and die.

The tuberactinomycin family of antibiotics are of clinical importance as they are effective against MDR *M. tuberculosis*. Viomycin was the first member of this family to be isolated from the soil bacteria *Streptomyces puniceus*. It binds to bases A1492, A1493 and G1491 of helix 44 of the large subunit, and A1913 and C1914 of H69 which are located at the end of helix 69 in the small subunit (Stanley *et al*, 2010). This binding forces bases A1492 and A1493 to permanently flip up, even when the nucleotide sequence doesn't match that of the mRNA so the bacteria encodes the wrong protein. Mutations in the antibiotic binding site confer resistance, including the demethylation of C1409 which reduces the binding affinity of viomycin to *M. tuberculosis* 2 - 8 fold. As a result the antibiotic is sterically blocked and no longer able make a triple hydrogen bond with the binding site (Stanley *et al*, 2010). Paromycin has a slightly lower binding site than viomycin, but a similar mechanism of action.

The aminoglycosides also inhibit protein synthesis through steric blocking of the tRNA binding sites. They are used to treat Gram-negative infections, including MDR tuberculosis, and a minority of Gram-positive infections.

Examples within this family include kanamycin, tobramycin and gentamicin. Aminoglycoside resistance can occur through a variety of different mechanisms, but amongst the most prevalent are the AME's. The biological function of these enzymes is not known, but there are over 100 AME's which each confer resistance to aminoglycosides through the catalysis of specific chemical modifications to prevent binding. The most prevalent AME is AAC(6')-Ib (see fluoroquinolone resistance), but other subclasses of AME's include aminoglycoside *O*-nucleotidyltransferases (ANTs), and aminoglycoside *O*-phosphotransferases (APHs). (Garneau-Tsodikova & Labby, 2016)

Interestingly, aminoglycoside use results in ototoxicity in a subset of the population, due to build up in the inner ear fluid. An A1555G mutation in the 12S eukaryotic ribosomal subunit has been shown to be responsible for this susceptibility (Garneau-Tsodikova & Labby, 2016). Other mutations in the decoding centre such as A1490G and C1410U restore the bacterial base-pairing so the aminoglycoside is able to form Watson-Crick base-pairing and bind with greater affinity (Garneau-Tsodikova & Labby, 2016).

50S subunit

The 50S subunit consists of ~3000 nucleotides and up to 35 proteins (Yonath, 2005). The catalytic site is known as the peptidyl-transferase centre (PTC), where peptide bonds are formed if the codon on the mRNA matches the anticodon on the tRNA. A productive twist towards the *P site* occurs and a

bond is made between the incoming amino acid and the end of the peptide chain. The translocation of the mRNA and tRNA through the ribosome and into solution is catalysed by Elongation factor G.

Antibiotics that target the PTC of the 50S subunit work in one of two ways: they sterically block the PTC to prevent substrate binding, or they prevent the formation of peptide bonds. A diverse range of compounds are able to prevent substrate binding including clindamycin, which reversibly bind to both the *A* and *P sites*, and chloramphenicol which only targets the *A site*. Chloramphenicol is an effective treatment for the common causes of bacterial meningitis: *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*. However, it is only prescribed in the absence of safer alternatives as chloramphenicol use has been associated with serious side effects such as bone marrow suppression. Resistance is acquired resistance through binding-site mutations and chloramphenicol acetyltransferases (CATs), which replace the hydroxyl group at the C3 position with an acetyl group from acetyl-CoA (Schwarz *et al*, 2004). The plasmid-mediated *cat* genes enable genetic material to be transferred horizontally. In the presence of short DNA uptake sequences (DUS's) for example, *N. meningitides* Type IV pilin are able to bind to DNA and integrate the genetic material into the genome by homologous recombination. Organisms carrying the *cmlA* gene, located on Tn1696, also show decreased susceptibility to chloramphenicol (Schwarz *et al*, 2004). The gene encodes a

transmembrane transport protein which exports chloramphenicol out of the cell.

Different cell types may contain a drug binding site, but the structure of the ribosome itself may prevent binding. For example, chloramphenicol and the related pyrrolidine anisomycin bind to the same aromatic residues in the prokaryotic 50S and eukaryotic 80S ribosomes respectively: namely C2452, G2447, C2501, and G2061. However, the position of nucleotide U2504 differs between the two ribosomes and sterically blocks the binding of anisomycin to the eubacterial ribosome. Thus, anisomycin can only be prescribed for the treatment of eukaryotic fungal infections (Bulkley *et al*, 2010).

Another important class of antibiotics that bind at the *P site* to prevent the formation of peptide bonds are the macrolides. These natural products are derived from *Saccharopolyspora erythraea*, and are mainly used to treat Gram-positive infections such as *Chlamydia trachomatis* and Streptococci, and select Gram-negative organisms including *Bordetella pertussis*. Due to their wide antimicrobial spectrum, they are commonly prescribed for patients with a penicillin allergy. The most commonly prescribed macrolide is erythromycin, and resistance mainly occurs through one of two mechanisms. High-level resistance is conferred by the *erm* genes, which encode an Erythromycin-resistant methylase that dimethylates an adenine residue on the 23S rRNA. This prevents macrolide binding to the *P site*. Low level

resistance is provided by the chromosomally-mediated *mefE* genes, which encode a macrolide-specific efflux pump (Pechère, 2001)

The oxazolidinones are a class of synthetic compounds with antimicrobial properties which were discovered during compound screening in the 1970's, but weren't further developed until the 1990's. Linezolid was the first compound of this class to be approved in 2000, and is prescribed for the treatment of MDR Gram-positive infections including VRE and MRSA. Though it was originally thought that linezolid had a novel mechanism of action, it is now known to bind at the *A site* in a similar fashion to other drugs acting at the catalytic site. It interacts with many nucleotides in the PTC, including G2061, A2451, C2452, A2503, U2504, G2505, U2506, and U258 in such a way that amino acid transfer between the *A* and *P sites* is blocked (Long & Vester, 2012). The binding site of linezolid overlaps with others that bind at the PTC so cross-resistance is common.

Subtle mutations can occur in the PTC binding pocket can give rise to linezolid resistance, but mutations in distal nucleotides not involved in direct binding can also result in resistance. Examples include mutations G2576U and G2447U, which sterically block linezolid binding (Long & Vester, 2012) However, the most powerful means of resistance is *cfr* gene acquisition which is also a prime example of evolutionary pressure driving a change in function. The *cfr* gene was first discovered in *Staphylococcus sciuri*, but as linezolid is a synthetic compound it is clear that the *cfr* gene serves another

function within the cell. There are lots of natural methyl groups on the ribosome, and the housekeeping methyltransferase RlmN methylates the C2 position. However, the methyltransferase encoded by the *cfr* gene is able to change the specificity of this methylation to the C8 position. This sterically blocks linezolid binding and gives rise to resistance (Long & Vester, 2012).

Streptogramins

The streptogramin antibiotics are comprised of several different subclasses which are prescribed for the treatment of VRSA and VRE. Several streptogramins are in clinical use including the combination antibiotic quinupristin-dalfopristin. These two antibiotics work synergistically to inhibit tRNA rotatory motion: dalfopristin is a modified version of pristinamycin IA and targets the *P site* to inhibit peptidyl transfer, creating a binding site for quinupristin. Mechanisms of dalfoprisitin resistance include the *vat* genes, which encode acetyltransferases that modify the antibiotic to reduce binding affinity. The *vga* genes encode an ABC transporter which actively transports the drug out of the cell (Hershberger *et al*, 2004). Conversely, quinupristin targets the peptide exit channel preventing elongation of the polypeptide chain. Mechanisms of quinupristin resistance include the plasmid mediated *vgb* gene which encodes the Virginamycin B lyase. This enzyme dimethylates an adenine residue on the 23S rRNA, not unlike the *erm* genes, so that quinupristin binds with decreased affinity (Hershberger *et al*, 2004). It is also common to see multiple forms of resistance together. For example, ABC

efflux genes and acyltransferase genes are commonly co-located on a "resistance pair" operon.

RESEARCH PROJECTS

It is clear that the “golden era” of antibiotic discovery has passed and as the discovery of novel compounds becomes increasingly difficult, tackling AMR requires a multi-disciplined approach. While research and development was once seen as the responsibility of the pharmaceutical companies, this area is now being led by universities, small biotechnology companies, and charities such as Antibiotic Research UK (ANTRUK). These companies also carry the cost of failed compounds which never make it to market. Whilst the pharmaceutical companies have moved towards investing in compounds at the point at which they enter clinical trials. Here, they have expertise in the approval process and the capital to fund expensive clinical trials.

The Universities of Birmingham and Nottingham are involved in numerous projects for antimicrobial discovery and development, including several collaborations with industry. Working with groups at both universities, research took very different approaches to tackling AMR. At the University of Birmingham, my research focused on understanding the mechanisms of novobiocin resistance in *Klebsiella pneumoniae* ECL8. As exemplified above, resistance to an antibiotic normally occurs by multiple different mechanisms so to maintain the long-term use of an antibiotic, it is important to fully understand the mechanisms of resistance. In doing so, it may be possible to design derivatives which are able to overcome resistance.

My research at the University of Nottingham focused on developing an assay for the HTS of IscR inhibition in *Yersinia pseudotuberculosis*. IscR has been identified as a novel anti-virulence target in a number of bacterial species, including *Y. pseudotuberculosis*. The IscR protein is highly conserved between *Yersinia* spp., so it is anticipated that inhibitors could also be effective against the more virulent *Yersinia pestis*. As there is a transition towards personalised medicine and targeted treatment plans, there will be an increased demand for antimicrobials which target specific bacterial pathogens. Unlike broad-spectrum antibiotics, anti-virulence drugs disarm rather than kill bacteria, so resistance is less likely as there is less selective pressure on the organism (Allen *et al*., 2014). Furthermore, anti-virulence drugs are less likely to disrupt the normal bacteria so the risk of secondary infections is reduced.

Novobiocin resistance in *Klebsiella pneumoniae* ECL8

Abstract

Novobiocin is an aminocoumarin antibiotic which binds to the GyrB subunit of DNA gyrase to competitively inhibit ATP hydrolysis and prevent DNA synthesis. Transposon Directed Insertion-site Sequencing (TraDIS) was previously used to identify 59 genes whose inactivation gave rise to novobiocin resistance in Multi-Drug Resistant *Klebsiella pneumonia* ECL8. The *ramR* gene is a known regulator of antimicrobial resistance and represented 46% of transposon insertions within the mutant population, whilst previously uncharacterised mechanisms of resistance *hscB* and *fre* represented <2% gene inactivation. Novobiocin resistant mutants were generated by exposing the transposon-library to 750 µg ml^{-1} novobiocin. Eighty mutants were screened for the presence of the Tn*5* transposon within the *ramR*, *hscB* and *fre* genes using colony PCR. Interestingly, none of the mutants screened contained a transposon insertion within the *ramR* gene, suggesting that this mechanism of novobiocin resistance is not as prevalent as the TraDIS data suggests. To determine whether inactivation of *ramR*, *hscB* and *fre* gives rise to novobiocin resistance, the Red-recombinase system was selected to create null mutants *in vivo*. Towards this aim, a novel red-recombinase plasmid pDS1 was generated. Restriction-free cloning was used to replace the *bla* cassette of pKD46 with the *kan* cassette from donor plasmid pDOC-K, as *K. pneumoniae* is intrinsically resistant to ampicillin. This plasmid will be used for future validation of the TraDIS dataset.

