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# *ANTIMICROBIALS AND ANTIMICROBIAL RESISTANCE*

MRes Thesis

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## **ABBREVIATIONS**

ART – Antiretroviral therapy

CF – Cystic fibrosis

CNS – Central nervous system

CYP – Cytochrome P450

DCS – D-cycloserine

FDA – Food and Drug Administration

HTS – High throughput screening

ICU – Intensive care unit

IC50 – Inhibitory concentration that gives 50 % growth inhibition

IHF – Integration host factor

MIC99 – Minimum inhibitory concentration that gives 99 % growth inhibition

ORF – Open reading frame

SDM – Site-directed mutagenesis

TB - Tuberculosis

# 1. INTRODUCTION

Antimicrobial resistance is rapidly emerging as a problem for industrialised countries because current healthcare regimes are unable to properly contain and treat resistance. Access to healthcare, with good sanitation lowered the threat of many infectious diseases in industrialised nations, however increase in drug resistance is now becoming a global crisis and is threatening to revert health-care to the state it was in the pre-antibiotics era<sup>1</sup>. The current global crisis of increasing antibiotic resistance has been heralded as an “apocalyptic” threat<sup>2</sup>.

## 1. The Antibiotic Age

Antibiotics exploit the differences between prokaryotic and eukaryotic cells and can be divided into different categories based on their target. Cell wall biosynthesis is targeted by  $\beta$ -lactam antibiotics and vancomycin. Moving inside the cell, DNA replication is prevented by Quinolones, and DNA transcription is inhibited when rifampicin binds to bacterial RNA polymerase. Protein synthesis, namely the bacterial 70S-ribosome, is inhibited by macrolides, aminoglycosides and tetracyclines. There is also a new class containing oxazolidinones which act on the initiation step during protein synthesis. Lastly, antibiotics target key metabolic processes such as the biosynthesis of tetrahydrofolic acid which is inhibited by sulphonamides and trimethoprim<sup>3</sup>. Some antibacterial agents have multiple cellular targets, such as triclosan at bactericidal concentrations<sup>4</sup>. At sub-lethal bacteriostatic concentrations triclosan specifically targets fatty acid biosynthesis<sup>4,5</sup>.

The first naturally occurring antibiotic to be used clinically was discovered in 1928 by Alexander Fleming. At the time, the clinical potential was unforeseen and initially lead to the dismissal of

Penicillin by Fleming and his peers shortly after its discovery. It wasn't until 1939 that it was re-discovered and mass produced as a cure for gram-positive bacterial diseases. Typically, infections caused by *Staphylococcus*, *Streptococcus*, *Clostridium*, and *Listeria* are responsive to penicillin. *P. aeruginosa* infections can be treated with penicillins from a more recent class, such as piperacillin<sup>6</sup>. The discovery of penicillin revolutionised the treatment of infectious disease, this can be seen in the rapid decline in mortality rates due to infectious disease between the periods 1938 to 1952 in the USA<sup>7</sup>.

The discovery of penicillin engendered the biotechnology industry we know today. Efforts to mass produce the chemical, produced by *Penicillium* mould as a secondary metabolite, lead to development of large-scale fermenters that are still used today<sup>8,9</sup>. The majority of current antibiotics originate from natural biological sources, and are usually secondary metabolites, produced by bacteria or mould that grows in complex environments such as soil where competition for available nutrients is high<sup>10</sup>. For example, *Pseudomonas fluorescens* produces a phenazine antibiotic in the rhizosphere of wheat in order to suppress the fungal infection caused by *Gaeumannomyces graminis* var. *tritici*<sup>11</sup>. Production of antibacterial agents provides a fitness advantage by killing off competition, however contention exists over this as a major role, as antibiotics are not naturally produced at such high concentrations seen in therapeutic use<sup>10,12</sup>. A few antibiotics, such as sulphonamides, trimethoprim and nitroimidazoles are of synthetic origin<sup>2</sup>.

Following on from penicillin, many more antibiotics have been discovered, the last major class being lipopeptides in the early 2000s, and before that, quinolones in 1960s<sup>13</sup>. The long break between the introduction of quinolones and the present shows that all the "low-hanging fruit" has been picked, and discovery of new antibiotics has slowed down<sup>2</sup>. The absence of new effective antibiotics combined with the rapid rise in bacterial resistance to current antibacterial

therapeutics is a huge problem, and if not addressed could lead to the end of the antibiotic era<sup>2,10,13,14</sup>.

## **2. The Rise in Antimicrobial Resistance**

Antimicrobial resistance (AMR) is the abolishment of sensitivity to current antibacterial and antifungal agents. For the purpose of this study, AMR will refer only to bacterial resistance to antibiotics. AMR is either acquired or intrinsic. Often antibiotic resistance strategies are common and widely distributed in nature due to the natural production of antibiotics as secondary metabolites<sup>10</sup>. This is not an issue until bacteria are subjected to antibiotics in certain environments, such as during therapeutic treatment. For example, bacteria such as *P. aeruginosa* and *M. tuberculosis* naturally have inherent resistance to a wide range of antibiotics<sup>3</sup>. Some bacterial species are completely sensitive to antibiotics but will acquire resistance by genetic modifications of their genome or by horizontal gene transfer of mobile elements encoding resistance genes. Resistance arises through selective pressure, simple Darwinian evolution. Resistance may exist in a population of cells before antibiotic is present in the environment, and with the exception of a high fitness cost the resistant bacteria are no different than the sensitive cells. Once an antibiotic selective pressure is introduced however the resistance increases in frequency as sensitive cells die and the resistant ones can thrive. Fleming himself warned against the emergence of resistance to penicillin in 1945, having witnessed how easily cells become insensitive to the antibiotic at non-lethal concentrations<sup>15</sup>. Resistance has been reported to nearly all antibiotics developed<sup>13</sup>.

Acquisition of resistance by horizontal gene transfer sees transposons, plasmids, resistance islands, prophages, and integrons containing resistance genes carried from resistant bacteria into sensitive ones.<sup>3</sup> These DNA elements are integrated into the

bacterial genome at specific sites using recombinases and integrases. Resistance brings with it a fitness cost for the cell but these are quickly negated through additional compensatory mutations<sup>3</sup>.

Intrinsic resistance is a natural insensitivity that is found in every bacterium of a species, without any additional genetic mutation. The prime example for intrinsic resistance is that exhibited by *P. aeruginosa*, a multidrug resistant opportunistic pathogen. It has a high level of intrinsic resistance due to restricted outer membrane permeability, a  $\beta$ -lactamase encoded on the bacterial chromosome, and the presence of, and overproduction of multi-drug-resistant (MDR) efflux pumps<sup>3,16</sup>. Of these, the tripartite MexAB-OprM and MexXY-OprM pumps have very broad specificity for their substrates. Fluoroquinolones, tetracycline, macrolides, trimethoprim and  $\beta$ -lactams (excluding imipenem) are all transported by the constitutively-expressed MexAB-OprM, and in addition to the above, aminoglycosides are transported by MexXY-OprM<sup>17</sup>. *P. aeruginosa* efflux pumps export detergents, disinfectants, organic solvents and dyes in addition to antibiotics<sup>3</sup>. With the exception of MexAB-OprM, multi-drug efflux pumps are very tightly regulated<sup>17</sup>. Another example of intrinsic resistance is that of *Mycoplasma*, as they are naturally resistant to  $\beta$ -lactams as the cell wall inherently lacks the peptidoglycan target<sup>3</sup>.

*P. aeruginosa* is also a model organism for physiological resistance as it readily forms sessile biofilms<sup>3,18</sup>. The expression of this type of intrinsic resistance is dependent on the environmental growth conditions. Sessile biofilms can be seen in the lungs of infected cystic fibrosis individuals, and the bacteria within often cannot be cleared with antibiotics leading to persistent or recurring infections<sup>19,20</sup>. Biofilms offer protection from antibiotics as they are a complex assembly of stationary-phase bacteria surrounded by an organised extracellular matrix that is difficult for antibiotics to

penetrate. Extracellular DNA, found in the biofilm matrix, plays a key role in biofilm maturation and structure, but also conveys protection from aminoglycoside antibiotics by binding to and sequestering them<sup>21</sup>. During biofilm growth resistance genes are expressed that are repressed during planktonic growth<sup>22</sup>. In addition to this, a high number of bacteria within the biofilm exhibit a low metabolic state, possibly due to the limited access to nutrients for growth. The bacteria are persistors as they cannot be cleared by antibiotics that target active growth, such as  $\beta$ -lactams.

Antimicrobial resistance is rapidly on the rise due to worldwide overuse and misuse of antibiotics<sup>13</sup>. Use of antibiotics is not regulated in many countries and they are available for purchase online and prescription-free over the counter<sup>1,23</sup>. Easy access to cheap antibiotics leads to inappropriate usage because the public sees them as a quick and simple alternative to a wide range of illnesses<sup>1,13,24</sup>. Another key contributor to antibiotic resistance is incorrect prescriptions. This includes incorrect duration of treatment, type of antibiotics, and unnecessary use. Inappropriate prescriptions occur in up to 50% of cases<sup>13</sup>. Often the need for rapid treatment means that there is limited time to determine the bacterial cause of infection and administer the most suitable antibiotics accordingly. In intensive care units (ICUs) in the US 30-60% of antibiotics administered are inappropriate because of this need to treat patients with bacterial infections quickly<sup>25</sup>. Patients in the ICU are prescribed more antibiotics than necessary as there are often no defining clinical symptoms of infection<sup>25</sup>. Aside from developing resistance, inappropriate usage of antibiotics can have negative effects on the patient, such as destroying the natural microflora that plays a key role in protection from disease.

Extensive use of antibiotics for agricultural practices is a massive driver for resistance. They are used throughout the developing world as growth promoters in livestock to give maximum yields of healthy,



quality product<sup>13,23,26</sup>. It is estimated that 80% of all antibiotics sold in the US go to livestock, which are then eaten, resulting in transfer of both antibiotics and resistant bacteria to humans<sup>26–28</sup>. These bacteria then cause infections. Antibiotics excreted by the animals are spread to the environment through ground water, surface run off and use of excrement as fertilizer, exposing the environmental microbiome to low levels of antibiotics and encouraging the development of resistance<sup>13,26</sup>. Again, these can then be picked up by humans and cause disease that is hard to treat. Antibacterial agents, such as triclosan, are also used widely as preservatives, disinfectants and antiseptics and again these contribute to the antibiotic resistance crisis. For example, triclosan can be found in many household items such as plastics, as well as in personal health-care items like toothpastes, but triclosan-resistance has been reported since 2000<sup>4</sup>.

### 3. Tackling the AMR Crisis

AMR is both a clinical and a socioeconomic problem that is estimated to result in a loss of \$100 trillion USD by 2050 and cause the deaths of 10 million people per year<sup>3,29</sup>. At present 700,000 deaths each year globally are due to antibiotic-resistant infections<sup>29</sup>. Avoiding this crisis can be achieved through a combination of policy and research.

One excellent example of policy preventing the spread of disease-causing bacteria is of the instructions implemented to prevent spread of multi-drug resistant *C. difficile* in hospitals. *C. difficile* forms highly resistant spores, which can be transported easily across hospitals to vulnerable immune-compromised patients. It was recommended that new hand-washing procedures, isolation of patients and thorough ward sterilisation with diluted bleach should be implemented, and as a result nosocomial *C.difficile* infections were reduced significantly<sup>30</sup>. Preventing the spread of antibiotic resistance at the transmission step is fast and produces instant results.

However, care is needed when using disinfectants as there have been reports of increased tolerance to antiseptics as a result of AMR. Quinolone resistance, in the form of gyrase mutants in *E. coli* and *Salmonella* decreases susceptibility to the disinfective agent triclosan by upregulation of the stress response<sup>31</sup>.