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen frequently associated with infections ranging from UTI's and cholecystitis, to pneumonia, meningitis and septicaemia. In healthcare settings, mortality rates can be as high as 50% (WHO, 2014), with the immunocompromised and extremes of age at greatest risk. *K. pneumoniae* infection can be difficult to treat due to the presence of a thick capsular polysaccharide, and the carriage of plasmid-associated resistance genes. Though β-lactams remain the first line treatments for *K. pneumoniae* infection, all strains express one or more of the LEN, OKP or SHV-1 β-lactamases which confer resistance to penicillins and early generations of cephlosporins (Hæggman *et al*, 2004). KPC producing strains are increasingly prevalent and present a significant challenge to clinicians. KPC's are Class A β-lactamases which encode resistance to all β-lactams, cephlosporins and carbapenems, leaving few treatment options (Arnold *et al*, 2011). In such cases, novobiocin may be prescribed intravenously as a drug of last resort despite extensive side-effects including nausea, vomiting, diarrhoea, pancytopenia, and haemolytic anaemia. Novobiocin is naturally produced by *Streptomyces spheroids* and belongs to the aminocoumarin family of antibiotics. Unlike the fluoroquinolones and other antibiotic compounds which target the GyrA subunit of type-II DNA topoisomerases, novobiocin preferentially binds to the GyrB subunit of DNA gyrase. Here, ATP binding is competitively inhibited thorough the formation of hydrogen bonds with GyrB residues, preventing DNA synthesis.

Novobiocin resistance is widespread. Within nature, *S. spheroids* possesses the self-resistance *nov* gene locus encoding a novobiocin-resistant GyrB subunit (Ivanisevic *et al*, 1995). Similarly, most novobiocin resistance arises due to target-site mutations resulting in an altered GyrB structure. Point mutations in the R136 residue are the most prevalent resistance mechanism, specifically R136H, R136L and R136S. This amino acid forms two hydrogen bonds with the coumarin ring of novobiocin to sterically block antibiotic binding. Experimental mutations D73E, G77A, G77S, I78A, I78L, T165A and T165V have also been shown to significantly increase the IC_{50} of novobiocin (Gross *et al*, 2003) Other mechanisms of novobiocin resistance include the MexAB-OprM pump which actively transports novobiocin out of the cell in *P. aeruginosa* (Poole, 2001) Though OmpA has been implicated in novobiocin resistance in *A. baumannii* (Kwon *et al*, 2017), it is not believed to contribute towards resistance in *K. pneumoniae* (Lobet *et al*, 2009).

In the absence of new antibiotics, it is crucial that we maintain the utility of currently available drugs. One way to do this is to gain a thorough understanding of all resistance mechanisms, their spread, and the conditions necessary for their persistence within a population. Transposon Directed Insertion-site Sequencing (TraDIS) is a technique which combines transposon mutagenesis with next generation sequencing (NGS), and can be used to assay whole genomes for novel drug targets (Langridge *et al*, 2009). As little is known about novobiocin resistance in *K. pneumoniae*, TraDIS was used to sequence 12,576 novobiocin-resistant mutants and identify the genes whose

inactivation was essential for resistance (unpublished data). The results predicted that 46% of all Tn*5* transposon insertions were located within the AraC family transcriptional regulator *ramR*, which is a known regulator of the multi-drug resistant phenotype in *K. pneumoniae* (De Majumdar *et al*., 2014). Normally, *ramR* represses expression of *ramA*, but inactivation of this gene causes the upregulation of *ramA* and subsequent upregulation of the AcrAB-TolC efflux system (Hentscke *et al*, 2010). Others genes predicted to be implicated in novobiocin resistance included the Fe-S protein assembly co-chaperone *hscB*, and NAD(P)H-flavin reductase *fre*, which together represented less than 2% of all transposon insertions. Neither of these genes are known to be involved in antibiotic resistance.

This study aimed to expand on previous work and validate these resistance mechanisms in two ways. Firstly, by replicating the original TraDIS experiment to generate resistant mutants which were screened for the presence of Tn*5* transposon within the *ramR*, *hscB* and *fre* genes. Secondly, by creating *K. pneumoniae* null mutants for each of the candidate resistance genes. The Red system described by Datsenko & Wanner (2000) was chosen because of the high frequency of homologous recombination with as little as 40 bp homologous DNA sequence. The system encodes three components: γ binds to the endogenous RecBCD and SbcCD nucleases to prevent the digestion of the linear DNA fragments, *exo* is an exonuclease which degrades linear double stranded DNA and β binds to the single stranded DNA created by *exo* and promotes annealing to a complementary target within the cell.

MATERIALS AND METHODS

Strains and growth conditions

A list of the bacterial strains and plasmids used in this study is detailed in Table 1. Bacteria were grown at 37°C in sterile Luria-Bertani (LB) agar or broth at 200 RPM, supplemented with antibiotics at the following concentrations: 750 µg ml^{-1} novobiocin, 50 µg ml^{-1} kanamycin, 30 µg ml^{-1} chloramphenicol, 100 µg ml^{-1} carbenicillin. Competent cells were recovered in sterile Brain Heart Infusion (BHI) following electroporation. *K. pneumoniae* ECL8 was kindly provided by Dr. Michelle Buckner.

Table 1. Bacterial strains and plasmids used in research project 1

Strain or plasmid	Genotypic or phenotypic description	Antibiotic resistance	Source
ECL8	Wild-type *Klebsiella pneumoniae* ECL8	MDR	Dr. Michelle Buckner, The University of Birmingham, UK
ECL8::Tn*5*	*Klebsiella pneumoniae* ECL8 transposon library	MDR	unpublished data
MG1655	*Escherichia coli* MG1655 was used as a host for plasmids	N/A	Dr. Jack Bryant, The University of Birmingham, UK
DH5α	*Escherichia coli* [fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17] was used as a host for plasmids.	N/A	NEB
pDOC-K	Kanamycin donor plasmid	Kanamycin	(Lee *et al*., 2009)
pKD46	Red-recombinase helper plasmid	Carbenicillin	(Datsenko & Wanner, 2000)
pDS1	Red-recombinase helper plasmid	Kanamycin	This study
pKD3	Template plasmid containing FRT-flanked *cat* cassette	Chloramphenicol	(Datsenko & Wanner, 2000)

DNA manipulations

Chromosomal DNA was extracted using the RTP Bacteria DNA Mini Kit (Stratec) and plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen). DNA concentrations were measured using the Qubit 2.0 fluorometer. Agarose gel electrophoresis was performed as per Ausubel *et al*. (1989), and subsequent DNA purification was performed using the QIAquick Polymerase Chain Reaction (PCR) Purification Kit (Qiagen). Competent cells were prepared and electrophorated according to Sambrook *et al*. (1989). Restriction endonucleases were used according to manufacturer's instructions (Promega). PCR amplifications were performed in accordance with Ausubel *et al*. (1989), using *Taq* polymerase (Phusion, New England Biolabs) and primers synthesised by Eurogentec (Table 2).

Restriction free cloning

Restriction free (RF) cloning was performed as per Van Den Ent & Löwe (2006) using the thermal cycler conditions detailed in Table 3.

Table 2. **Primers used for amplification of target DNA in research project 1**

The nucleotide sequence, restriction sites and corresponding description of each primer are indicated.

Identifier	5' – 3' sequence	Restriction Sites	Description
A	ATC TAA AGT ATA TAT GAG TAA ACT TGG TCT GAC Aga tga gta ttc aac att tcc gtg tc	N/A	*kan* RF cloning F
B	GAT AAA TGC TTC AAT AAT ATT GAA AAA GGA AGA GTt tac caa tgc tta atc agt gag gc	N/A	*kan* RF cloning R
C	TCC TCA ACC CCA CGG TCA CGC CGC GCG TGG GGT TTT AAT TTA GAT GCA TGA CCC TGA GGT TAC Tat ggt gta ggc tgg agc tgc ttc	N/A	*hscB* disruption cassette F
D	TCA GGC CGG GCT CAC TAA TTT GTA ATA AGG CCA TAA TTG CTT CCG AAA ATT AAA AAT CGA GCA GTT TTT Cca tat gaa tat cct cct tag	N/A	*hscB* disruption cassette R
E	ATC TTT AAA CAA CAG TAA GCG GGG CTA ACG CCG CTG TTT TGC TCT ATT GAC CCG ACA GAG GGG ACG Cat ggt gta ggc tgg agc tgc ttc	N/A	*fre* disruption cassette F
F	CAG GTT TAG TTG CCG TTC TTC CCG CCT GTC AGG GGC GGG TTT TTT ATT TTC AGA TAA ACG CAA ACG CAT Cca tat gaa tat cct cct tag	N/A	*fre* disruption cassette R
G	GCC GTT GCA GCG ACC TGG TCA GAC GTG CCA AGA TCG GCG GTT TGT TTA AAC CTG Cgt ggt gta ggc tgg agc tgc ttc	N/A	*ramR* disruption cassette F
H	ATA CGG TGA GCG CAG GGA TGC AGT GTT TCC GGC GTC ATT AGG CGT CCG CCT CAT GCA Gca tat gaa tat cct cct tag	N/A	*ramR* disruption cassette R
I	CTG GAC TTC GTC AAA GAA GG	N/A	*hscB* F
J	CAG CAG ATA GCG GCC TTG AT	N/A	*hscB* R
K	GGC TGG ATG CCA CCA ACA AAT GG	N/A	*fre* F
L	AGC GAA AGG GTA TCT CAA CG	N/A	*fre* R
M	CGA TGA AAG TCG TCA AGA CG	N/A	*ramR* F
N	TAA ACA CGT CGT ACT CTG GC	N/A	*ramR* F
O	TTA TAC GCA AGG CGA CAA GG	N/A	*cat* F
P	GAT CTT CCG TCA CAG GTA GG	N/A	*cat* R
Q	GAA CAA GAT GGA TTG CAC GC	N/A	*kan* F
R	GCC TTC TAT CGC CTT CTT GA	N/A	*kan* R

Table 3. PCR parameters for restriction free cloning

PCR Run	Description	Temperature	Cycles	Time (Minutes)
Primary PCR	Initial Denaturation	98°C	1	4
	Denaturation	98°C	} 35	0.5
	Annealing	65/55°C		0.5
	Extension	72°C		1.5
	Final Extension	72°C	1	7
	Hold	4°C	∞	∞
Secondary PCR	Initial Denaturation	98°C	1	0.5
	Denaturation	98°C	} 15	0.13
	Extension	72°C		3.5
	Final Extension	72°C		8
	Hold	4°C	∞	∞

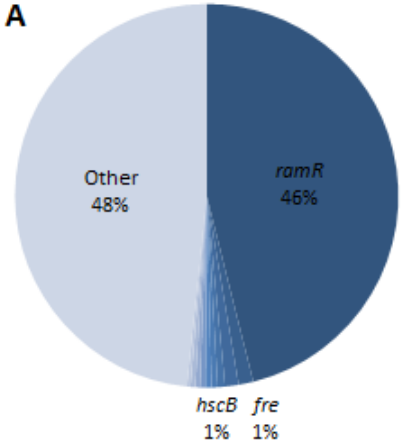
Gene Disruption

Knockouts were created using an adapted version of the method described by Datsenko & Wanner (2000). ECL8 transformants carrying the Red recombinase expression plasmid pDS1 were grown in LB supplemented with kanamycin, 0.5 % L-arabinose and 0.7 mM EDTA until the culture reached an OD_{600} 0.4. Electrocompetent cells were shocked with gene disruption cassettes amplified from template plasmid pKD3 using the BioRad GenePulser II (1.4 kV, 25 µF, and 200 Ω). Transformed cells were selected for on LB agar supplemented with chloramphenicol. Colonies were screened for the presence of the chloramphenicol resistance gene *cat* using primers O and P (Table 2).

RESULTS

Transposon inactivation of novobiocin resistant mutants

A



	Gene	UIP	Reads
■	*ramR*	96	5796
■	*mutL*	6	156
■	*me*	21	143
■	*fre*	36	78
■	*pgaA*	1	65
■	*hscA*	40	62
■	*hscB*	32	56
■	*pgm*	29	51
■	*mioC*	9	50
■	*BN373_44791*	32	40
■	Other	5684	6079
Total:	**2599**	**5986**	**12576**

B

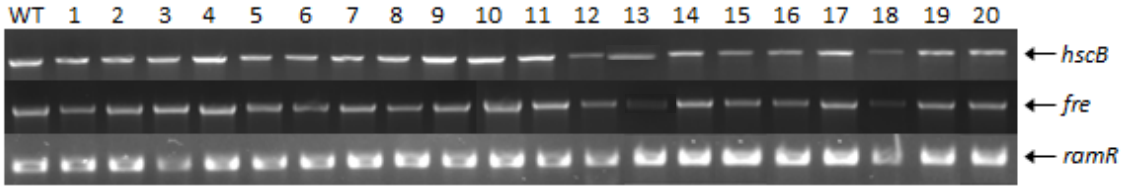


Figure 3: Locating the Tn*5* transposon within novobiocin resistant mutants

(A) Distribution of the Tn*5* transposon within 12,576 novobiocin resistant mutants, including the number of unique insertion points (UIP) and sequencing reads for the top 10 genes. This data was obtained by pooling the mutants and extracting DNA using the QIAamp DNA blood mini kit. Samples were fragmented by sonication, concentrated and prepared for NGS using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) and KAPA Library Quantification Kit for Illumina Platforms (KAPA Biosystems), as per manufacturer's instructions. NGS was performed on the Illumina MiSeq and data was analysed using in-house scripts (B) Screening of 80 novobiocin resistant mutants for the presence of the *hscB*, *fre* and *ramR* genes using colony PCR. Samples were run on a 1% agarose gel, and show gene sizes comparable with the wild-type (860 bp, 989 bp, and 713bp respectively) indicating the absence of a 1221 bp Tn*5* transposon insertion. Data for mutants 1-20 shown.

NGS of 12,576 novobiocin resistant mutants predicted 59 genes whose inactivation was essential for antibiotic resistance. Gene essentiality was calculated using the $\log_2$ likelihood ratio (LR). Firstly, data was normalised by dividing the number of transposon insertions by the length of a gene to calculate the insertion index. The insertion index was then plotted against gene frequency, and a gamma distribution was applied to the resultant bimodal peak to determine the LR. Genes with an LR greater than 3.6 were predicted to be essential, and those with an LR less than -3.6 were non-essential. Those with an LR between -3.6 and 3.6 were classed as indeterminate.

Of the mutants sequenced, 46% contained a transposon insertion within the *ramR* gene which equates to a $\log_2$ likelihood score of 173.5. A further 1% contained transposon insertions within the *hscB* gene and 1% within the *fre* gene (Fig. 3A). As previously uncharacterised mechanisms of antibiotic resistance, genes *hscB* and *fre* were selected for further investigation. With $\log_2$ likelihood scores of 4.8 and 6.2 respectively challenge, they also challenge the LR cut-off to ensure that transposon inactivation of these genes resulted in novobiocin resistance.

To test the reproducibility of the original TraDIS data set 80 novobiocin resistant mutants were generated by exposing the ECL8::Tn*5* library to LB agar supplemented with a lethal concentration of novobiocin. The Minimum Inhibitory Concentration (MIC) of novobiocin was determined to be 750 µg

ml^{-1} (data not shown). The mutants were screened for the presence of Tn*5* within the *ramR, hscB* and *fre* genes by colony PCR, using primers C-H which bound to the 5' and 3' ends of the gene (Table 2). Figure 3B shows that all fragments were comparable with the wild-type (WT) strain and that the 1.2 kB Tn*5* transposon was not located within genes *ramR*, *hscB*, and *fre* (only mutants 1 - 20 shown). These screening results suggest the distribution of transposon insertions differs from the original TraDIS dataset, and that mutations in *ramR* might not be as prevalent as first thought.

Constructing the Red-recombinase plasmid pDS1

Though transposon insertion were not located within genes *ramR*, *hscB* or *fre*, earlier research indicated that inactivation of these genes gave rise to novobiocin resistance. To validate these resistance mechanisms, the Red-recombinase system was selected to generate null mutants.

Traditional red-recombinase plasmid pKD46 (Datsenko & Wanner, 2000) is unsuitable for making gene knockouts in ECL8, due to the presence of the β-lactamase resistance gene *ampH* which confers resistance to ampicillin (Fookes *et al*, 2013). The Red helper plasmid is derived from pINT-ts and comprised of *araC-*P_{araB}, the lambda red recombineering system (γ, β, and *exo*) and 2,154 nucleotides of phage λ. The kanamycin resistance (*kan*) cassette was amplified from donor plasmid pDOC-K using primers A & B (Table 2) which contained 35 nt homology to pKD46 and 24 nt homology to the *kan* insert sequence. The mega-primer was cloned in to pKD46 to replace

the ampicillin resistance (*bla*) cassette using restriction free cloning. The resultant Red recombinase plasmid was designated pDS1 (Fig. 4A) and transformed into chemically-competent DH5α. Transformants were selectively recovered on kanamycin.

A test digest was performed to confirm the uptake of the *kan* cassette. PstI cuts into *kan* to produce three 3731, 3484 & 247 bp amplicons respectively (Fig. 4A). Figure 4B shows that of the 10 candidates screened, all but one contained the resistance cassette. As PstI cuts into the *kan* cassette, the absence of a third band in transformant 4 indicates that cloning was unsuccessful. Plasmid to profile sequencing of pDS1 was performed in both directions using primers Q and R, to confirm the presence of the *kan* cassette (Fig. 4C, reverse orientation shown). To determine whether replacement of the *bla* cassette with the *kan* cassette had adversely affected the expression of the phage λ recombination genes *exo, bet* and *gam* in pDS1*,* samples were visualised on a 12% SDS-PAGE gel (Laemmli, 1970). The protein expression of pDS1 was compared with that of pDS1 induced with 0.5 % L-arabinose, though expression of the Red system was indeterminate (Fig. 4D). Together, this data confirms that pDS1 contains the *kan* cassette and is suitable for use during targeted gene deletion.

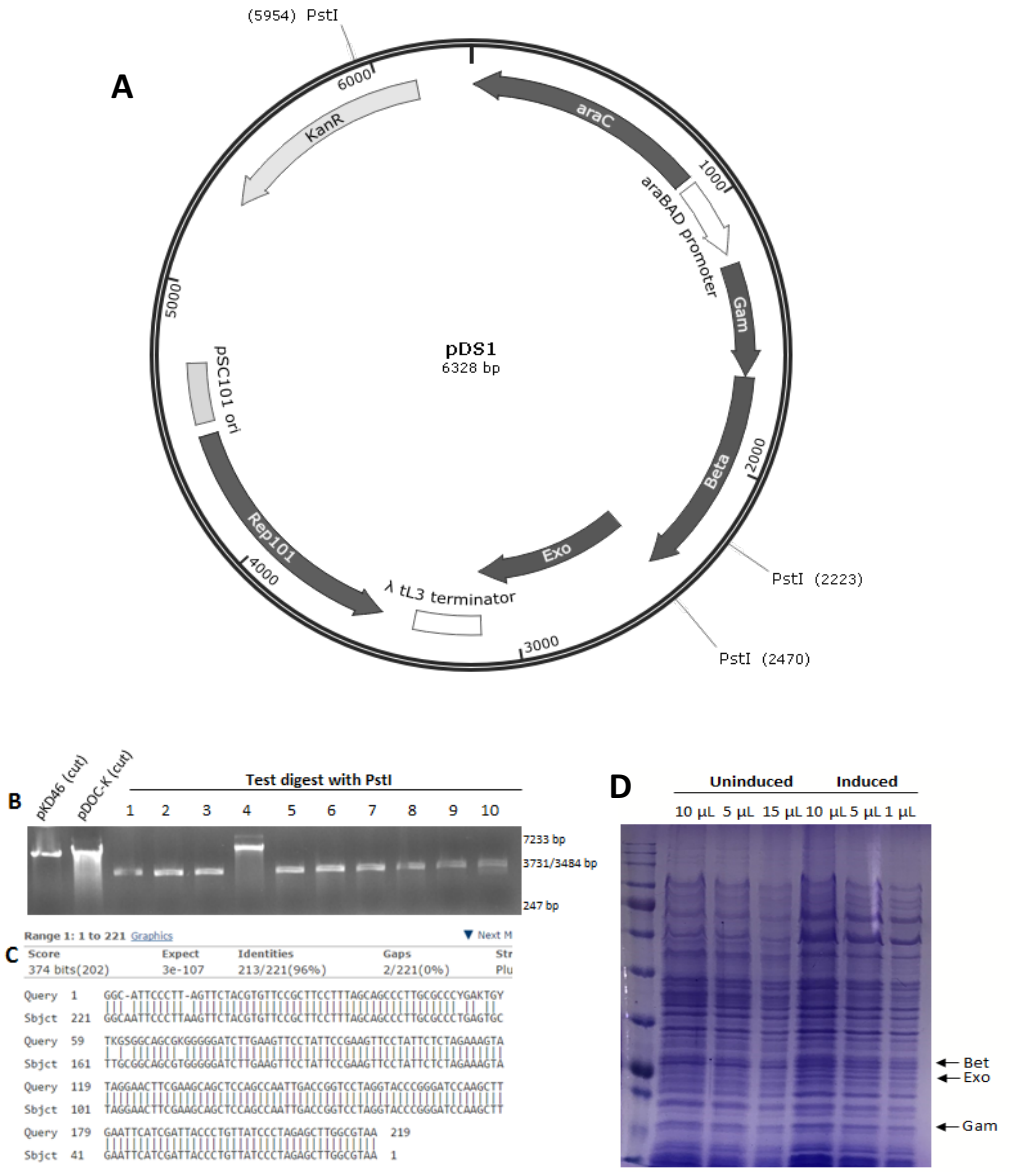


Figure 4: Construction of the Red helper plasmid pDS1

(A) Plasmid map of the red recombinase expression plasmid pDS1, including PstI restriction sites. pDS1 was created by replacing the *bla* cassette of pKD46 with the *kan* cassette from pDOC-K (B) Test digest confirms the presence of the *kan* cassette in pDS1. All transformants produced three 3731 bp, 3484 bp and 247 bp fragments as expected when digested with PstI, except transformant 4 (C) A BLAST alignment of plasmid-to-profile Sanger sequencing of pDS1 with pDOC-K, using reverse primer R specific for the *kan* resistance gene (Table 2). The two 221 nt sequences are analogous (alignment 96%, E-value 3 x 10^{-107}) (D) SDS-Page gel comparing the expression of Exo (24 kDa), Bet (28 kDa) and Gam (16 kDa) between uninduced ECL8 harbouring pDS1, and cells induced with 0.5 % L-arabinose. Protein expression was indeterminate.

The Red-recombinase system

Gene disruption cassettes were created to replace the gene of interest with a selectable antibiotic resistance cassette (Figure 5). The chloramphenicol resistance (*cat*) cassette was amplified from pKD3 by PCR, using primers C – H. These primers contain 20 nt homology to *cat* gene and ~ 60 nt homology to gene to be disrupted. To avoid the introduction of PCR errors, a high-fidelity DNA polymerase with proof-reading ability was used. The *cat* cassette was successfully amplified from pKD3, to create disruption cassettes for the targeted deletion of the *ramR*, *hscB* and *fre* genes (data not shown).