It is important to increase global surveillance and globally-accessible databases for genetic fingerprinting profiles of drug-resistant bacteria. Identifying bacteria can be done by a number of ways, such as Multilocus sequence typing (MLST), PCR ribotyping, multilocus variable tandem repeat analysis (MVLA)<sup>32</sup>. Keeping identifiable information in world-wide databases will aid tracking the spread of resistance across the globe, and data can be used to predict the origin of resistance and also highlight potentially problematic pathogens<sup>3</sup>.

A vast amount of research is being undertaken to confront the global issue of AMR. There is a strong focus on finding new drug targets in order to develop new antibiotics<sup>2</sup>. Current antibiotics in use target essential genes and disrupt bacterial growth, thus giving a strong selective pressure for development of resistance. A new approach is to screen for drugs that disrupt bacterial virulence rather than bacterial growth as antivirulence agents should generate a far weaker selective pressure for the development of resistance than any current bactericidal or bacteriostatic antibiotics<sup>3</sup>. Antivirulence drugs target secreted virulence factors and bacterial quorum-sensing, the mechanism for bacterial communication that enables attachment and colonisation of surfaces through the formation of complex bacterial communities called biofilms. Examples of current antivirulence drugs include Virstatin, which halts expression of the cholera toxin and the co-regulated pilus in *Vibrio cholerae*, and B81-2, an inhibitor of type IV secretion in *Brucella abortus*<sup>33,34</sup>. Targeting virulence of pathogens instead of growth should allow bacteria to remain within its host long enough for the adaptive immune system to destroy it, thus conferring protection for the next time. It is worth

mentioning that mechanisms of resistance to antivirulence drugs have already been reported, both in clinical isolates and laboratories<sup>33–36</sup>. However, resistance seen today by traditional antibiotics arises because cells are either killed directly or growth is perturbed, thus resistant cells can reproduce rapidly in a cell-free environment. This level and spread of resistance is unlikely to occur upon use of antivirulence drugs, as cells will still grow in a competitive, well-mixed population and the selection for resistance is low<sup>37</sup>.

Two important areas of AMR research are finding new drug targets for exploitation, and drug discovery. The first project undertaken during this research year involved the former, and the second project, the latter. *P. aeruginosa* is a multi-drug resistant pathogen that secretes a barrage of virulence factors into its environment. One key virulence factor is the aminopeptidase autotransporter AaaA, which generates a supply of arginine for growth in oxygen-limited environments such as chronic wound infections<sup>38</sup>. The expression of virulence factors must be tightly controlled. During the first project, the regulation of AaaA was investigated using a transcriptional reporter to find potential new drug targets and to understand how and when expression occurs during infection. In addition, AaaA was to be investigated as a target for novel antivirulence drugs. In order to carry out structural studies of AaaA for inhibitor binding site predictions, a soluble, active, truncated version was needed. This was also to be used for cell-free inhibitor screens against potential antivirulence agents. *P. aeruginosa* is a common human pathogen that causes a broad spectrum of diseases. It is vital to find effective antibiotics against it as it is commonly picked up in hospitals by immunocompromised patients.

The second project aimed to use high-throughput screening methods to find current drugs for repurposing as antimycobacterial agents. The FDA library contains over 1200 different small molecule compounds, all licenced for use in a wide range of illnesses. These

illnesses are not necessarily infection-related, and the library includes drugs used to treat depression, cancer, and arthritis to name a few. One objective during this project was to find appropriate drugs that would act as adjuncts to lower the dose needed for D-cycloserine, a second-line drug used to treat TB resistant to front-line antibiotics. The current treatment for drug-resistant tuberculosis is long, costly and has serious side effects. By lowering the concentration of D-cycloserine required, the negative side-effects should be reduced too. It is important to find new antibiotics to treat tuberculosis, as there has been a steady rise in the number of multi-drug resistant and extensively drug resistant cases.

**2. INVESTIGATING THE REGULATION OF THE  
ARGININE-SPECIFIC PROTEASE AAAA IN  
*PSEUDOMONAS AERUGINOSA* AND CREATING A  
SOLUBLE TRUNCATED VERSION FOR CELL-FREE  
INHIBITOR SCREENS**

## 2.1. ABSTRACT

*Pseudomonas aeruginosa* is a Gram-negative ubiquitous human pathogen that infects wounds, burns, eyes and the lungs of cystic fibrosis patients. Its intrinsic resistance to many antibiotics makes *P. aeruginosa* notoriously difficult to treat. The gene *pa0328* encodes an autotransporter, AaaA, with arginine-specific aminopeptidase activity and an important virulence factor. AaaA remains attached to the surface of bacteria where it aids the uptake of nitrogen in environments with limited free oxygen and nitrogen availability but plenty of peptides with an N-terminal arginine. AaaA allows *P. aeruginosa* to evade the host immune system, grow, and establish biofilms under anoxic conditions. The regulation of such a virulence factor is complex and it is important to understand which signals result in *aaaA* expression or repression. These signals could be used as targets for, or mimicked by, novel antivirulence drugs. We hypothesise that regulators IHF and RpoN bind directly to the promoter of *aaaA* to alter its expression. By specifically mutating these sites the control of said regulators can be determined using a transcriptional reporter. AaaA is a key virulence factor for chronic wound infections and its mutation perturbs virulence. Additionally, AaaA sits on the surface of *P. aeruginosa* and is easily accessible to antimicrobials making it an ideal target for antivirulence drugs. There is a need for a high-throughput inhibitor screen to be developed, and for this, a soluble truncated version of AaaA must be cloned, which was the second aim of this project. Mutagenesis of the IHF and RpoN binding sites was attempted using site-directed mutagenesis, but proved repeatedly unsuccessful. Truncating *aaaA* was more successful but not fully achieved in the time frame.

## 2.2. INTRODUCTION

*Pseudomonas aeruginosa* is an important multidrug-resistant pathogen that infects cystic fibrosis (CF) sufferers, wounds, eyes, and thermal injuries. It is an opportunistic pathogen and a common cause of hospital-acquired infection <sup>38</sup>.

*P. aeruginosa* readily forms biofilms; bacterial communities adhered to a surface and a common cause of corrosion and infection. Biofilms commonly grow on industrial surfaces resulting in biofouling - examples of this can be found from washing machines to the Mir space station <sup>39</sup>. From a clinical perspective, biofilms provide increased protection from antibiotics and clearance by the host immune system. Microcolonies show increased resistance to antibiotics due to the physical barrier between interior cells and the environment <sup>40</sup>. In addition, antibiotics targeting active metabolism are less effective on bacteria further within the biofilm as these cells have less access to nutrients and so exhibit a low metabolic state. <sup>40</sup>.

*P. aeruginosa* is notorious as the primary cause of morbidity in CF sufferers <sup>38</sup>. Cystic fibrosis patients contract lung infections intermittently during childhood, eventually these persist to become chronic lung infections that can survive for decades <sup>41</sup>. Studies show that 80 % of CF patients are infected with *P. aeruginosa* by adulthood <sup>42</sup>. These chronic lung infections are, in part, due to failure of antibiotic treatment to clear the bacteria. As the infection continues through the generations strains become mucoid, a phenotype associated with increased antibiotic tolerance and increased morbidity and mortality in CF patients <sup>41</sup>. Formation of immobile biofilms from planktonic cells in the CF lungs increases resistance to antibiotics 1000-fold <sup>40,42</sup>. It is therefore vital to investigate the biology of *P. aeruginosa* to better understand how to treat these infections and prevent persistence of the disease.

AaaA is an important virulence factor of *P. aeruginosa*. It is an autotransporter with arginine-specific aminopeptidase activity and is one of the 20 most-produced enzymes in biofilms<sup>38</sup>. It has previously been shown to provide a strong virulence advantage in chronic wound infection. The bacterial load of *P. aeruginosa*  $\Delta$ aaaA in a mouse chronic wound model was far lower than the wild type at both 2 and 8 days post-infection, demonstrating its key role in immune evasion<sup>38</sup>. The catalytic domain of AaaA cleaves the amino terminus arginine from extracellular peptides providing *P. aeruginosa* with an alternate nitrogen source when environmental nitrogen is low. This enables the bacterium to grow and provides a selective advantage. It can also aid survival in hypoxic environments, such as within biofilms, as it provides arginine as an energy source through substrate level phosphorylation<sup>43</sup>.

Autotransporters are a large superfamily of virulence factors found in Gram-negative bacteria<sup>44</sup>. They are characterised by an N-terminal signal peptide to allow passage across the inner membrane, a central passenger domain that confers enzymatic function, and a 12-stranded C-terminal  $\beta$ -barrel domain for translocation across the outer membrane. Autotransporters, as the name suggests, were thought to translocate the extracellular passenger domain independently of ATP or other secretion systems using the covalently-linked C-terminal  $\beta$ -barrel. However, recent evidence suggests that chaperone proteins, in particular the Bam complex, are required for insertion of the  $\beta$ -barrel into the outer membrane and subsequent translocation<sup>44</sup>. Unlike many autotransporters, AaaA remains attached to the cell surface via its membrane-embedded  $\beta$ -barrel after crossing the outer membrane<sup>38,45</sup>.

It is important to look for inhibitors of AaaA as it is such a crucial virulence factor. AaaA is an ideal target for antimicrobials as it is surface-associated and therefore easier for compounds to access. To screen for potential AaaA inhibitors, a cell free inhibitor assay is required. This project aimed to develop and express a version of AaaA which is soluble, active, and can easily be purified with a polyhistidine tag. Wild-type AaaA is a membrane-associated protein, and these are notoriously hard to



purify in large quantities. To overcome this, the membrane-bound  $\beta$ -barrel domain is not included in the truncated version as it is not necessary for catalytic function of AaaA. This should make the protein easier to purify in large quantities. The truncated version (tAaaA3) includes the N-terminal signal peptide to direct the peptide to the periplasm where it should fold into its fully-functional form, and not form toxic cytoplasmic inclusion bodies when overexpressed. Previous versions created by Daniella Spencer (University of Nottingham) did not contain the N-terminal signal peptide, were highly toxic, and readily formed inclusion bodies in the cytoplasm (unpublished data). Overall, tAaaA3 retains the catalytic passenger domain of AaaA in the periplasm where it is predicted to be easier to purify and less toxic to the cell than a cytosol-localised version. The Purified tAaaA3 can then be used in a high-throughput antimicrobial screen, or for structural studies to investigate inhibitor binding sites.

Bacteria must tightly control the expression of genes in response to environmental signals and their changing intracellular metabolism in order to survive. This is commonly achieved by regulatory proteins that bind to promoters and positively or negatively affect transcription. In one recent study, the promoter region of *aaaA* was shown to be negatively regulated by RpoN, RhIR, MvaT and MvaU, and positively regulated by ArgR using a bioluminescent transcriptional reporter<sup>46</sup>. The promoter region of *aaaA* also contains putative binding sites for a number of these regulators, such as ArgR and RpoN, as well as a site for an additional transcriptional regulator, IHF<sup>46</sup>. Although the regulatory effect has been shown, it is not yet established if this is caused by direct binding of these regulatory proteins to the *aaaA* promoter region<sup>46</sup>.