ECL8 harbouring the Red-recombinase plasmid pDS1 were grown in the presence of L-arabinose to induce expression of the Red system. Electrocompetent cells were then transformed with the disruption cassettes. Transformants were plated onto agar plated supplemented with chloramphenicol to select for the uptake of the disruption cassette. Resultant candidates were screened for the presence of the 1.1 kbp *cat* cassette using check primers I - N. Figure 6 shows that none of the chloramphenicol resistant candidates contained the disruption cassette, and that in-frame deletions were unsuccessful.

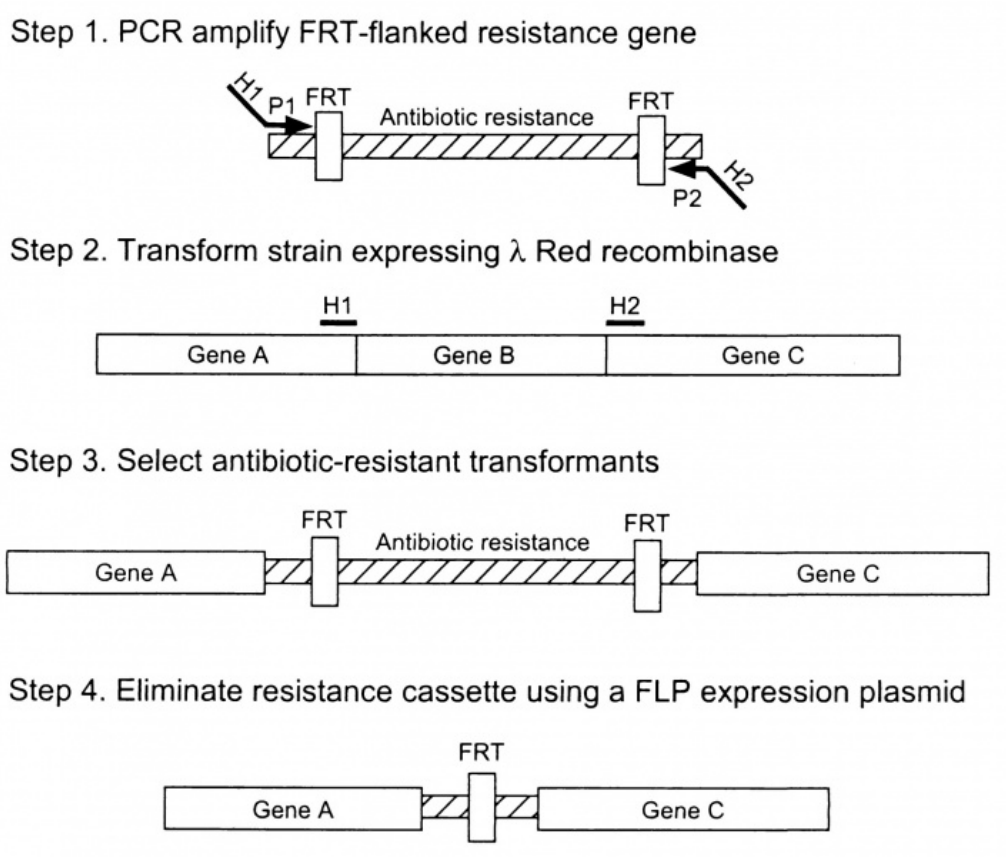


Figure 5: Overview of the Red-recombinase system

Step 1: To generate gene disruption cassettes, the flippase recognition target (FRT)-flanked chloramphenicol resistance (*cat*) cassette was amplified from pKD3 using primers C – H. **Step 2:** ECL8 harbouring pDS1 were grown in the presence of 0.5 % L-arabinose to induce expression of the Red system. Cells were electroporated with the disruption cassettes and recovered in BHI. **Step 3:** Antibiotic-resistant transformants were selected on 30 µg ml^{-1} chloramphenicol. **Step 4:** Resistance cassette eliminated using FLP expression plasmid pCP20. Figure taken from Datsenko & Wanner (2000)

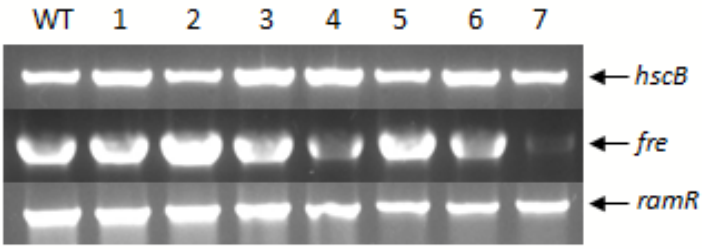


Figure 6: PCR to confirm the presence of the gene disruption cassette

PCR amplification of ECL8 genes *hscB*, *fre*, and *ramR* following transformation with gene disruption cassettes. Results are comparable to WT for all genes (*hscB* 860 bp, *fre* 989 bp, *ramR* 713 bp), indicating the absence of the 1.1 kbp disruption cassette.

DISCUSSION

The definition of an essential gene is debated in the literature. Only viable organisms can be sequenced, so genes which are essential for the survival of the organism are indicated by the absence of transposon insertions within that gene, which render those organisms none viable. To define an essential gene the ECL8::Tn*5* library used within this study used an LR < -3.6, which is more stringent than the cut-off of -2 used in the original TraDIS study (Langridge *et al*, 2009). This gives us greater confidence that the genes originally defined as essential are truly required for the survival or the organism. Correctly defining the essential gene list is imperative, because this list was compared against that of the novobiocin resistant mutants to identify the genes whose inactivation was required for resistance. Conversely, these genes are indicated by the presence of transposon insertions within the dataset. Here, an LR > 3.6 was used to define essential genes. However, as with any statistical tool, a level of error exists and though TraDIS has generated many large datasets to date, there is little *in vivo* validation to substantiate these predictions.

Of the 5006 genes located on the ECL8 chromosome, the inactivation of 59 genes was predicted to confer resistance to novobiocin. These included genes involved in DNA binding, metabolism and biofilm production, as well as several genes of unknown function which might represent novel resistance mechanisms. It would be interesting to explore the function of these uncharacterised genes in future work. The majority of the novobiocin

resistant mutants (46%), however, contained a Tn*5* insertion within the *ramR* gene. Surprisingly, it was not possible to locate a Tn*5* transposon within this gene, despite screening 80 mutants. This suggests that novocbiocin resistance occurring due to *ramR* inactivation might not be as prevalent as first thought.

Genes *hscB* and *fre* were also selected to challenge the sensitivity of the cut-off, with $\log_2$ likelihood scores of 4.8 and 6.2 respectively. When 80 mutants were screened, neither of these genes were found to contain a Tn*5* insertion but this is unsurprising given the low frequency of these insertions in the original dataset. Further attempts to determine the *actual* location of the transposon within the 80 mutants proved unsuccessful. A DNA concentration of 2-3 µg is required for Sanger sequencing, and neither commercial DNA extraction kits nor traditional phenol-chloroform extraction were able to yield a DNA concentration >1.3 µg. Further work is needed to identify the inactivated genes responsible for novobiocin resistance in these mutants. From here, the frequency of mutations would be compared against the original dataset to determine the reproducibility of the data.

The difference in the frequency of *ramR* insertions might be random, or it could be attributable to gaps in the original sequencing data which have created a bias. For instance, sequencing only mapped 4991 genes to the ECL8 reference genome (Fookes *et al*, 2013), implying that there are differences between the reference genome and the ECL8 strain used within this study. To

complete the genome, *de novo* sequencing is required. Furthermore, though the library contained an average insertion site every 17.18 bp, the flow cell only generated 5 million nucleotide sequencing reads. Langridge *et al* (2009) recommend between 7 – 11 million reads to represent the whole genome, so further sequencing is required to increase the density of the library.

Though transposable elements insert randomly within a gene, the Tn*5* transposon is reported to have a bias towards sequences with a high guanine-cytosine content (Green *et al*., 2012). It is also known that insertions at the 3' end do not always result in the inactivation of that gene. Thus, false positive results may occur whereby a functional gene contains a transposon insertion, yet bioinformatic analysis predicts that resistance is attributable to the transposon. DNA from lethal insertions might also be sequenced and appear as false positives. Repeating the experiment in liquid media and using flow cytometry to sort dead and live cells would remove any dead cells prior to sequencing.

As many resistance genes are located on plasmids, it is possible that novobiocin resistance was attributable to mutations on its 206,103 bp plasmid or second-site mutations elsewhere on the chromosome, rather than the insertion of a transposon within the gene. Similarly, NGS is unable to identify multiple transposon insertions within the same chromosome. Where genes are located on the same operon, polar mutations can occur where disruption of one gene may affect the expression of a neighbouring gene.

This may result in a false positive result whereby the transposon is detected in the first gene, and attributed to resistance, when in fact the resistance is actually caused by the altered expression of the second gene.

Further attempts to create null mutants to validate this data *in vivo* were unsuccessful. Historically, knockouts were created using restriction enzymes and ligases to re-order linear pieces of DNA. The Red-recombinase system was chosen because it enables sequence modifications such as insertions, deletions and point mutations at any point along the chromosome independent of restriction sites. As cloning occurs *in vivo* there is a high frequency of recombination events (estimated to be ~ 1 positive clone from every 10^5 colonies) (Datsenko & Wanner, 2000). Though chloramphenicol resistant colonies were observed, screening revealed the absence of the *cat* disruption cassette. It is possible that chloramphenicol resistance arose due to an increase in the expression of other chloramphenicol acetyltransferases (Martinez & Baquero, 2000) or because the Red system was not expressed following induction with L-arabinose. It was unclear whether proteins Exo, Bet and Gam were expressed using SDS-PAGE. A western blot using antibodies specific for the three proteins would provide a clearer indication of expression. Alternatively, there are many other methods of chromosomal gene inactivation including suicide vectors (Skrzypek *et al*, 1993) and gene doctoring (Lee *et al*, 2009).

One of the main limitations of this study is that only those resistance mechanisms arising from gene inactivation would be detected, and not those which require gene up-regulation. Future work should focus on these resistance mechanisms too, in order to gain a complete understanding of novobiocin resistance in *K. pneumoniae*. TraDIS has proved a useful tool for advancing the understanding of serum resistance in uropathogenic *E. coli* ST131 (Phan *et al*, 2013), and bloodstream infection in *Acinetobacter baumannii* (Subashchandrabose *et al*, 2015). TraDIS may also be used to increase our understanding of antimicrobial resistance, through much work is required to validate the huge datasets generated using this tool.

FUTURE PERSPECTIVES

Once null mutants have been confirmed and exposed to a growth-inhibitory concentration of novobiocin to confirm resistance, complementation plasmids should be used to restore gene function. From here, the mechanisms of resistance could be explored. Whilst inactivation of *ramR* is known to increase expression of multi-drug efflux pumps, it is not clear how inhibition of Fe-S protein assembly (*hscB*), or riboflavin reduction (*fre*) results in novobiocin resistance. Towards this aim, creating a TraDIS library in each of the Δ*hscB* and Δ*fre* strains would reveal those genes which are up- or down-regulated by inactivation of *hscB* or *fre*.

It would also be interesting to determine the genes which are advantageous for ECL8 survival under novobiocin stress. This could be done by exposing the

transposon library to a stressful, but not inhibitory, concentration of novobiocin. Sequencing of the library following over several passages would identify the essential genes which drop out of the mutant population over time. These genes could then be explored further as potential novobiocin resistance mechanisms.