IHF is a global regulator conserved between Gram-negative bacteria and belongs to the DNA-binding protein family DNABII<sup>47</sup>. It regulates many genes, including those involved in the biosynthesis of known virulence factors<sup>48,49</sup>. IHF has two subunits, IHF $\alpha$  and IHF $\beta$ , which form two  $\beta$ sheet arms and are joined at one end<sup>47</sup>. The

heterodimer binds DNA specifically, creating a sharp 180° bend and facilitating interactions between DNA and other DNA binding proteins, like RNA polymerase, by opening up the major groove<sup>47,50</sup>. RpoN is an alternative sigma factor which also regulates the expression of virulence factors in *P. aeruginosa*<sup>51</sup>. When *rpoN* mutants were challenged with a mouse burn-wound model, they were 100-fold less virulent, demonstrating its importance to the pathogen<sup>51</sup>. Like IHF, RpoN binds specifically to a consensus sequence in the promoter region of target genes<sup>52,53</sup>. It has been shown to regulate aliginatase, rhamnolipid, and lipase biosynthesis as well as quorum sensing pathways<sup>52,54</sup>.

In order to further understand the regulation of *aaaA*, the aim of this project was to identify IHF and RpoN as two regulators that bind directly to their respective predicted binding sites in the promoter region of *aaaA*. The effect of IHF and RpoN on expression was to be assessed using a bioluminescent transcriptional reporter fused with the *aaaA* promoter region. Inactivation of IHF and RpoN binding motifs within the promoter region of the reporter was to be carried out by engineering mutations in their binding consensus sequences using site-directed mutagenesis. The transcriptional reporters could then be used to compare transcription in the activated and inactivated promoter regions.

This project aimed to determine the role of IHF and RpoN in regulating *aaaA* expression, and to create an expression system for a truncated *AaaA* version for inhibitor screens.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Bacterial Strains and Culture conditions

Strains were cultured in Lysogeny broth (LB) or grown on LB agar plates at 37 °C. Liquid cultures were shaken at 250rpm and were used directly for plasmid purification. Where appropriate, ampicillin was used at a working concentration of 100 µg/ml and tetracycline was used at 30 µg/ml. All stock antibiotics were stored at -20 °C and were prepared at 50 mg/ml. Stocks of *E. coli* strains were made from LB broth and 25% glycerol, and stored at -80°C.

### 2.3.2 Strain List

TABLE 1. LIST OF STRAINS, PLASMIDS AND OLIGONUCLEOTIDES USED IN THIS STUDY

Strain	Features	Reference
<i>E. coli</i>		
S17-1 $\lambda$ pir	Strain for maintenance and mobilization of R6K suicide plasmids .	<sup>55</sup>
DH5 $\alpha$	Cloning strain.	<sup>56</sup>
Plasmid	Description	Origin
pMiniCTX:aaaA':lux	miniCTX plasmid carrying an <i>aaaA'</i> - <i>lux</i> fusion, Tc <sup>R</sup>	<sup>46</sup>
pET21a	Bacterial expression vector with T7lac promoter, Amp <sup>R</sup> , restriction enzyme cloning	Novagene
pET21a:aaaA	pET21a plus full length <i>aaaA</i>	Kindly given by Daniella Spencer (university of Nottingham)
pGEM-T-Easy	Linearised vector containing 3'-T overhangs for easy ligation of PCR products with terminal polyadenosine tails. Amp <sup>R</sup> .	Promega
pGEM-T-Easy:taaaA3	pGEM-T-Easy plus <i>taaaA3</i>	This study

Oligonucleotides	Sequence	Details
SVAAAAF	5' -TATCATATGTTCAAACCATTAGCT-GTCGCTG-3'	Forward for amplification of truncated <i>aaaA</i> . <i>NdeI</i> site.
aaaAt2R	5' -TATGAATTCTTAATGGTGGTGG-TGATGATGGTCGTGCAGGCGGGTCAGT-3'	Reverse for amplification of truncated <i>aaaA</i> . <i>EcoRI</i> site.
IHF_SDM_F	5' -CGCTGCCTCTCTCTGCTTCGCCGT-CACCGCCGCCGCGCAGTTACAACG-3'	Lightening Quickchange SDM
IHF_SDM_R	5' -CGTTGTAACTGGCGGCGGCGGTGA-CGGCGAAGCAGAGAGAGGCAGCG-3'	Lightening Quickchange SDM
RpoN_SDM_F	5' -CGTGCTCCAGAGACATGAAACTAT-CCGCATCCTAATTTCCGCG-3'	Lightening Quickchange SDM
RpoN_SDM_R	5' -CGCGGAAATTAGGATGCGGATAGT-TTCATGTCTCTGGAGCACG-3'	Lightening Quickchange SDM
Q5_SDM_IHF_F	5' -CTTCGCCGTCACCGCCGCTGCCAG-3'	Q5 SDM single mutation IHF binding site
Q5_SDM_IHF_R	5' -CAGAGAGAGGCAGCGGCC-3'	Q5 SDM single mutation IHF binding site
Q5_SDM_IHF_F2	5' -CGCCGCCAGTTACAACGTAGCGAC-3'	Q5 SDM double mutation IHF binding site
Q5_SDM_IHF_F2	5' -GCGGTGACGGCGAAGCAGGAGA-3'	Q5 SDM double mutation IHF binding site
Q5_SDM_RpoN_F	5' -GACATGAAACAATCCGCATCCT-GCTTTC-3'	Q5 SDM single mutation RpoN binding site
Q5_SDM_RpoN_R	5' -TCTGGAGCACGAATTGAC-3'	Q5 single mutation RpoN binding site
Q5_SDM_RpoN_F2	5' -ATCCTAATTTCCGCGGCGGAGCAT-3'	Q5 SDM double mutation RpoN binding site
Q5_SDM_RpoN_R2	5' -GCGGATTGTTTCATGTCTCTGGAG-CACGAATTGAC-3'	Q5 SDM double mutation RpoN binding site

### 2.3.2. DNA Manipulation

Plasmids were constructed by digestion of DNA with restriction endonucleases and then ligation into the target vector backbone. Agarose gel electrophoresis was carried out in 1X TAE buffer (80 mM Tris-acetate pH 7.8, 19 mM EDTA) using a standard method<sup>57</sup>. DNA amplification by PCR was used to amplify fragments of DNA for cloning. Q5 DNA Polymerase (NEB) was used for accurate amplification of the DNA sequence. 2X Q5 Master Mix, nuclease-free water, 1.25 µl of each 10 mM oligonucleotide, and up to 100 ng of DNA template were added to a total volume of 25 µl. PCR conditions were followed as recommended by NEB. PCR clean-up was carried out using the Wizard SV Gel and PCR Clean-Up System (Promega). Digestion was carried out by mixing 1 µg DNA with 1 µl of each restriction enzyme, 2 µl of 10X buffer and nuclease-free water to a total volume of 20 µl. For double-digestion with NdeI, the enzyme was added alone and the reaction was incubated at 37 °C for 1 h before the second restriction endonuclease was added and incubation continued for a further 1 h. Single-digest controls were used when appropriate. Restriction digest fragments were isolated from agarose gel using the Monarch DNA Gel Extraction kit (NEB). A-tailing was necessary for cloning DNA fragments into the vector P-GEM-T-Easy. To do this, 10 mM dATP, 0.2 µl Taq, 1 µl Standard Taq Buffer, 7.8 µl cleaned-up DNA fragment, and nuclease-free water to a total volume of 10 µl was incubated at 72 °C for 20 min. For ligation of DNA fragments to the target vector, DNA was mixed together at a ratio of 5:1 insert:vector with 50 ng vector DNA. This was incubated at 16 °C with 1 µl T4 DNA ligase, T4 DNA ligase 10X buffer, and nuclease-free water to a total volume of 10 µl for 16 h. The resulting ligation mix was dialysed for 1 h and then transformed into electrocompetent *E. coli* DH5α cells and grown overnight on selective LB agar. Plasmid DNA was prepared using a plasmid purification kit (Qiagen). All DNA sequencing was performed by Source Bioscience.

### 2.3.3. Site-Directed Mutagenesis

Site-directed mutagenesis (SDM) was carried out using two different methods.

#### QuikChange Lightning Site-Directed Mutagenesis Kit (Aligent)

Primers were designed according to the QuikChange Lightning protocol. A PCR reaction mixture was prepared containing *Pfu* Buffer 10X, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 50 ng template DNA, 1 µl 10 mM each dNTP, 1 µl *Pfu* DNA Polymerase, and nuclease-free water to 50 µl. PCR was performed under the following thermocycling conditions:

Temperature (°C)	Time (s)
95	30
95	30
68	60
72	60 / kb
4	Stored indefinitely

Steps 2 to 4 were repeated for 12-16 cycles. The reaction mixture was then treated with 2 µl DpnI and 10X DpnI Buffer (NEB). After gentle mixing the reaction was incubated at 37 °C for 20 min for digestion of parental DNA. 15 µl of the reaction was then used to transform commercially competent *E. coli TOP10* cells.

### Q5 Site-Directed Mutagenesis Kit (NEB)

The primers for site-directed mutagenesis were designed using the web-based NEBaseChanger® tool (<http://nebasechanger.neb.com>) according to the recommendations in the Q5 SDM protocol. Briefly, PCR amplification was carried out on reactions containing 50ng plasmid DNA, 1.25 µl of each 10 mM oligonucleotide, 12.5 µl Q5 Hot Start High-Fidelity 2X Master Mix, and nuclease-free water to a 25 µl total volume. The following thermocycling conditions were used:

Temperature (°C)	Time
98	30 s
98	10 s
65	30 s
72	12 min
72	4 min

Steps 2 to 4 were repeated for 25 cycles. The PCR reaction product was then dialysed to remove excess salts and then treated with Kinase, ligase and DpnI to leave only the synthesised plasmid. This was then transformed into chemically competent *E. coli* NEB5α by heat shock procedure and the cells were grown on tetracycline 30 µg/ml LB agar overnight at 37 °C.

## 2.4. RESULTS AND DISCUSSION

### 2.4.1. *In silico* analysis of *aaaA*

*In silico* analysis of the *aaaA* promoter region was carried out using BPROM (SoftBerry) a promoter prediction resource, the PRODORIC database and PRODORIC Virtual Footprint binding site prediction tool (<http://www.prodoric.de>). Promoter prediction was attempted with BPROM (SoftBerry) but this returned no promoter sites. The promoter was located instead by querying the PRODORIC database. PRODORIC Virtual Footprint is a prediction tool which utilises an extensive database of information on gene regulation in a number of prokaryotic organisms. Such information is taken from scientific literature and computational analysis and can be used to predict the regulation of a target prokaryotic gene<sup>58</sup>. A 500 bp region directly upstream of the *aaaA* open reading frame (ORF) was input into the Virtual Footprint algorithm to determine the binding sites for putative regulatory proteins. Sequence analysis gave two predicted binding sites for Integrated host factor (IHF); which can be found between the bases i) -253 to -238 relative to the start codon of *aaaA* on the (+) strand and ii) -148 to -133 on the (-) strand (Figure 1a). The latter lies on the correct strand and fit with the IHF binding consensus sequence WCARNWNNTTTR described in previously published literature, where A/T is represented by a W, and A/G is indicated by the letter R (Figure 1b). As a result this predicted binding sequence was chosen for site-directed mutagenesis<sup>47,59</sup>.

### 2.4.2. Mutating binding sites in the promoter region of *aaaA*

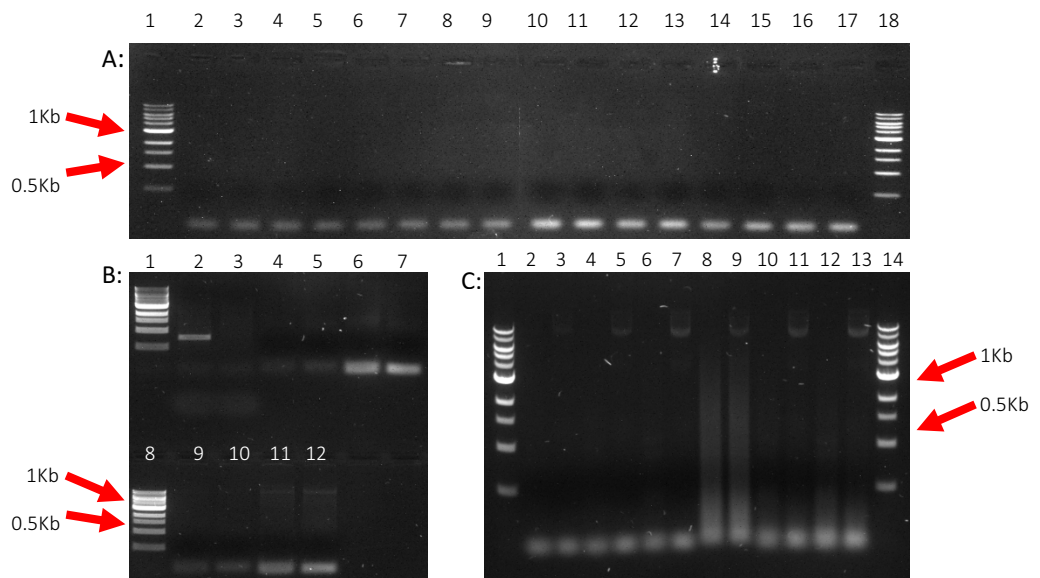
To further understand the regulation of *aaaA*, substitution mutations were to be made in the 500bp upstream region using site-directed mutagenesis. The 500bp upstream region of *aaaA* has previously been cloned onto a plasmid encoding the *lux* operon, called miniCTX:paaaA:lux<sup>46</sup>. Transcription of the *lux* genes is subsequently under the control of the *aaaA* promoter and provides a simple,



[illegible]

25

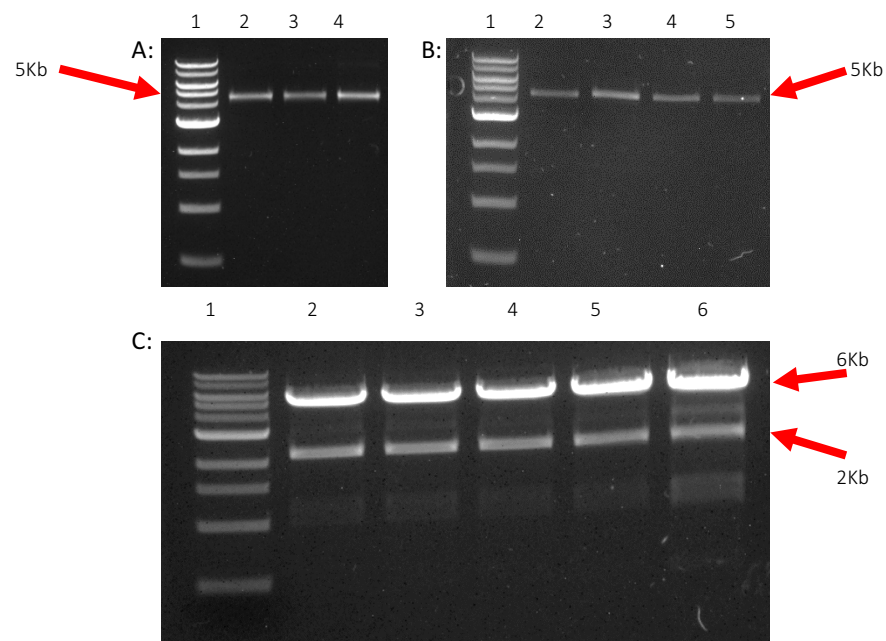
Initially, oligonucleotides were designed to be <40 bp, fully overlapping, and for use with the QuikChange Lightning SDM protocol (Table 2). Using the QuikChange Lightning SDM method the plasmid was amplified with the mutant primers and the PCR reaction digested with DpnI to remove methylated template DNA. However, when the results of each step were run on a 1 % agarose gel there was no DNA fragment representing the miniCTX:paaaA:lux plasmid following DpnI digestion, but there was a strong DNA fragment corresponding to primer-dimers formed due to their complementary nature. A range of DMSO concentrations and template DNA concentration was included with the PCR mix to prevent this from happening, but still this did not produce a synthetic plasmid (Figure 2). Consequently, the QuikChange Lightning SDM kit was replaced with the NEB Q5 SDM kit as the oligonucleotide design did not contain overlapping regions (Figure 1c).



**Figure 2. Optimisation of QuikChange Lightning SDM method.**

DMSO was added to the PCR reaction for both IHF and RpoN site mutation (A). A range of DNA template concentration was also tested, covering 10 ng (lanes 4 and 5), 25 ng (lanes 6 and 7), 50 ng (lanes 9 and 10) and 100 ng (lanes 11 and 12) (B); and 100-200 ng (C) (lanes 2 and 3 100 ng, 4 and 5 is 150 ng, 6 and 7 200 ng DNA for IHF site mutation; lanes 8 and 9 100 ng, 10 and 11 is 150 ng, 12 and 13 is 200 ng DNA for RpoN site mutation). Had the PCR reaction worked a DNA fragment would be seen at 12.8 kb but only primer-dimer pairs can be seen following DpnI digestion.

Primers to introduce the mutation using the Q5 SDM kit were designed using NEBaseChanger® online resource (<http://nebasechanger.neb.com>). Having followed the Q5 SDM protocol to completion, tetracycline-resistant colonies were isolated and their plasmids extracted and purified. Unfortunately, the purified plasmid DNA was unable to be sequenced, and following digestion with Nde1 and EcoR1 it became clear that the isolated DNA was not in fact the expected MiniCTX:paaaA:lux vector (Figure 3). Consequently a new stock of MiniCTX:paaaA:lux plasmid DNA was prepared, verified by digestion with Nde1 and EcoR1, and Q5 SDM was repeated (Figure 3c). Clearly before attempting any point mutagenesis or cloning, all materials to be used during the process should be verified as the expected ones to avoid wasting time and resources. However, there was only very limited time available to carry out this project and so materials were not checked as this would have taken up some of the little time available.



**Figure 3. MiniCTX:paaaA:lux Diagnostic digestion revealed incorrect DNA vector was being used.** Digestion of MiniCTX:paaaA:lux stocks with Nde1 (lanes 2-4, A) and double-digestion with Nde1 and EcoR1 (lanes 2-5, B) revealed a plasmid that gave one DNA fragment at 5Kb. Expected DNA fragments following a double-digest of MiniCTX:paaaA:lux should be ~2 kB and ~6 kB. MiniCTX:paaaA:lux was amplified using S17-1  $\lambda$ pir from a different glycerol stock and then a sample was double-digested with Nde1 and EcoR1. The resulting DNA fragments separated at the expected sizes for MiniCTX:paaaA:lux (lanes 2-6, C).

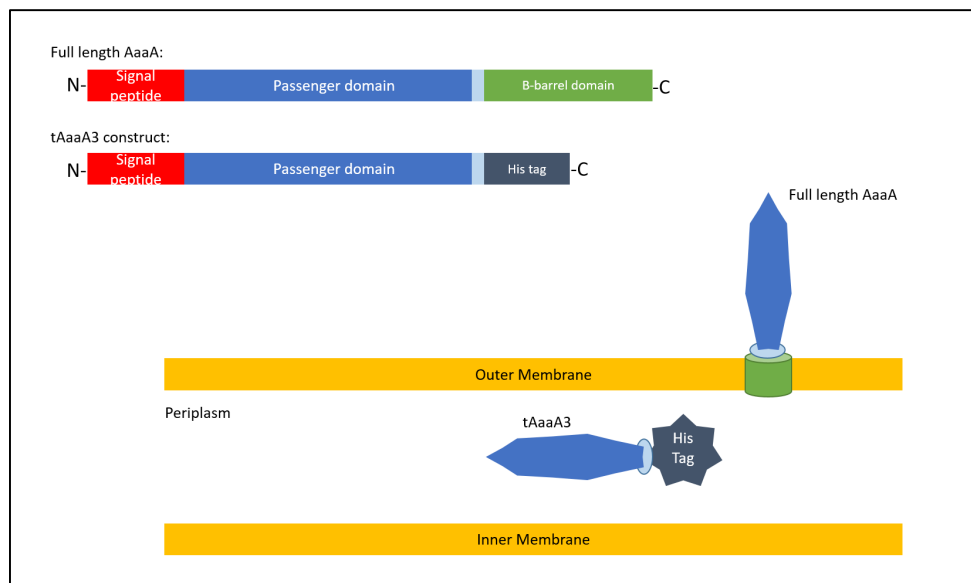
The Q5 SDM method was completed using the MiniCTX:paaaA:lux vector and the plasmids from tetracycline-resistant colonies were purified. However, sequence analysis revealed that although the plasmids were correct, they did not carry the desired mutation. This could be for a number of reasons. Firstly, the primer-binding to the DNA may have been weak, or the annealing temperature was wrong. This can be solved by performing the PCR on a number of reactions with an annealing temperature gradient. Alternatively, the primers can be redesigned to overlap each other, and the extra bases will increase affinity for the DNA. If there was incomplete digestion of the parental DNA, this could be fixed by increasing the KDL digestion time from 5 min to 20 min although it is more likely that the DpnI enzyme was not functional. Lastly, the template plasmid could simply be too large to amplify with this method as it is 12,822 bp. The only way to overcome this would be to mutate *paaaA* in a smaller vector and then clone it back into MiniCTX:lux. An alternative method to tackle the overall project question is through the use of gel mobility shift assays which have been used to show IHF binding to the *phtD* operon in *P. syringae*<sup>47</sup>.

#### **2.4.3. Cloning a soluble truncated version of the AaaA protein**

It is notoriously difficult to obtain high quantities of purified, functional membrane proteins. For this reason, a truncated version of AaaA (tAaaA3) was designed to exclude the membrane-embedded  $\beta$ -barrel domain, and include a polyhistidine tag for additional purification using a nickel column.

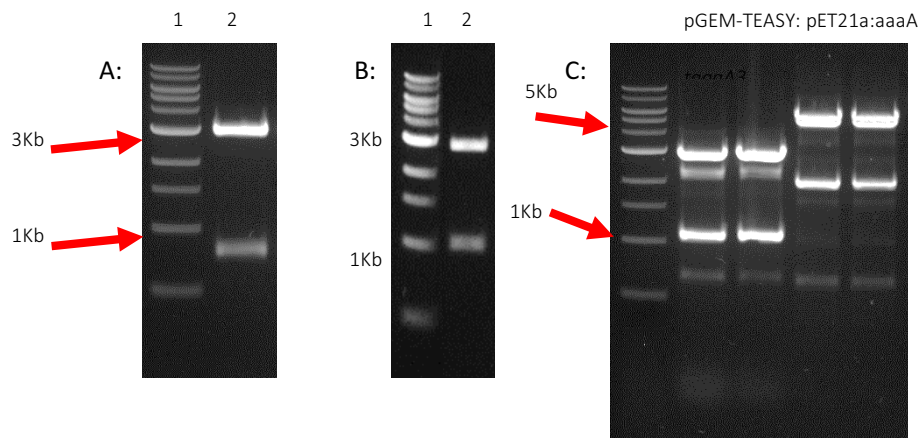
Using the plasmid pET21a:aaaA a truncated version of *aaaA* was PCR amplified. The oligonucleotides used have either an NdeI restriction site or an EcoR1 site and were designed to include the N-terminal signal peptide of AaaA and end the truncated protein on hydrophilic amino acids to prevent the formation of inclusion bodies.

Inclusion of the signal peptide aimed to target tAaaA3 to the periplasm, where it will be less toxic to the cell (Figure 4).