A live host model could also be used to further increase our understanding of *K. pneumoniae* infection. TraDIS has been used in a similar way to determine the virulence genes essential for intestinal colonisation of livestock by *Salmonella* Typhimurium (Chaudhuri *et al*, 2013). However, there are many limitations with whole organism models: they are costly, and ethics limit the size and duration of study. Kill studies only allow the study of the infection at the time of death, which does not necessarily represent the whole infection process. An alternative approach would be to develop an *ex vivo* infection model such as the porcine lung model developed by Harrison *et al*, (2014) for the study of *P. aeruginosa* infection.

Finally, ECL8 also contains a 206,102 bp plasmid and the function of the majority of these genes is not known. Though the plasmid was not included in this study, many resistance mechanisms are plasmid mediated so it would be interesting to determine whether any of these genes involved in novobiocin resistance. These may present future anti-virulence targets as inactivation of these genes would decrease the virulence of *K. pneumoniae*.

Targeting IscR to inhibit Type III Secretion in *Yersinia pseudotuberculosis*

Abstract

Yersinia pseudotuberculosis is a Gram-negative enteropathogen and a model organism for the study of *Yersinia pestis*: the zoonotic pathogen responsible for the pneumonic, bubonic and septicemic plagues. Virulence factors including a Type III Secretion system (T3SS) and Yersinia outer proteins (Yops) are encoded on a 70 kB virulence plasmid (pYV). The Iron-sulphur cluster regulator (IscR) has been shown to bind upstream of T3SS master-regulator *LcrF* to increase Yop expression, and therefore represents an attractive virulence target. To develop a screening assay for IscR inhibitors, *Y. pseudotuberculosis* YpIII cut::attTn7(lux::Tc2) was transformed with pYV-Gm^R to create a *lux* reporter strain. Growth curves performed over 20 hours confirm this transformation had no adverse effect on growth, though the light emission of the reporter strain increased. This unexpected side-effect increases the overall sensitivity of the assay. Piericidin A1 was selected as a positive control to show that the assay can detect the increased growth phenotype that occurs when the T3SS is inhibited. Conversely, both WT pYV+/- strains and the *lux* reporter displayed concentration dependent growth inhibition, indicating that piericidin A1 has a bactericidal effect at concentrations above 0.04 μg/μL. We also developed an IscR expression vector which will be used for future structural studies. With further

development, these tools will be used to identify novel IscR inhibitors for future commercial development.

INTRODUCTION

The Yersinia genus includes three human pathogens: *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are enteropathogens acquired through the ingestion of contaminated food or water, whilst *Yersinia pestis* is the etiological agent of the pneumonic, bubonic and septicemic plagues. *Y. pestis* is believed to have diverged from *Y. pseudotuberuclosis* as recently as 5,000 years ago (Rasmussen *et al*, 2015), though it is probable that early *Y. pestis* strains did not contain the virulence factors associated with disease outbreaks. One pandemic that epitomises the devastation this organism can cause is the Black Death, which wiped out up to 50% of the European population between 347–1351 AD (Rasmussen *et al*, 2015).

Given their shared homology, *Y. pseudotuberculosis* is considered to be a model organism for the study of *Y. pestis*. Many of the virulence factors known to facilitate adhesion and colonisation in both organisms are located on a large 70 kb virulence plasmid (pYV) which encodes the structural components of the Type III Secretion System (T3SS) and Yersinia outer proteins H, E, A/O, M & J/P (Yops). Upon cell contact, the basal body of the T3SS assembles at the bacterial cell membrane and proton motive force is used to transport Yops through a hollow protein complex directly into the host cell cytoplasm. Here, Yops H, E, & A/O disorder actin cytoskeleton

arrangement and prevent further assembly, whilst YopM and YopJ modulate host signalling pathways (Trosky *et al*, 2008). Ultimately, these processes result in the death of the infected cell through induced apoptosis.

The resultant consequences to the host can be devastating: whilst gastroenteritis caused by *Y. pseudotuberculosis* is rarely fatal, the rapid development of *Y. pestis* infection carries a 100% mortality rate if left untreated. Symptoms are plague-dependent but include fever, gangrene and the characteristic lymph node swellings known as buboes. Contrary to popular belief 1000-2000 cases of this re-emerging disease are reported each year (CDC, 2017), and the first MDR *Y. pestis* strain was isolated from Madagascar in 1995 (Galimand *et al*, 1997). Given that there is no licenced vaccine for *Y. pestis* infection, there exists a need for novel treatments in the event of future outbreaks. Here, we discuss a novel anti-virulence drug target which would reduce pathogenicity in both *Y. pseudotuberculosis* and *Y. pestis*.

The Rrf2-family transcription factor, iron-sulphur cluster regulator (IscR) controls the formation of iron-sulphur clusters through regulation of the *isc* operon. Recently, IscR has also been shown to control T3SS expression and Yop secretion in *Y. pseudotuberculosis*, where an Δ*IscR* mutant was shown to be less pathogenic in mice (Miller *et al*, 2014). Other studies have also found IscR to be a global regulator of virulence in *Vibrio vulnificus* (Lim & Choi, 2014) and *P. aeruginosa* (Kim *et al*, 2009). IscR exists in two forms: holo-IscR

binds to both Type I and Type II operator sequences, whereas apo-IscR binds only to Type II sequences. It is believed that both holo-IscR and apo-IscR bind to a Type II motif in the *yscW-lcrF* operon, leading to increased expression of *LcrF* and subsequent transcription of the *Yop* genes (Miller *et al*, 2014). IscR therefore represents a system specific target for virulence through suppression of the T3SS expression. As the *Y. pseudotuberculosis* IscR protein shares 100% homology with that of *Y. pestis*, it is likely that inhibitors would also be effective treatments for the plague.

This project aimed to develop two tools in parallel that will be used to validate IscR inhibitors identified through *in silico* screening of a large compound library. Firstly, a lux-based screening assay was developed (Figure 7) based on Pan *et al*. (2009), who used a similar tool to identify several T3SS inhibitors. Light producing cells are used as an indirect measure of target inhibition: if a compound inhibits IscR and reduces T3SS expression, light output will be observed from an increased number of cells. Conversely, in the absence of IscR inhibition cells exhibit a slow-growth phenotype due to the burden of T3SS and Yop expression, and the light output from a decreased number of cells is reduced. Secondly, the construction of an expression vector with a 6 x histidine tag will be used for future IscR protein expression and purification, to determine the binding of any lead compounds identified through screening. Together, these tools will be used for the characterisation and development of IscR inhibitors.

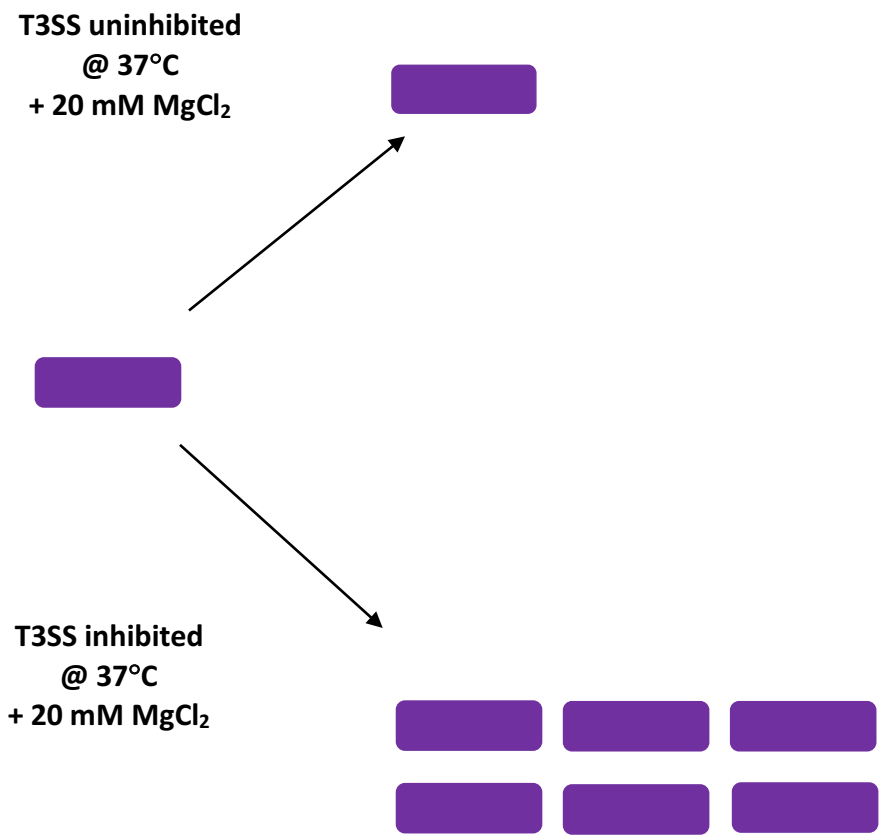


Figure 7. Lux-based screening assay

Lux based screening assay based on the burden associated with T3SS expression. The assay is performed at 37°C in the presence of a calcium chelating agent to enable assembly of the T3SS and Yop secretion. In the absence of IscR inhibition, the T3SS is expressed at a fitness cost to the bacteria resulting in a slow growth phenotype. The reduction in the number of viable cells results is measured as a decrease in luminescence from the *lux* reporter strain. Conversely, when IscR and subsequent expression of the T3SS are inhibited, an increase in luminescence from the reporter strain will be observed due to an increased number of cells. Figure adapted from Pan *et al*. (2009)

MATERIALS AND METHODS

Strains and growth conditions

The bacterial strains and plasmids used in this study are detailed in Table 4. *Y. pseudotuberculosis* strains were grown at 30°C in Yersinia Luria-Bertani (YLB) agar or broth supplemented with the appropriate antibiotics: 15 μg ml^{-1} nalidixic acid, 10 μg ml^{-1} gentamicin, 50 μg ml^{-1} kanamycin and 10 μg ml^{-1} tetracycline. Yop induction assays were performed at both 22°C and 37°C in a low calcium media prepared by supplementing BHI with 20 mM $MgCl_2$. *E. coli* strains were grown at 37°C in LB agar or broth.

DNA manipulations

Chromosomal DNA was extracted using the Wizard genomic DNA Purification Kit (Promega). Plasmid DNA was isolated using GenElute plasmid mini and midi prep kits (Sigma). Agarose gel electrophoresis was performed as per Ausubel *et al*. (1989), and subsequent DNA purification was performed using the Wizard SV Gel and PCR Clean-up System (Promega). Competent cells were prepared and electrophorated according to Sambrook *et al*. (1989) Restriction endonucleases and T4 ligase were used according to manufacturer's instructions (Promega). PCR amplifications were performed in accordance with Ausubel *et al*. (1989), using primers synthesised by Eurofins Genomics (Table 5).

Type III secretion assay

Yop secretion was visualised using the method previously described by Miller *et al.* (2014) Samples were resuspended in 30 µl Phosphate Buffered Saline (PBS) and boiled for 10 minutes prior to visualisation on a 12% SDS-PAGE gel (Laemmli, 1970).

Growth curves

Overnight cultures of each strain were diluted to OD_{600} 0.01 and incubated for 4 hours at 30°C. Cells were pelleted at 12,000 x *g*, washed twice with YLB, diluted to a final concentration of OD_{600} 0.006, and triplicate 200 µl aliquots of each culture were transferred into a UV-sterilised black 96-well microtiter plate. Bioluminescence and turbidity were measured every 30 minutes over 40 cycles on a combined luminometer-spectrophotometer (Tecan Infinite F200 Pro). Luminescence is displayed as relative light units (RLU) divided by OD_{600}.