**Figure 4. Cartoon of Full length AaaA compared to truncated version tAaaA3.** AaaA sits tethered to the outer membrane of *Pseudomonas aeruginosa*. The truncated version, tAaaA3, does not encode the  $\beta$ -barrel domain but does include a polyhistidine tag to aid purification. It is thought that by including the signal peptide tAaaA3 will be targeted to the periplasm and therefore will not be toxic to the cells during over-expression.

The purified PCR product was dialysed, a poly-A tail was introduced and the resultant fragment was ligated into pGEM-T-Easy. Following a second dialysis step, pGEM-T-Easy:taaaA3 was transformed into electrocompetent *E. coli DH5 $\alpha$* . Successful transformants were ampicillin resistant. On the first attempt the taaaA3 fragment was significantly shorter than expected due to the reverse primer annealing to a different position on the gene. After altering the primer and repeating, plasmids from successful clones and were shown by digestion with EcoR1 and Nde1 (Figure 5a, 5b). Sequencing confirmed that the correct fragment had been cloned into pGEM-T-Easy.



**Figure 5. Cloning taaaA3 into pGEM-T-Easy.** The first attempt to clone taaaA3 into pGEM-T-Easy was unsuccessful, shown by the observed DNA fragment at around 800 bp in lane 2 (A) following digestion of the plasmid with Nde1 and EcoR1. After changing the primers, the next attempt gave a DNA fragment of expected 1.2kb size (lane 2, B). The DNA fragments in (C) corresponded to plasmid DNA digested by Nde1 and EcoR1 and the fragments at sizes indicated by the red arrows were gel extracted for ligation together to create pET21a:taaaA3.

The plasmids pGEM-T-Easy:taaaA3 and pET21a:aaaA were digested using the enzymes Nde1 and EcoR1 and the DNA fragments were separated on a 0.8% agarose gel (Figure 5c). Both taaaA3 and pET21a vector backbone were gel extracted and purified, then ligated together overnight and transformed into *E.coli DH5α*. However, this did not produce any successful transformants. It was decided that the excision of taaA3 using Nde1 and EcoR1 may be causing problems due to similar restriction sites close by in the multiple cloning region of pGEM-T-Easy. As a result, the PCR product of taaaA3 was digested and ligated directly into the previously digested pET21a vector. Following transformation this produced ampicillin-resistant colonies, however these potential transformants were unable to grow in liquid culture. This meant that the plasmid could not be extracted could not be verified. It is likely that the cloning procedure was inefficient. In future, this cloning procedure should be repeated missing out the pGEM-T-Easy step, and the taaaA3 insert should be directly ligated to the pET21a vector

backbone. Literature shows that each pET expression system enables overexpression of a protein, but has varying degrees of effectiveness depending on the protein in question. It is recommended that if protein expression is inadequate in one pET system, then another, such as pET28a, may be preferential <sup>61</sup>.

Continuation of this study should also focus on developing other types of high-throughput inhibitor screens, for example, those used in 96 pin-lid biofilm assays <sup>62</sup>. In addition to screening potential AaaA inhibitors for their ability to penetrate and affect *P. aeruginosa* biofilms, 96 pin-lid biofilm assays can be used to further understand the role AaaA has in biofilm formation by comparing growth of mutant and wild-type in the presence and absence of its substrate and under a variety of environmental conditions.

The outcome from this project was the creation of the vector pGEM-T-Easy:taaaA3, which is the penultimate step in the cloning procedure for creating the expression vector pET21a:taaaA3. AaaA is an important virulence factor in *P. aeruginosa*, and when absent alters biofilm formation *in vivo* and *in vitro* <sup>38</sup>. The truncated version, tAaaA3, is designed to be a soluble, active version that will not form toxic cytoplasmic inclusion bodies when overexpressed. Obtaining tAaaA3 will provide a functional version of AaaA for structural studies and inhibitor binding site predictions. Understanding the regulation of *aaaA* will help determine its role in the cell and ultimately aid the use of inhibitors in *P. aeruginosa* infection treatment and prevention. However, in the time frame given the IHF and RpoN predicted binding sites were unable to be mutated by site-directed mutagenesis in the plasmid MiniCTX:paaaA:lux. This process may be more successful in a smaller vector.

#### **ACKNOWLEDGEMENTS**

I would like to thank Dr Kim Hardie, Daniella Spencer, James Brown and lab C75 for all their help during this project.

### **3. ASSESSING FDA-APPROVED DRUGS FOR THEIR ABILITY TO ACT AS ANTITUBERCULAR AGENTS**



### 3.1 ABSTRACT

Tuberculosis is the leading cause of morbidity and mortality across the globe. It presents itself as a pulmonary infection but if left untreated can spread to other organs. Current TB therapy is long and expensive, and resistance to front-line antituberculars is increasing; there are growing numbers of multi drug-resistant and extensively drug-resistant cases. Second-line drugs used for drug-resistant TB, such as D-cycloserine, have serious side-effects giving a poor quality of life and often result in the treatment discontinuation. Part of this study aimed to screen a sub-inhibitory concentration of D-cycloserine against the FDA library to find compounds that act synergistically with D-cycloserine. Ultimately these could reduce the therapeutic dosage of D-cycloserine and thus the side-effects. From a library of over 1200 compounds, 50 hits were found. In a previous study the drug GBR12909 (Vanoxerine) showed potential for repurposing as a new antitubercular. The genes MSMEG\_2240 and MSMEG\_2038 were then found mutated in highly vanoxerine-resistant strains of *Mycobacterium smegmatis*, highlighting them as potential targets. The second arm of this study aimed to establish whether these genes are targeted by vanoxerine and therefore solve the mode-of-action of the drug. Instead, the study revealed that vanoxerine is not targeting, but is in fact activated by these enzymes.

### 3.2. INTRODUCTION

Tuberculosis (TB) is a common and serious disease that has been recorded throughout history. Indeed, evidence exists as far back as the ancient Egyptians whose mummified skeletons and soft tissue contain ancient *Mycobacterium tuberculosis* complex DNA <sup>63</sup>. TB is caused by a group of bacteria, including *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, and the more well-known *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* <sup>64</sup>. The disease can either present in an active form, or lie dormant in an asymptomatic, latent form for many years <sup>64</sup>. Current treatment involves six to nine months of overseen therapy and a combination of four first-line drugs with adverse side-effects <sup>65</sup>.

TB is declining in industrialised countries due to access to effective treatments and improvements in healthcare <sup>66</sup>. Nevertheless, TB remains the leading cause of both death and morbidity across the world and in 2015 the World Health Organisation estimated that there were 10.4 million new cases of TB globally <sup>67,68</sup>. In 2008 one third of the world's population was estimated to be carrying latent TB, and in one tenth of those people the disease will become active during their lifetime <sup>66</sup>. This, coupled with the rise of multiply drug-resistant (MDR) and extensively drug-resistant (XDR) strains has increased the pressure to search for new antitubercular drugs. Second-line therapies for MDR and XDR strains have serious side-effects; D-cycloserine (DCS) is notorious for causing psychiatric problems such as anxiety, depression, delirium, euphoria and hallucinations in up to 50% of patients as well as serious seizures in 2% <sup>69,70</sup>.

New drugs are estimated to cost \$1.5 billion and take up to 17 years to reach patients; with only 10-30% successfully making it through clinical trials <sup>71-73</sup>. Research into re-purposing current drugs for alternative therapies, such as new antimicrobials, is growing - its appeal

coming from the decreased time frame and cost for trialling these drugs to treat diseases<sup>73</sup>. Repurposing drugs for TB therapy is well-established; previous work has shown that specific cephalosporins will act synergistically with first-line drugs rifampicin and ethambutol, and certain non-steroidal anti-inflammatory drugs and anti-arthritic drugs have promising antitubercular effects<sup>74–76</sup>. High-throughput screening (HTS) is a useful tool for drug-repurposing as it allows a massive number of compounds to be tested for their potency against specific bacteria under a short time frame. This study aimed to screen the FDA library against *M. bovis* BCG and to compare it with a second screen containing a sub-inhibitory concentration of D-cycloserine in order to find compounds that act as adjuncts for DCS. Ultimately, these could be used to lower the dose of DCS given to patients and therefore reduce the toxic side-effects.

A second arm of this project involves the mode-of-action studies of Vanoxerine (GBR12909). Vanoxerine was discovered in a previous screen of the FDA library by Panchali Kanvatirth (University of Birmingham) as having potential as an antimycobacterial drug (unpublished data). Vanoxerine (GBR-12909) was developed to be a treatment for Parkinson's disease, but was abandoned during clinical trials due to a lack of efficacy. It has also been involved in clinical trials for depression and cocaine addiction where it was established as safe for humans in a tolerance study and phase 1 trial<sup>77</sup>. During a project carried out by Christopher Burke (University of Birmingham) two genes, MSMEG\_2038 and MSMEG\_2240, were identified in the model *M. smegmatis* as conferring high-levels of resistance to vanoxerine (unpublished data). Both genes encode enzymes with a similar function and were found to contain non-synonymous mutations in two mutants growing in high concentrations of vanoxerine, suggesting that they are the drug targets. This project aimed to discover the mode-of-action of vanoxerine by over-expressing the genes in *M. smegmatis* and determining the new minimum inhibitory concentration (MIC); a higher MIC than the wild-type would confirm that the hypothesis is correct.

### 3.3. MATERIALS AND METHODS

#### 3.3.1. List of Strains

**TABLE 3. STRAINS AND PLASMIDS USED DURING THIS STUDY.**

Strain	Features	Reference
<i>M. smegmatis</i> MC <sup>2</sup> 155	Wild-type <i>M. smegmatis</i>	<sup>78</sup>
<i>M. bovis</i> BCG	Wild-type <i>M. bovis</i> BCG	<sup>79</sup>
<i>E. coli</i> TOP10	Electrocompetent <i>E. coli</i> cells.	<sup>80</sup>
Plasmid	Description	Origin
pSNT3eGFP	Plasmid containing gene for enhanced green fluorescence protein under the control of a constitutively expressed promoter. Hyg <sup>R</sup> .	Unpublished. Panchali Kanvatirth (University of Birmingham)
pTIC-2240	pTIC expression plasmid containing the gene MSMEG_2240 under the control of anhydrotetracycline-inducible promoter. Kan <sup>R</sup> .	Unpublished. Christopher Burke (University of Birmingham)
pTIC-2038	pTIC expression plasmid containing the gene MSMEG_2038 under the control of anhydrotetracycline-inducible promoter. Kan <sup>R</sup>	Unpublished. Christopher Burke (University of Birmingham)
pVV16-2240	pVV16 plasmid containing gene MSMEG_2240 under the control of a constitutively expressed promoter. Kan <sup>R</sup> .	Unpublished. Christopher Burke (University of Birmingham)

### **3.3.3. Culturing *M. smegmatis* strains**

*M. smegmatis* was grown in Middlebrook 7H9 broth + glycerol 0.5% with 0.05% tween 80 and selective antibiotic. Hygromycin was used at 50 µg/ml and Kanamycin at 50 µg/ml to maintain plasmids. Liquid cultures were incubated at 37 °C and shaken at 220 rpm. Cultures were passaged every 5 days by adding 100 µl into 10 ml broth and were passaged a maximum of 5 times. At this point, a new colony was selected from an agar plate and used to begin a novel liquid culture.

### **3.3.4. Culturing *M. bovis* BCG strains**

Liquid cultures were made from Middlebrook 7H11 broth + 0.5 % glycerol with 0.05% Tween 80 and the correct selective antibiotic. Kanamycin was added at a concentration of 25 µg/ml. Cultures were incubated at 37 °C and 5% CO<sub>2</sub>, and passaged every 10 days by inoculating 10 ml broth with 100 µl of the previous culture. After 5 passages, the next culture was started using a new colony from an agar plate.