Table 4. Bacterial strains and plasmids used in research project 2

Strain or plasmid	Genotypic or phenotypic description	Antibiotic resistance	Source
YpIII	Wild-type *Yersinia pseudotuberculosis*	Nalidixic acid	(Rosqvist et al., 1988)
pYV+	YpIII containing virulence plasmid pYV	Nalidixic acid	The University of Nottingham, UK
pYV-	YpIII lacking virulence plasmid pYV	Nalidixic acid	The University of Nottingham, UK
JP140	YpIII cut::attTn7(lux::Tc2)	Nalidixic acid, Tetracycline	The University of Nottingham, UK
DS1	YpIII cut::attTn7(lux::Tc2) pYV::cut::YopJGmGfpMut3	Nalidixic acid, Tetracycline, Gentamicin	This study
DH5α	*Escherichia coli* [fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17] Host for plasmids	N/A	Thermo Fisher
pYV-Gm^R	pYV::cut::YopJGmGfpMut3	Gentamicin	The University of Nottingham, UK
pET24b(+)	pET-24b(+)AgrB1Syn N-X	Kanamycin	EMD Biosciences

Table 5. Primers used for amplification of target DNA in research project 2

Target DNA was amplified using the primers listed below. The nucleotide sequence for each primer is detailed, and restriction sites, where applicable, are underlined.

Identifier	5' – 3' Sequence	Restriction Sites	Description
S	CCTCTAGACGATTATGGTGCACTCTC	XbaI	Gm F XbaI
T	GATCTAGACGATTATGGTGCACTCTC	N/A	Gm Out R
U	TCGATCGTCGAACCTTCACT	N/A	YopJ Out F
V	GATCAGCGATGAGATGTCTG	N/A	YopJ Out R
W	CTTTACATATGAGACTGACATC	NdeI	IscR F NdeI
X	TTCCTCGAGTGCGCGCAGATTG	XhoI	IscR R XhoI

MIC determination

The MIC of piericidin A1 was determined using an adapted version of the macrodilution method described by Andrews (2001). Briefly, 200 μl aliquots of each culture were transferred into a UV-sterilised black 96-well microtiter plate, inoculated with either 0.005, 0.01, 0.02, 0.04, 0.08, or 0.16 μg/μL of a 100 μg/100 μL piericidin A1 solution dissolved in Dimethyl Sulfoxide (Sigma). Bioluminescence and turbidity were measured on the Tecan Infinite F200 Pro as above. The MIC was determined to be the lowest concentration of antibiotic at which there was no visible growth.

RESULTS

Construction of reporter strain DS1

Bioluminescence was selected as an indirect measure of T3SS inhibition in our screening assay, as light output is a stable and selectable marker of cell growth. The luciferase gene cassette (*lux*) containing strain JP140 was transformed with pYV-Gm^R, which encodes an aminoglycoside-(3)-N-acetyltransferase, to ensure the maintenance of pYV over the duration of the assay. It is known that the virulence plasmid is carried at a fitness cost to the population, and though most bacteria lose the plasmid over time a sub-set of the population retain a functional pYV (unpublished data). The introduction of an antibiotic resistance marker allows the application of a selective pressure which ensures the plasmid is maintained over the duration of the assay.

Transformants were screened by colony PCR for the presence of pYV-Gm^R using primers S & T (Table 5), which bound within the gentamicin resistance cassette. Plasmid uptake was identified by the presence of a 638 bp fragment on a 1% agarose gel and all but one of the ten colonies screened contained pYV-Gm^R (Figure 8). To confirm the absence of WT pYV in these colonies, further screening was performed using primer pair U & V (Table 5) which bound within YopJ. Colonies which only harboured pYV-Gm^R did not produce a 2443 bp fragment corresponding to the WT virulence plasmid. Colony four was the only transformant to produce a single 4536 bp band, indicating the

presence of pYV-Gm^R only (data not shown). Colony 4 was designated reporter strain DS1, and will be used in further assay development studies.

Testing reporter strain

To determine whether transformation with pYV-Gm^R had adversely affected DS1, the growth and *lux* expression profile of the reporter strain were compared with that of parent strain JP140 over 20 hours. Figure 9A shows that transformation with the plasmid has not adversely affected the growth rate of DS1, which aligns with both that of JP140 and WT YpIII. Interestingly, DS1 produced up to 2.5 fold more Relative Light Units (RLU)/OD_{600} compared to JP140 at 22°C and 37°C, although both strains are approximately 15% more luminescent at 37°C (Figure 9B). A Yop induction assay was also performed to confirm that T3SS assembly and Yop secretion remained functional following plasmid transformation. When the T3SS was induced at 37°C in a low calcium media, the secretion of Yops B, D & E were detected in both strains, but at a reduced quantity in DS1 (Figure 9C) Taken together these results suggest that whilst transformation with pYV-Gm^R has reduced the Yop expression profile of DS1, the luminescence of the strain has increased which will increase the overall sensitivity of the assay.

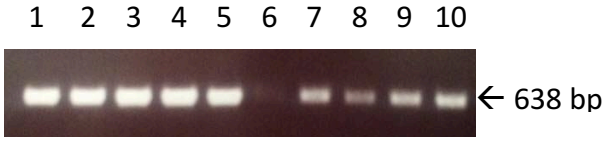


Figure 8. Transformation of JP140 with pYV-Gm^R to produce reporter strain DS1

Colony PCR of 10 JP140 colonies which were transformed with pYV-Gm^R and plated onto YLB supplemented with gentamicin to select for the uptake of the plasmid. A 1% agarose gel was used to visualise PCR amplifications using primers that bind within the gentamycin resistance cassette. Of the 10 transformed colonies screened, all contained pYV-Gm^R except colony six.

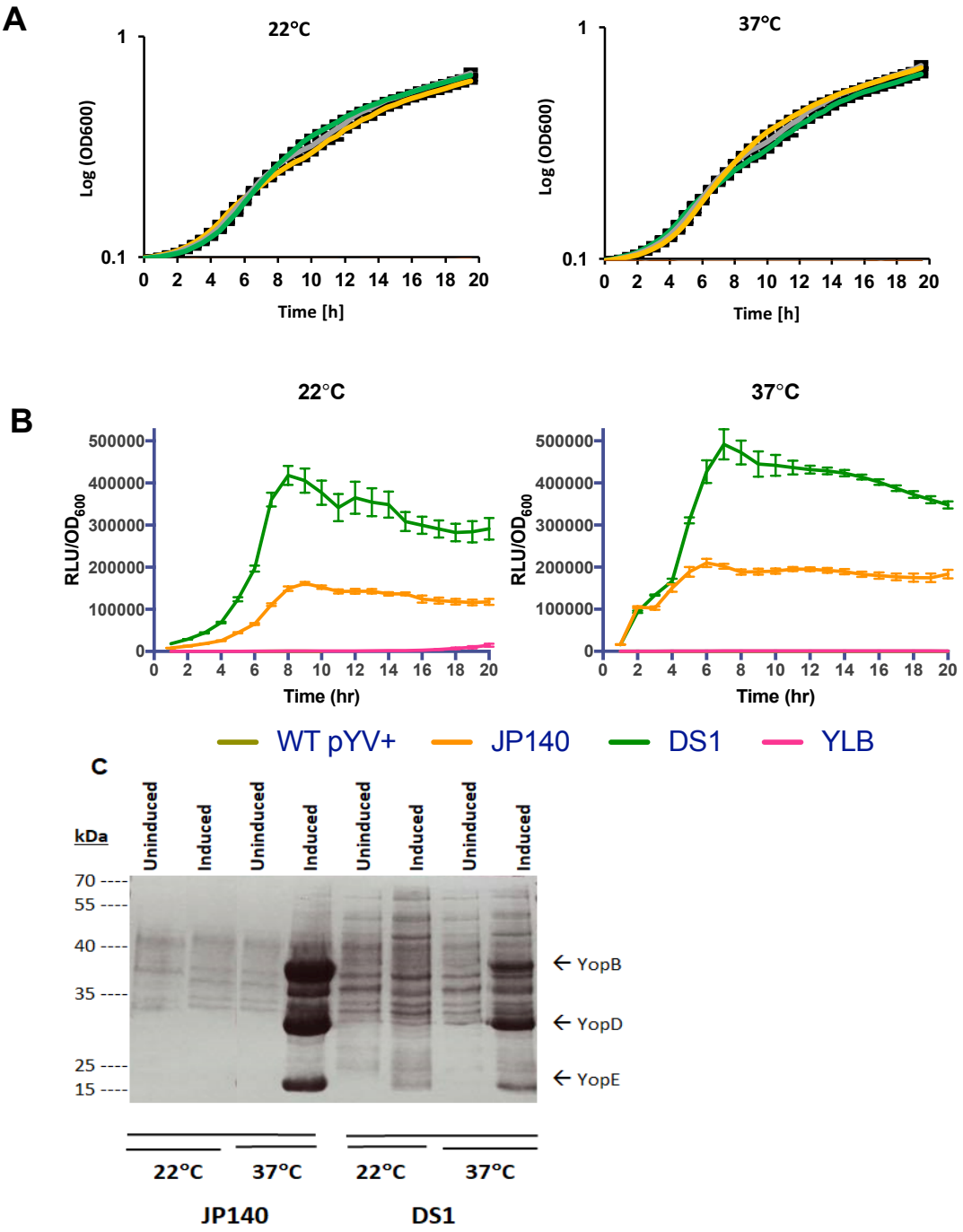


Figure 9. Growth rate, luminescence and Yop expression of DS1

(A) Growth curve displaying OD_{600} vs Time over 20 hours at 22°C and 37°C for all strains (B) Growth curve displaying RLU/OD_{600} ± SD over 20 hours at 22°C and 37°C for all strains (C) Yop induction assay comparing secretion of YopB (40 kDa), YopD (30 kDa) and YopE (25 kDa) in YLB vs low calcium media at 22°C and 37°C. Yop production is observed in low calcium media at 37°C for both strains, though the level of Yop expression is reduced in the reporter strain.

MIC Determination

To determine if the *lux* reporter strain can accurately detect IscR inhibition, the growth rate of DS1 was monitored in the presence of known T3SS inhibitors which display the same phenotype. Piericidin A1 (Sigma) is a ubiquinone mimetic (Figure 10A) produced by *Actinobacteria* which inhibits Complex I of NADH-ubiquinone oxidoreductase so it is unable to reduce coenzyme Q, thus blocking electron transport. Piericidin A1 has recently been shown to inhibit Yop secretion in *Y. pseudotuberculosis* by preventing the formation of YscF polymers and subsequent T3SS needle tip formation (Morgan *et al*, 2017).

We predicted that the addition of piericidin A1 would increase the number of cells in strains possessing a functional pYV, due to the inhibition of the T3SS. Conversely, figure 10B shows that piericidin A1 actually inhibits growth in a concentration dependent manner in both pYV+ and pYV- strains. At a concentration of 0.08 μg/μL, growth is completely inhibited in WT $pYV^{+/-}$ strains, but DS1 is slightly more resistant with an MIC of 0.16 μg/μL. This data suggests that at concentrations above 0.04 μg/μL, piericidin A1 has a bactericidal effect on cells. Further Yop induction assays are required to determine the effect of piericidin A1 concentration on Yop secretion.