### **3.3.5. *M. smegmatis* Liquid MIC99 Tests and *M. bovis* BCG Liquid MIC99 Tests**

Liquid MIC99 Tests were carried out using 96-well flat-bottom plates. Initially each well contained 100 µl of broth + 0.05% tween, apart from column 2 which contained 200 µl plus the highest concentration of drug. This was then serially diluted 9 times across the plate. The range of concentrations for Vanoxerine began at 400 µM, DCS began at 400 µg/ml, and EMK compounds began at 800 µM. A stock of each compound was kept at 10 mM. Cells were taken from an exponentially growing culture and diluted to an initial OD<sub>600</sub> of 0.1. 100 µl of the culture was added to each well to give a final OD<sub>600</sub> of 0.05. When needed, 50 ng/ml anhydrotetracycline was added to induce the expression of genes cloned on to the pTIC expression vector. This was then wrapped in tin

foil to prevent evaporation and incubated at 37 °C for 24 h. The fluorescence of GFP-expressing strains was measured immediately by excitation at 485 nm and emission at 520 nm with an automated microtitre-plate reader, whereas wild-type strains were incubated with 30 µl 0.02% resazurin in each well for 4 h (*M. smegmatis*) and 24 h (*M. bovis* BCG) using a previously published protocol, then fluorescence was measured by excitation at 530 nm and emission at 590 nm<sup>81</sup>. The output was given as raw data for each microtitre well and as a % survival when compared with positive and negative controls, accompanied by a Z-prime value. The Z-prime value assesses the quality and reproducibility of the assay, it is a standard statistical measure used in high throughput screening. It relies upon the means ( $\mu$ ) and standard deviations ( $\sigma$ ) of the positive (p) and negative (n) controls:

$$\text{Z-prime} = 1 - (3(\sigma_p + \sigma_n)) / (\mu_p - \mu_n)$$

The Z-prime value determines the scale of separation between the positive and negative controls in relation to the standard deviations. Assays were only considered of good enough quality if the Z-prime value was above 0.5, meaning that there are 12 standard deviations between the mean of the positive and the mean of the negative controls. Graphpad Prism was used for analysis and to determine the MIC99, IC50 (the concentration that gives 50% survival) and highest non-inhibitory concentration (NIC) for each data set using results given as % survival.

### **3.3.6. *M. smegmatis* Solid MIC Tests and *M. bovis* BCG Solid MIC Tests**

Cell growth was tested against a range of concentrations of Vanoxerine, from 0 µM to 100 µM. *M. smegmatis* was grown on 7H9 agar + 0.5% glycerol and 7H11 agar + 0.5% glycerol + 10% OADC was used for *M. bovis* BCG. Plasmid-selective antibiotic was included in the media when required. 2 µl of cells at OD 0.1, 0.01, 0.001 and 0.0001

were spotted onto the plate in a clockwise manner and the plates were incubated at 37 °C for 4 days (*M. smegmatis*) or 37 °C + 5 CO<sub>2</sub> for 10 days (*M. bovis* BCG). The MIC was determined as the plate where there was no growth for the inoculum of lowest cell concentration.

### **3.3.7. *M. bovis* BCG Vanoxerine-resistant Mutant Generation**

Plates were made using the same media used for solid MIC testing and containing 0X, 1X, 2.5X and 5X, and 10X the MIC of Vanoxerine. 10<sup>8</sup> cells were spread on each plate, which was then incubated at 37 °C + 5% CO<sub>2</sub> for a minimum of 2 weeks or until the plates dried out.

### **3.3.8. Transformation of plasmids into *M. smegmatis* and *M. smegmatis* pSNT3eGFP**

Electrocompetent cells were made by growing a 50 ml culture to OD 0.5, then cells were serially washed with 10% glycerol (v/v) and harvested, each time resuspending in a smaller volume of 10% glycerol. 100 µl of electrocompetent cells were added to a 2mm electroporation cuvette with 6 µl of the plasmid DNA. This was left on ice for 30 minutes then electroporated at 1800V, and returned to the ice for 15 min before 250 µl tryptic-soy broth (TSB) was added and the cells. They were then incubated for 4 h at 37 °C before spreading on TSB-agar plate with the appropriate antibiotics. Cells were also spread onto a TSB-only plate to act as a control. Plates were kept at 37 °C and successful transformants were grown in a liquid culture and then stored at -80 °C in 25% glycerol.

### **3.3.9. Second-line drug screen against the FDA library**

Two screens were carried out using *M. bovis* BCG against the FDA library. 96-well microtiter plates were used; each well containing 10 µl 400 µM of each compound to which 100 µl *M. bovis* BCG cell culture at OD<sub>600</sub> 0.05 was added. An inhibitory concentration of isoniazid was

added to the negative control wells. Both screens were carried out simultaneously, with the second screen also containing 6.25 µg/ml DCS in the cell culture. The 96-well plates were then incubated at 37 °C for 48 h in air-tight containers, then 30 µl 0.02% resazurin was added to each well and the plates were replaced for a further 24 h<sup>81</sup>. Once the plates had been read, the two data sets were compared for significant changes in cell survival.

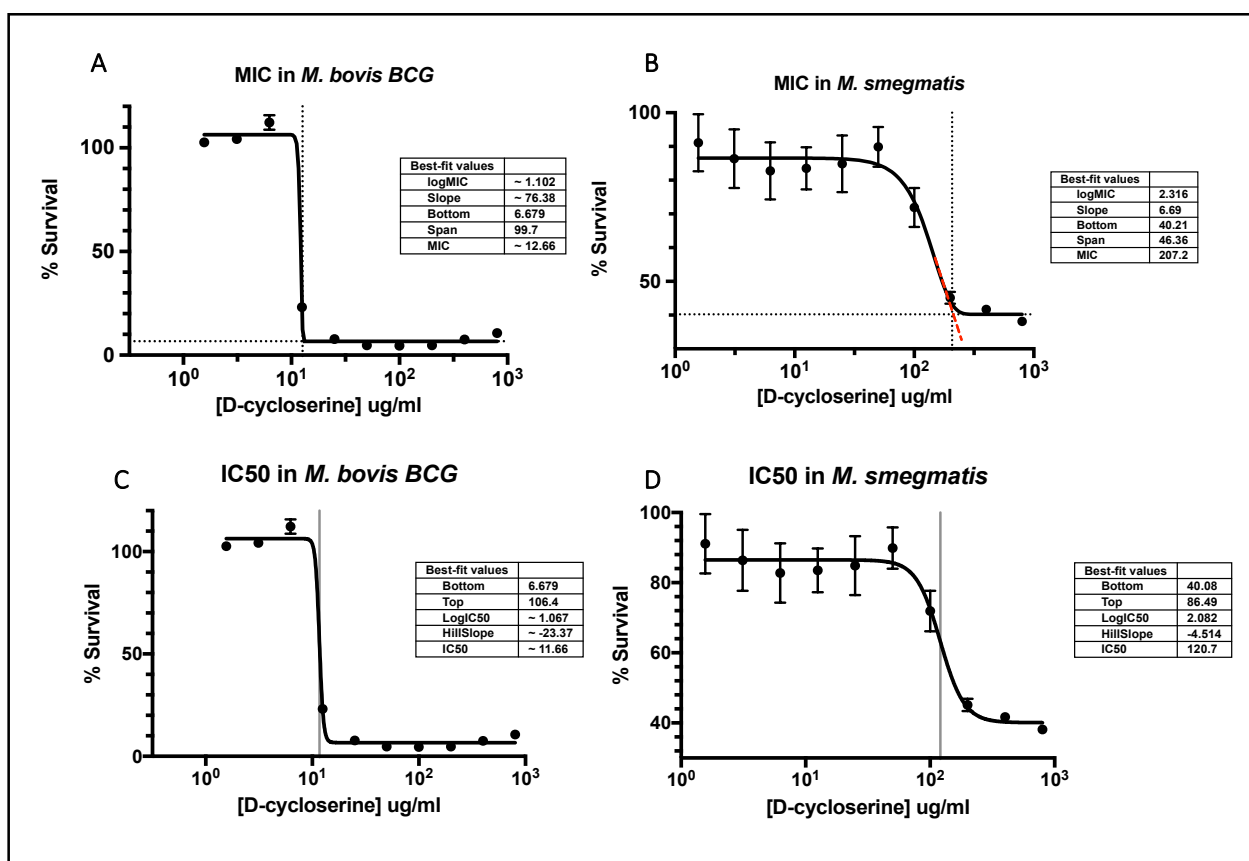


### 3.4. RESULTS

#### 3.4.1. FDA Compound Screen for DCS Adjuncts

##### **Determination of the D-cycloserine MIC99 and IC50 in *M. bovis* BCG and *M. smegmatis***

MIC can be defined as the minimum inhibitory concentration that completely retards bacterial growth. Typically it is determined visually, however for this study 96-well microtitre plates were used for experiments to make the data more comparable and as a result the process was automated and the MIC determined by an algorithm to make it accurate and reproducible<sup>82</sup>. As a result, any liquid MIC determined during this study will be defined as the MIC99 that gives 99% growth inhibition. Before performing the FDA-compound screen it was necessary to determine the MIC99 of DCS for *M. bovis* BCG and a sub-inhibitory concentration to use as a standard, the IC50. The MIC99 was determined as 12.66 µg/ml and the IC50 as 11.66 µg/ml in *M. bovis* BCG (Figure 6). As there was little difference between the MIC99 and the IC50, the concentration 6.25 µg/ml was chosen for the FDA screen as this was the last concentration tested that gave 100% survival.



**Figure 6.** Cell survival of *M. bovis* BCG (A, C) and *M. smegmatis* (B, D) grown in different concentrations of D-cycloserine. The minimum inhibitory concentration was determined as 12.66  $\mu\text{g/ml}$  in *M. bovis* BCG and 207.2  $\mu\text{g/ml}$  in *M. smegmatis*. The IC50, the concentration that gives 50% survival, was 11.66  $\mu\text{g/ml}$  in *M. bovis* BCG and 120.7  $\mu\text{g/ml}$  in *M. smegmatis*.

## BCG FDA Screen against D-cycloserine at Sub-Inhibitory Concentrations

Two screens against the FDA compound library were carried out simultaneously. Both used the same culture of *M. bovis* BCG, however, while screen A was cell-only screen B contained DCS at 6.25  $\mu\text{g/ml}$ . Table 4 shows a comparison between the two screens, listing all 50 of the compounds which give reduced survival in the presence of DCS. B/A ratios describe the relationship between cell survival in both assays. A B/A ratio of less than 1 means that cell survival is reduced when a sub-inhibitory concentration of DCS is present.

A common theme from these HTS results is the presence of other antibiotics. DCS lowers the minimum inhibitory concentration needed for many Rifamycins, including the first-line TB drugs rifampicin and rifabutin with B/A ratios of -0.68 and 0.03 respectively (Table 4)<sup>64</sup>. Of the other first-line tuberculosis drugs, cell survival % for streptomycin sulphate is lowered slightly when combined with DCS, shown by the B/A ratio of 0.91. Isoniazid and pyrazinamide do not appear to be affected as they gave B/A ratios of 1.02 and 1.00 respectively and cell survival was close to 100 % for both screens. Other antimycobacterial drugs appear to have synergy with DCS, such as dapson which had a B/A ratio of 0.70. Dapsone has been used to treat leprosy, caused by *Mycobacterium leprae*, since 1945, and has also been approved for use against dermatitis herpetiformis by the FDA<sup>83,84</sup>.