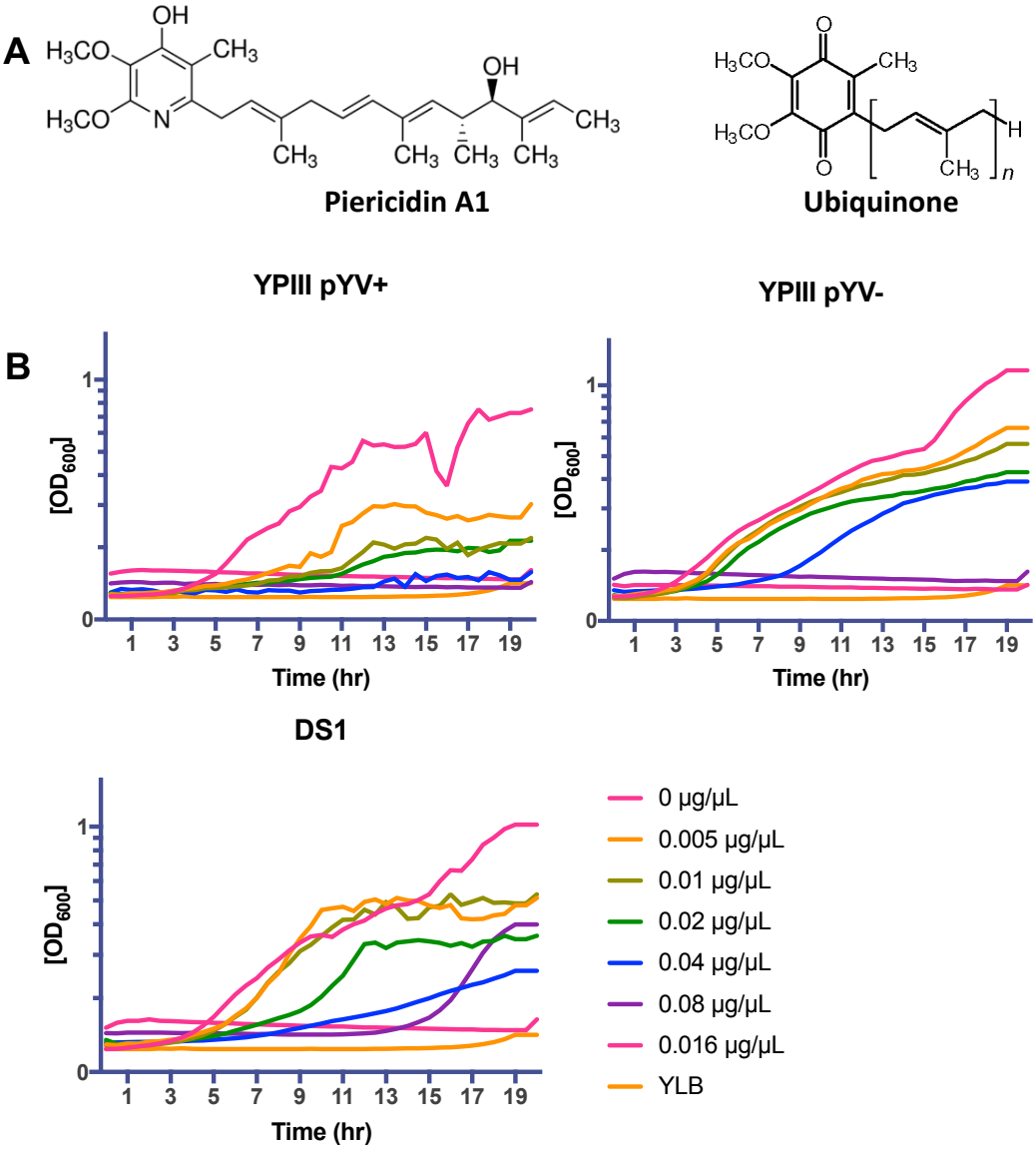


Figure 10. Piericidin A1

(A) Structural comparison of Piericidin A1 and ubiquinone (B) Growth curves showing the concentration dependent response of YpIII pYV$^{+/-}$ and DS1 strains to Piericidin A1 over 20 hours at 37°C. Growth is displayed as [OD_{600}].

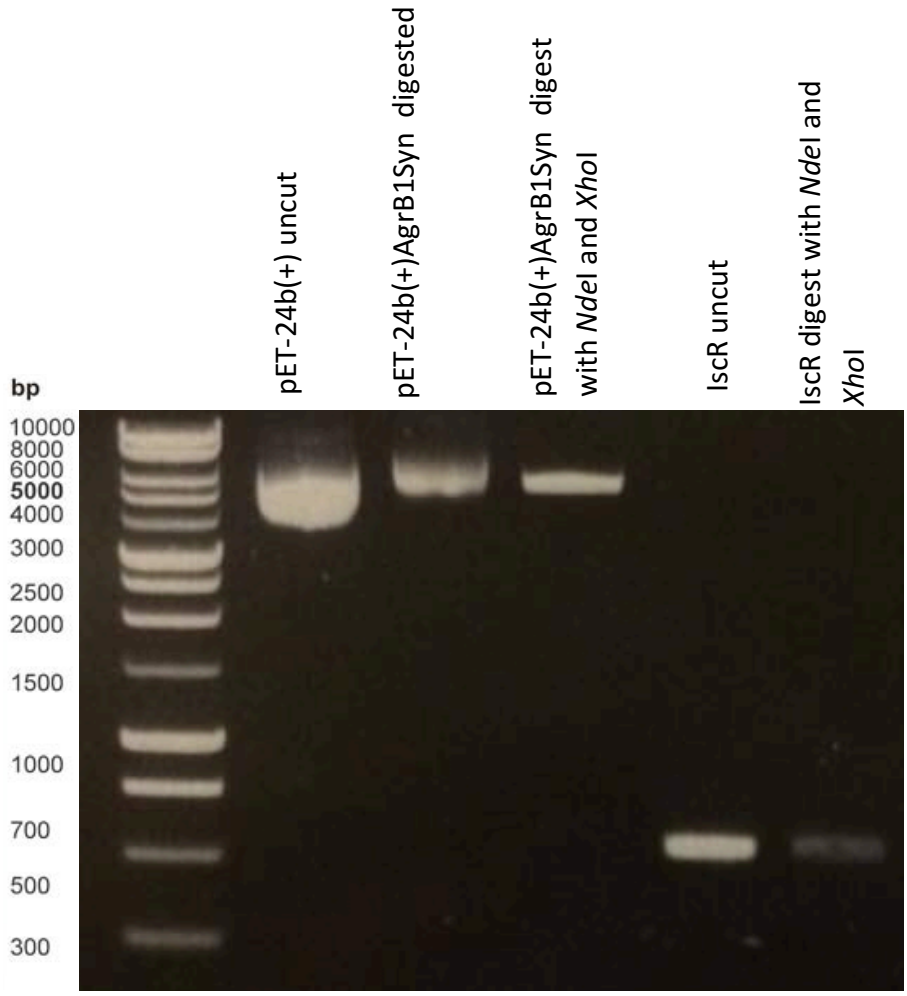


Figure 11. Expression vector constructon

Amplification, digestion and purification of vector and insert: 5.3 kB pET-24b(+) uncut, pET-24b(+)AgrB1Syn N-X uncut, pET-24b(+)AgrB1Syn double digest with *Nde*I and *Xho*I produces a 6159 bp and an 850 bp fragment (not visible with only 5 µl sample loaded), 509 bp IscR fragment amplified from YpIII, IscR fragment digested with *Nde*I and *Xho*I.

Constructing the expression vector

An expression vector was constructed to express and purify IscR for future structure studies with IscR inhibitors identified through *in silico* screening. IscR was amplified from WT YpIII using primers W & X (Table 5), which contain *Nde*I and *Xho*I engineered restriction sites respectively. The cleaned fragment was then digested with *Nde*I and *Xho*I and the resultant 495 bp fragment was excised for cloning into similarly digested pET-24b(+). Expression vector pET-24b(+) was selected due to the presence of a C-terminal hexa histidine-tag which will be used to isolate the recombinant protein during affinity purification. Figure 11 confirms that both vector and insert were isolated and digested effectively, in preparation for future ligation and transformation to complete construction of the expression vector.

DISCUSSION

We report on the development of a *lux* based screening assay for the identification of IscR inhibitors. A constitutively light reporter strain DS1 was constructed by transforming JP140 with the plasmid pYV-Gm^R, which contains an insertion within the YopJ gene. YopJ is a serine/threonine acetyltransferase which prevents the phosphyorlation of MAPK and IkB kinase-β signalling kinases to ultimately block cytokine secretion (Trosky *et al*, 2008) As YopJ is the last effector protein to be secreted, it was thought that inactivation of this gene would not impact expression of the T3SS or earlier Yop secretion. Though there appears to be no impact on growth rate of the reporter strain, our data suggests that transformation has altered the luminescence and Yop expression profiles of the reporter strain (Fig. 9A & B). Other studies have found that Δ*yopJ* strains are more virulent (Brodsky & Medzhitov, 2008) so it would be interesting to use the reporter strain for a survival assay in a macrophage cell line, and see if our strain behaves the same way.

Temperature is an important environmental parameter for Yop secretion: at temperatures below 30°C the pYV genes encoding the T3SS are expressed, but those of the Yop effector proteins are not. At approximately 37°C the DNA-binding protein LcrF, known as the master regulator of the T3SS, binds directly to promoters of the Yop genes to induce expression (Schwiesow *et al*, 2015). It is no coincidence that 37°C is also the core body temperature of the mammalian host. During the transition between the flea vector and host,

T3SS assembly and effector protein secretion only occurs upon host cell contact. As expression of the T3SS carries a fitness cost to the bacteria, this temperature-dependent switch enables the regulation of virulence. Accordingly, all growth curves were repeated at both 22°C and 37°C to observe the effect of Yop secretion on growth. At 37°C, there is both an increase in growth rate and luminescence of the reporter strain which aligns with the increase in virulence at higher temperatures (Fig. 9A & B).

Another environmental parameter important for Yop secretion is extracellular calcium. When millimolar concentrations of calcium are present mimicking host cell conditions, the injectisome assembles but Yop secretion is inhibited. *In vitro*, the addition of a calcium chelating agent triggers massive up-regulation of the genes encoding the T3SS, and subsequent Yop secretion (Dewoody *et al*, 2013). The low calcium response is conserved between all three pathogenic *Yersinia* species, but exactly how Ca^{2+} is able to regulate T3SS expression is not known (Schwiesow *et al*, 2015). In our Yop induction assays, samples were induced with $MgCl_2$ to mop up any Ca^{2+} ions. Figure 9C shows confirms Yop expression only occurs under induced conditions at 37°C as expected.

To determine the suitability of the reporter strain, T3SS inhibitor piericidin A1 was used as a positive control. Morgan *et al* (2017) found that a piericidin A1 concentration of 71 μM (0.029 μg/μL) was approximately twice the 50% minimum inhibitory concentration for Yop secretion. Due to material

constraints, it was not possible to repeat the experiment in triplicate or investigate the effect on Yop expression. However, we suggest that at concentrations above 0.08 μg/μL piericidin A1 has a bactericidal effect on cells. It is probable that piericidin A1 has a secondary target on the cell surface in addition to the T3SS. Whilst other T3SS inhibitors have been described in the literature (Pan *et al*, 2008), they are commercially unavailable and so cannot be used as alternative positive controls.

We also show the construction of a vector for the protein expression of IscR. Whilst restriction enzyme cloning is the backbone of molecular cloning, it relies on the use of restriction enzymes and sequence modifications can only be made at the appropriate cut sites. Attempts to ligate the vector and insert were unsuccessful, largely due to the absence of a selectable marker such as a blue-white screen. The insert and vector will be ligated in future work, and pET-24b(+)IscR will be cloned into an *E. coli* strain lacking T7 RNA polymerase expression such as NovaBlue which facilitates the uptake of the recombinant plasmid. Assuming that protein expression and purification of IscR generates crystals and a structure can be obtained, understanding how bind IscR-inhibitors bind to prevent Yop transcription could also enable the development of second-generation inhibitors.