A few antimicrobials also appear to have an increased killing effect on *M. bovis* BCG when used in conjunction with DCS, for example, there are two antifungal agents, Enilconazole and Clotrimazole listed as hits (Table 4)<sup>85,86</sup>. They gave the B/A ratios of 0.95 and 0.94 respectively. Pentamidine isethionate, with a B/A ratio of 0.90 is an antiparasitic drug used to treat African trypanosomiasis and leishmaniasis<sup>87,88</sup>. Interestingly, the  $\beta$ -lactamase inhibitor clavulanic acid had a B/A ratio of 0.80. Clavulanate is used as an adjuvant with  $\beta$ -lactam antibiotics to resensitise penicillin-resistant bacteria<sup>89</sup>.

As well as antimicrobials, DCS appears to increase bactericidal activity for a number of drugs licenced for other uses. The most dramatic change can be found with Auranofin, a gold-containing compound currently used as an antirheumatic, which had a B/A ratio of 0.17 (Table 4)<sup>73</sup>. Some drugs that appeared as hits in this screen affect the central nervous system, such as Protriptyline hydrochloride which is used as a tricyclic antidepressant, and zotepine, a second-generation antipsychotic drug used in the treatment of schizophrenia<sup>90,91</sup>. It is unlikely that either of these would be recommended for use with DCS as all three drugs affect the CNS<sup>69</sup>.

There are many other antibiotics in the FDA library that were not affected by addition of DCS, and these were found across a range of antibiotic classes. Such antibiotics include ceftazidime pentahydrate, a third-generation cephalosporin which gave a B/A ratio of 1.02 and the aminoglycoside sisomicin sulphate which had a B/A ratio of 0.98. Additionally, imipenem, a carbapenem  $\beta$ -lactam antibiotic, had a B/A ratio of 0.97, and dirithromycin, a macrolide antibiotic, gave a B/A ratio of 1.12<sup>92-95</sup>. The B/A ratios vary slightly between these antibiotics but, most importantly, all had a measured cell survival around 100 % (the level of the positive control) for both screens. This means that they had no effect on cell survival in the presence or absence of DCS.

Some compounds appeared to interact antagonistically with DCS. One such compound is the antibiotic vancomycin hydrochloride which gave a B/A ratio of 1.4. Vancomycin is a glycopeptide antibiotic often used as a last-resort antibiotic to treat serious drug-resistant gram-positive infections<sup>96</sup>. A B/A ratio of 1.4 suggests that cell survival increased when DCS was present in combination with vancomycin. The data shows cell survival was 11.09 % with vancomycin and 15.63 % with DCS and vancomycin together. Although these results show that cell growth is inhibited significantly in both conditions, it also suggests that the inhibitory properties of vancomycin are perturbed in the presence of low levels of DCS, highlighting problems that may occur if combining the drugs in a clinical setting. The most dramatic antagonistic effect is seen with methiothepin maleate, an antipsychotic<sup>97</sup>. In the presence of sub-inhibitory concentrations of DCS the cell survival went from 42.73% to 95.05%, giving a B/A ratio of 2.22 and completely negating any antimycobacterial effects.

**TABLE 4. TABLE LISTING THE POTENTIAL HITS FOUND DURING THE FDA LIBRARY SCREEN. THE COMPOUNDS RESPONSIBLE ARE LISTED IN THE FINAL COLUMN. HITS HAVE A B/A RATIO OF LESS THAN 1 AS % SURVIVAL IS GREATER WHEN DCS IS NOT PRESENT. NEGATIVE VALUES ARE SEEN WHEN MEASURED % SURVIVAL IS LESS THAN THE NEGATIVE CONTROL.**

% Survival		Ratio B/A	Drug	Description
A (Cells only)	B (D-CS Present)			Antibiotic Class
3.345159789	2.709215831	0.809891306	Gatifloxacin	Fluoroquinolones
8.48532827	4.177389132	0.49230731	Moxifloxacin	Fluoroquinolones
5.276818625	3.15829922	0.605635734	Nadifloxacin	Fluoroquinolones
10.72218526	6.803020839	0.634480815	Doxycycline hydrochloride	Tetracyclines
5.126184681	4.131587731	0.805977152	Meclocycline sulfosalicylate	Tetracyclines
8.059204371	5.784843072	0.717793321	Minocycline hydrochloride	Tetracyclines
12.51128205	11.19545544	0.894828794	Methacycline hydrochloride	Tetracyclines
2.953019428	0.717733235	0.243050631	Rifaximin	Rifamycins
1.35322159	0.044284017	0.032724882	Rifabutin	Rifamycins
1.027568633	-0.69561427	-0.676951639	Rifampicin	Rifamycins
1.02678388	-0.84931202	-0.827157532	Rifapentine	Rifamycins
13.89244676	9.443188916	0.679735476	Clarithromycin	Macrolides
15.66104429	14.31626745	0.914132365	Tylosin	Macrolides
12.14146477	11.15106421	0.918428247	Roxithromycin	Macrolides
81.93618609	51.12853184	0.624004293	Troleandomycin	Macrolides
12.29642263	7.471082767	0.607581814	Viomycin sulfate	Tuberactinomycin family
75.05533577	61.46806131	0.818969906	Sulfadoxine	Sulfonamides
88.36849787	68.15247091	0.771230388	Sulfamethoxypyridazine	Sulfonamides
81.81493374	65.5928139	0.801721775	Sulfadimethoxine	Sulfonamides
60.23188744	51.34704927	0.852489461	Sulfaquinoxaline sodium salt	Sulfonamides
99.04325997	89.73274304	0.905995452	Sulfapyridine	Sulfonamides
73.37461153	53.33636054	0.726904844	Sulfachloropyridazine	Sulfonamides
79.34903803	60.13122942	0.757806659	Sulfamerazine	Sulfonamides
65.3363437	50.32707292	0.770276849	Sulfadiazine	Sulfonamides
61.61298001	43.22194065	0.701507063	Dapsone	Sulfones
8.543664411	3.299002913	0.386134421	Apramycin	Aminoglycosides
15.84890867	10.31797564	0.651021206	Tobramycin	Aminoglycosides
3.080802853	1.808629833	0.58706445	Amikacin hydrate	Aminoglycosides

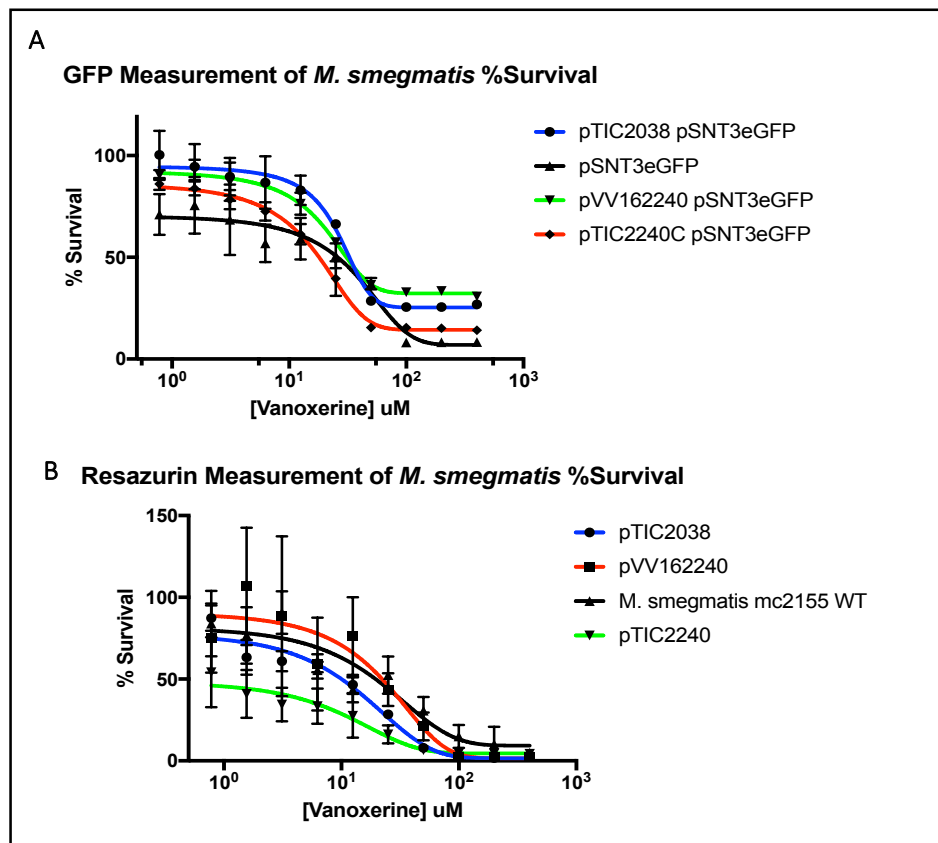
9.105023948	8.306152429	0.91226036	Streptomycin sulfate	Aminoglycosides
18.6606877	13.63130709	0.730482569	Gentamicine sulfate	Aminoglycosides
8.278133373	6.317405769	0.763143753	Dihydrostreptomycin sulfate	Aminoglycosides
44.14187837	37.30914166	0.845209652	Florfenicol	Phenicol
32.35296157	28.95862445	0.895084191	Thiamphenicol	Phenicol
4.857429464	3.100296499	0.638258676	Thiostrepton	Thiopeptides
15.50424322	7.419426622	0.478541682	Linezolid	Oxazolidinones
83.58896025	65.74426261	0.786518488	Sulfacetamide sodic hydrate	
<b>Not Currently Antibiotics</b>				
74.27600141	70.67780924	0.951556464	Enilconazole	Antifungal agent <sup>85</sup>
66.72212454	64.22889044	0.962632573	Salmeterol	
92.88983205	87.6520121	0.943612559	Protriptyline hydrochloride	Tricyclic antidepressant (TCA) <sup>90</sup>
29.35858164	4.96622702	0.169157594	Auranofin	antirheumatic agent <sup>73</sup>
58.6627251	46.90704673	0.799605655	Clavulanate potassium salt	b-lactam drug that inhibits b-lactamase <sup>89</sup>
89.56264909	78.94338766	0.881432031	Topotecan	
3.121447267	2.818781585	0.903036747	Pentamidine isethionate	Antiparasitic agent <sup>88</sup>
69.34561864	59.14401216	0.852887512	Amiodarone hydrochloride	
94.53179574	82.68924081	0.874724109	Cyclobenzaprine hydrochloride	
0.079280865	0.063049364	0.795265848	Doxorubicin hydrochloride	
93.18402104	82.76341653	0.888171766	Zotepine	antipsychotic <sup>91</sup>
48.87354515	42.72754438	0.87424688	Perhexiline maleate	
62.45088133	58.46523734	0.93617954	Clotrimazole	Antifungal agent <sup>86</sup>

### 3.4.2. Mode-of-Action Studies of Vanoxerine

A similar HTS screen had been performed by Panchali Kanvatirth (University of Birmingham) previously to find drugs within the FDA library that have antimycobacterial activity against *M. smegmatis* and *M. bovis* BCG. From this screen, the small-molecule compound vanoxerine showed promising results. Christopher Burke (University of Birmingham) raised two mutants in *M. smegmatis* against vanoxerine at 2.5X the MIC by inoculating plates containing vanoxerine at 0, 1, 2.5, 5 and 10X the MIC and incubating plates until growth appeared or the agar dried out, the same method as used during this study. The mutants' genomes were isolated and sequenced by Panchali Kanvatirth (University of Birmingham). Genes which contained non-synonymous mutations were thought to be potential targets. Two genes, MSMEG\_2038 and MSMEG\_2240 were found mutated in two separate vanoxerine-resistant mutants. They encode a cytochrome p450 and a monooxygenase, both have very similar functions and catalyse oxidation of their substrate<sup>98</sup>. As a result, their wild-type counterparts were suggested to be the targets of vanoxerine, and so by overexpressing them during cell survival assays their role should be explained further. The second arm of this project was to continue investigation into the mode of action of vanoxerine by overexpressing these genes in *M. smegmatis*.