However, there are a number of inherent problems with designing drugs that target a specific virulence factor such as Yop secretion. Firstly, optimised treatment plans require rapid diagnostic tools which are currently lacking.

Secondly, as a consequence of the limited number of infections that anti-virulence drugs could be used to treat, pharmaceutical companies are unlikely to get a return on their investment and are unwilling to invest. However, as diagnostics improve and there is a move towards personalised treatment, it is anticipated that there will be an increased demand for such drugs.

FUTURE PERSPECTIVES

Though the crystal structure of *Y. pseudotuberculosis* IscR is not available, a structure is available for both holo-IscR and apo-IscR in *E. coli* (Rajagopalan *et al*., 2013). Homology modelling using a bioinformatic tool such as Phyre2 could be used to predict the structure of IscR: multiple sequence alignments compare the IscR nucleotide sequence against that of other known IscR structures, to predict the secondary and tertiary structure of the protein (Kelley *et al*, 2015). Phyre2 can also be used to predict ligand binding sites and screen the Prestwick compound library for to identify compounds with a high binding affinity for IscR. This library is comprised of FDA-licensed compounds, so any putative inhibitors could expedite the regulatory aspect of bringing a new drug to market. The Centre for Biomolecular Sciences at the University of Nottingham also has access to a *de novo* library of drug-like compounds chosen for their properties, which could also be screened. Analysis will be performed using the parallel supercomputers at Nottingham High Performance Computing (HPC) or HPC Midlands Plus. It would also be interesting to look at the effect of nonsynonymous SNPs (nsSNPs) on IscR to

predict resistance mechanisms. Structural alterations could also be made to the compounds to increase the binding affinity.

From here, the MIC of lead compounds identified through *in silico* screening will be determined using standard methods (Andrews, 2001), and tested *in vivo* for IscR inhibition using the assay described above. IscR inhibitors will also be tested on *Y. pestis* in the Category 3 lab at The University of Nottingham. Testing will include clinical MDR strains.

Following successful construction of the IscR overexpression vector, the IscR protein will be purified. X-ray crystallography would be used to compare the structure of IscR with and without the ligand to prove function, and determine the residues essential for binding. However, it may be that the actual mode of IscR inhibition is through interaction with a regulator or subunits of an efflux pump, for example. Exposing a Δ*IscR* strain to the compounds and using RNA-Seq would enable whole genomes analysis of the genes up- or down-regulated in response to the compound. The construction of genomic reporter fusions using pMiniCTX-*lux* would enable quantification of the transcription of key virulence factors such as Yops.

Resistance mechanisms could be predicted using TraDIS to expose *Y. pseudotuberculosis* and *Y.pestis* transposon libraries to a growth-inhibitory concentration of the compound to generate resistant bacteria. Knockouts

created in a WT strain would be used to confirm these resistance mechanisms. RNA-Seq of the transcriptome in response to the compound would also enable the identification of possible resistance mechanisms such as efflux pumps.

Finally, standard murine models could be used to study the effects of the compound *in vivo,* specifically the pharmacokinetic properties such as PK/PD, and drug-drug interactions. Whole animal imaging will be used to study infection models and response to dosing.

CONCLUSION

The Future of AMR

To limit the spread of AMR, clinical recommendations include reducing unnecessary prescriptions, preventing over-the-counter and internet sales of antibiotics without prescription, and improving basic sanitation (O'Neill, 2016). Previous guidance to complete the full course of antibiotics to prevent resistance has recently been retracted, as taking antibiotics for too long has been shown to drive resistance (Llewelyn *et al*, 2017).

The link between AMR and the use of antimicrobial agents in agriculture has also been recognised, particularly their use as prophylaxes and growth promoters (World Health Organisation, 2014). Huge quantities of antibiotics are used in animal agriculture and in the USA animal agriculture represents u to 70% of the total antibiotic use. Whilst there are clearly times when antibiotics are required to maintain the health of an animal, much of this use would be unnecessary if it were not for intensive farming practices where animals are kept in close proximity in unsanitary conditions. Antibiotic use should be limited and the use of drugs of last resort, or related compounds in animal agriculture should be banned (O'Neill, 2016).

Vancomycin resistance in the clinic has been linked to the use of related glycopeptides in agriculture. Avoparcin is chemically similar to vancomycin, and was widely used as feed additives for livestock throughout the European

Union and Australia until a link was shown between the commensal VRE present as part of the normal flora of avoparcin-fed animals and VRE infections in a nearby hospital (Bates *et al*, 1994). The European commission banned the use of avoparcin in 1997, and since then the incidence of VRE has decreased dramatically. In Denmark, for example, more than 80% of poultry in Denmark carried VRE prior to the ban, but this fell to less than 5% within three years of the ban (Van den Bogaard *et al,* 2000). Antibiotic use is also limited to 50 mg / kg in Denmark.

The Future of Antibiotics

Whilst the topical antibiotic mupirocin was the only new class of antibiotic to be approved between 1962 and 2000, since then five new classes of drugs have been approved for clinical use in addition to numerous analogues of existing drug classes. However, neither the lipoglycopeptides, oxazolidinones, pleuromutilins, nor tiacumicins are effective against Gram-negative infections, whilst the diarylquinolines specifically target ATP synthase in *Mycobacterium tuberculosis*. There are also a number of candidates currently undergoing Phase I – III trials.

The search for novel antibiotics has led researchers to far corners of the natural world, with novel antimicrobial compounds discovered in marsupial pouches (Cheng & Belov, 2017), cockroach brains (Lee *et al*, 2012), and even marijuana (Appendino *et al*, 2008). In 2015, teixobactin was isolated from soil bacteria using the iChip. This cyclic depsipeptide binds to lipid II and lipid III

to inhibit cell wall biosynthesis of Gram-positive organisms and *M. tuberculosis*. Resistance is normally observed during the drug development process but boldly, researchers claim teixobactin works “without detectable resistance” (Ling *et al*, 2015). This claim remains to be substantiated.

Alternative Therapies

In addition to antibiotics, there are many other ways of tackling anti-microbial resistance, both in use and in development:

Vaccination

The principal of vaccination is developing adaptive immunity to a pathogen. Normally, this involves the administration of genetic material to stimulate the host immune system when memory B and T cells encounter the pathogen. Some contain an inactivated form of the pathogen such as the hepatitis A vaccine, whilst others include an attenuated from of the pathogen such as the Bacillus Calmette–Guérin (BCG) vaccine for *M. tuberculosis*. Some are comprised of genetic material rather than whole cells, such as inactivated toxins known to cause disease eg. tetanus vaccine, or protein subunits eg. Hepatitis A.

Smallpox was the vaccine created in 1796, and incidentally it was the first infectious disease to be eradicated in 1977. Other global eradication programmes are underway for poliomyelitis, dracunculiasis, yaws and malaria. Since 2006, the human papillomavirus (HPV) quadrivalent vaccine has been rolled out to all girls aged 12 – 13, and the incidence of HPV

infections and anogenital warts has reduced by more than 60% (Drolet *et al*, 2015). The vaccine targets 4 of the 13 types known to cause cancer: Types 6, 11, 16 and 18 so it is hoped that the vaccination will reduce the incidence of cervical, vulvar, vaginal and anal cancers.

Vaccinations can provide herd immunity and protect a population if ~70% of the population are vaccinated. However, we have seen how the rise of the anti-vaccination movement has had an effect. The link between the vaccination for measles, mumps and rubella (MMR) and autism has been disproved (Jain *et al*, 2015), and the original paper retracted (Wakefield *et al*, 1998), however the number of children not vaccinated has seen the incidence on measles rise. In 2013, there was a major measles epidemic in the Swansea area caused by low vaccination rates. Over 1,219 suspected cases were reported and 1 person died (McCartney, 2013).

However, vaccination acts as an evolutionary pressure on the pathogen, which evolves to out-compete the host in a phenomenon known as the Red-Queen hypothesis (Van Valen, 1974). Take *Streptococcus pneumoniae*, which is a major cause of pneumonia and meningitis in the extremes of age. The widespread use of pneumococcal vaccines could prevent 11.4 million days of antibiotic use due to a reduction in cases of pneumonae (Laxminarayan et al, 2016). However, whilst 92 *S. pneumoniae* serotypes are known, the two currently licenced vaccines are only 23- (Pneumovax) and 7-valent (Prevanr). Whilst this has resulted in a decrease in vaccine serotypes, the prevalence of

non-vaccine serovars such as MDR-serotype 19A has increased in a phenomenon known as “serotype-replacement” (Pichichero *et al*, 2007). New vaccines have been designed to offer protection against these emerging serotypes, including Synflorix (10-valent vaccine) and Prevenar 13 (13-valent vaccine). However, these are not long term solutions. Whilst it is not currently possible to include all serotypes in a single vaccine, vaccines should instead target surface proteins or other targets which are common to all serotypes.

Phage Therapy

Bacteriophages are viruses which infect almost every bacterium, reproducing and lysing the cell, releasing their progeny to continue the life cycle. Phage therapy was pioneered by Giorgi Eliava to treat the Red Army during the cold war, but lost favour with the advent of antibiotics. Phages have specific potential for biofilms, where other agents are unable to penetrate the polysaccharide layer coating the bacteria, and though phage therapies are still popular in Russia and Georgia none have been licenced for clinical use in the UK.

As a specific phage is needed for each bacterial strain poor diagnostics mean their application is limited. Phages normally bind to specific receptors on the cell surface, so unless this is a conserved receptor then resistance easy occurs through target-alteration.

Living antibiotics

The delta-proteobacterium *Bdellovibrio bacteriovorus* and other predatory bacteria are being developed as so-called living antibiotics. These obligate bacterial parasites use Type IV pili to reversibly attach to the Gram-negative cell wall (Evans *et al*, 2007). DD-endopeptidases Bd3459 & Bd0816 are secreted to decrosslink the cell wall and create a pore through which the bacterium can enter the cell. *B. bacteriovorus* also encodes its own self-protection mechanism from host endopeptidases. The bd3460 gene encodes the ankryin repeat protein which protects the cell from decrosslinking by Bd3459 & Bd0816 (Lambert *et al*, 2016).

The bacterium resides in the periplasmic space. This intracellular niche provides the bacterium with a nutrient-rich, competition-free environment. From here, *B. bacteriovorus* secretes hydrolytic enzymes to remodel the host cell wall and create an osmotically-stable predator-prey structure known as a bdelloplast. (Lambert *et al*, 2006). Following elongation, *B. bacteriovorus* divides and the progeny lyse the cell, to infect other cells and complete the life cycle.

B. bacteriovorus is under development as a novel therapeutic agent, for the targeted treatment of infection sites such as surgical wounds, diabetic foot ulcers, and burns, where is has been shown to pray on a variety of human pathogens including *E. coli*, *Salmonella spp*., *Legionella spp*., and *P. aeruginosa*. Towards this aim, *B. bacteriovorus* has recently been shown

killing ability *in vivo* for the first time, clearing Shigellosis in zebrafish larvae, increasing the overall survival rate of the host (Willis *et al*, 2016). It was also shown that *B. bacteriovorus* alone had no pathogenic effect on the zebrafish larvae. No resistance has been observed to date as cell attachment is mechanical not biochemical. What more, as *B. bacteriovorus* is a living organism it is able to adapt to host resistance mechanisms.

Summary

This body of research aimed to tackle two different areas of antimicrobial resistance. The first project explored novobiocin resistance mechanisms, and towards this aim RF cloning was used to make a novel Red-recombinase plasmid which will be used to make future null mutants. The second project developed a screening assay for IscR inhibitors, and a *lux* reporter strain was created to use luminescence as an indirect measure of IscR inhibition. Both of these projects pave the way for future research into AMR.

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