### GFP-Expressing Strains Were Chosen for the Cell Survival Assay

The plasmid pSNT3eGFP was transformed into *M. smegmatis* and used for reading the cell survival in liquid cultures. This was compared with growing wild-type *M. smegmatis* and then adding resazurin, a compound that fluoresces when metabolised. The former was shown to give the most accurate results, as shown in Figure 7.



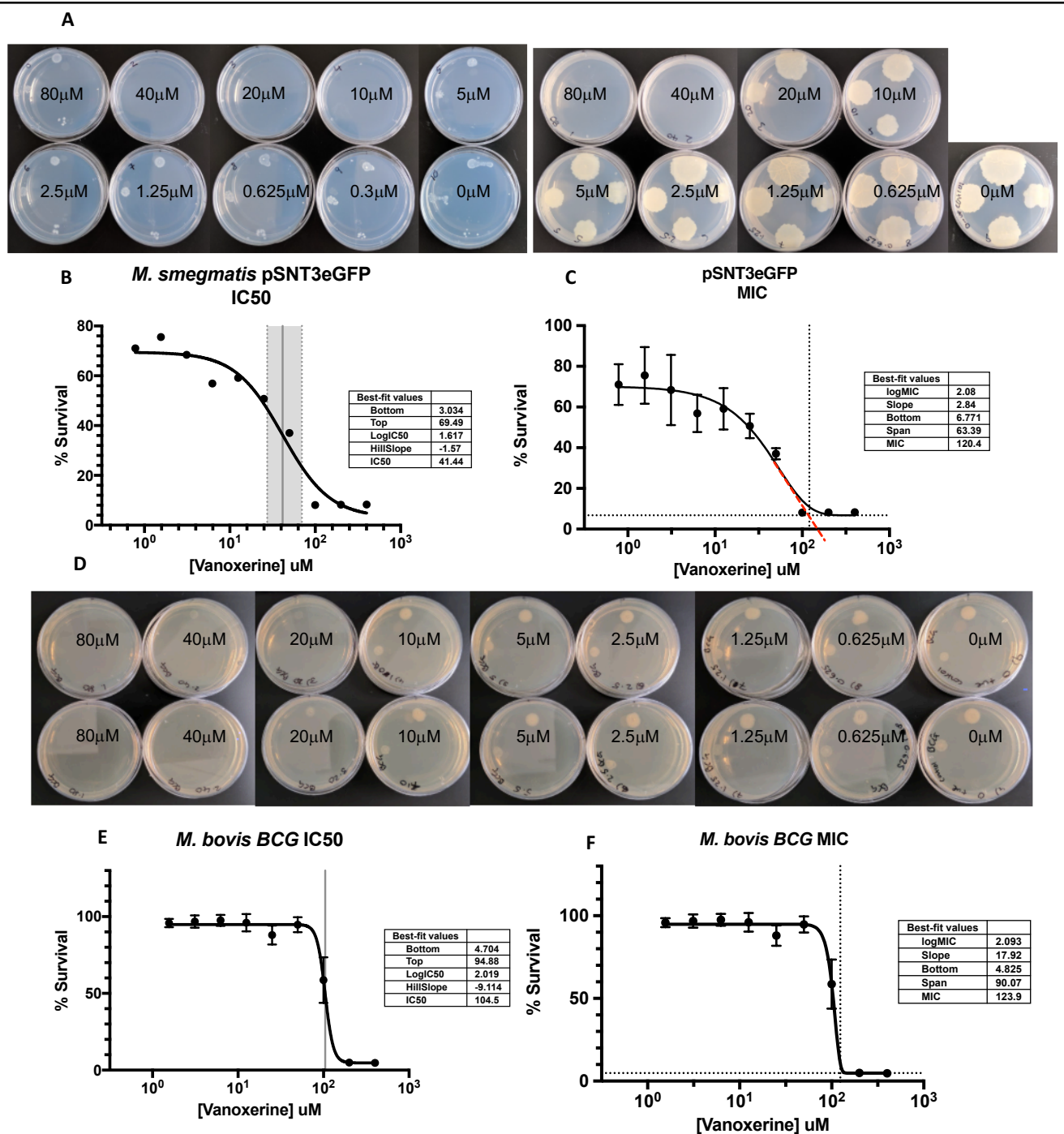
**Figure 7. Comparison of 96-well broth microdilution survival tests.**

The strain *M. smegmatis* pSNT3eGFP was grown in a range of vanoxerine concentrations, after 24 h cell survival was measured using GFP before resazurin was added and the plate was incubated for a further 4 h. Cell survival was then measured using red-ampex end-point assay. The error bars in A are not as large as the error bars in B, showing that there was greater reliability with the GFP method.

### **MIC Determination for Vanoxerine in *M. smegmatis* and *M. bovis* BCG**

The MIC<sub>99</sub> of vanoxerine in liquid *M. smegmatis* culture was determined as 120.4  $\mu$ M and in solid agar it is significantly lower, between 5 and 10  $\mu$ M. This pattern was repeated with *M. bovis* BCG; the liquid MIC<sub>99</sub> was 123.9  $\mu$ M and the solid MIC<sub>99</sub>, between 10 and 20  $\mu$ M (Figure 8).



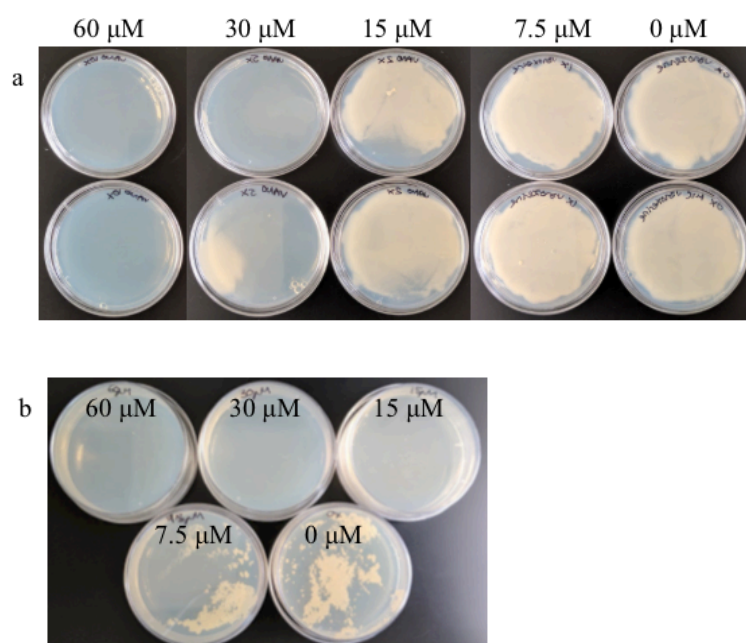


**Figure 8. MIC and IC50 of vanoxerine for *M. smegmatis* and *M.***

***bovis* BCG in both solid and liquid media.** A: two *M. smegmatis* pSNT3eGFP solid MIC tests against vanoxerine. Small colonies on the first MIC test made determining the MIC more difficult, so the test was repeated using 4x as many cells. The MIC is between 5 and 10  $\mu$ m. B, c: corresponding liquid MIC tests gave an MIC of 120.4 and IC50 of 41.44. D: solid MIC tests for *M. bovis* BCG show the MIC as between 10 and 20  $\mu$ m. E, f: the MIC and IC50 was determined as 123.9  $\mu$ m and 104.5  $\mu$ m respectively for *M. bovis* BCG.

### Growing *M. bovis* BCG in High Concentrations of Vanoxerine did not Yield any Vanoxerine-resistant Mutants

Generation of vanoxerine-insensitive mutants would allow a more clinically-relevant investigation into revealing the target of vanoxerine. Despite repeated attempts there were no mutants on 5X and 10X MIC99 media, and any growth on the other plates was seen as a film (Figure 9.A). On the third attempt fewer cells were used to inoculate the plate to prevent film formation (Figure 9.B).

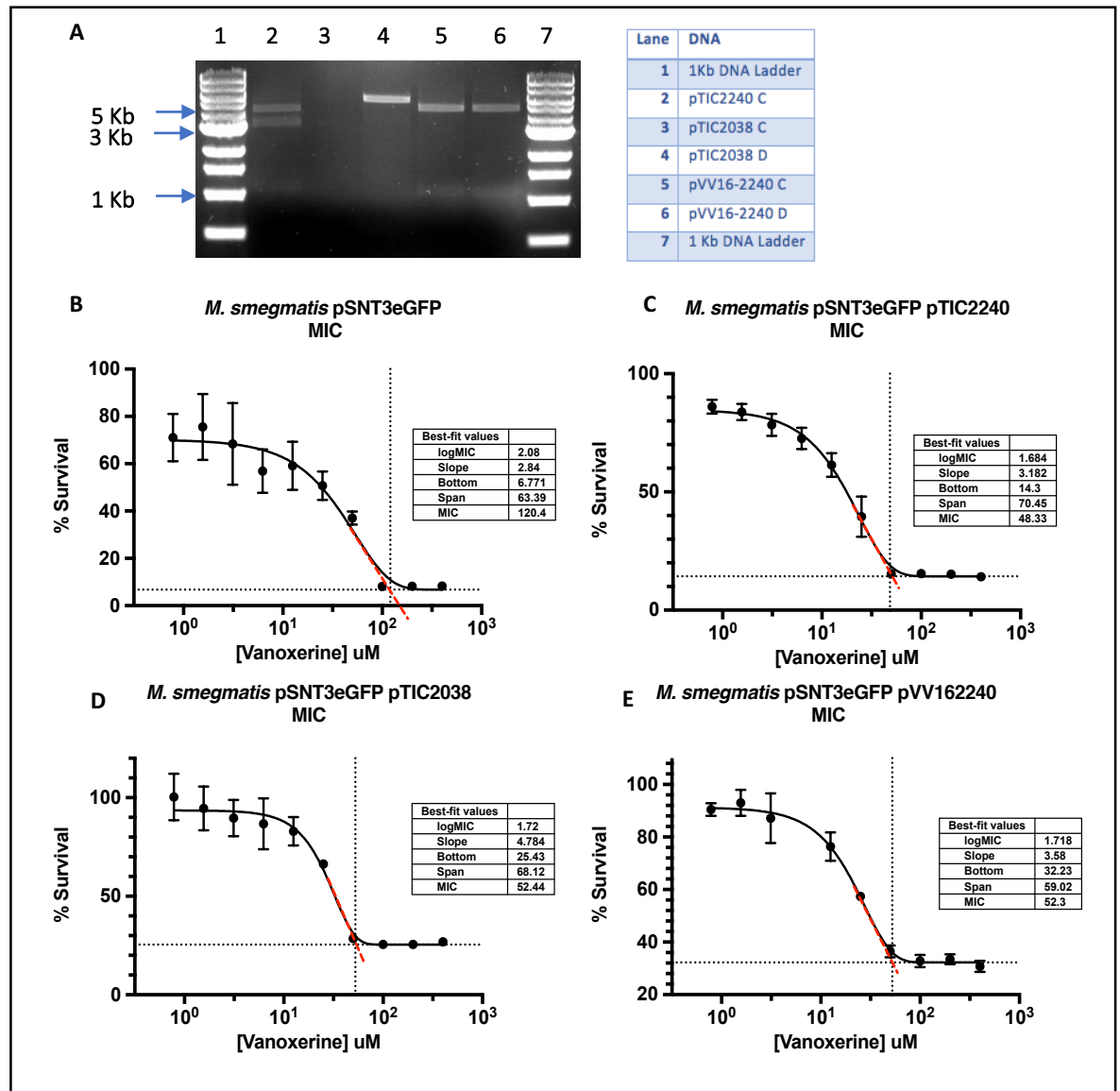


**Figure 9. Attempts to generate vanoxerine-resistant mutants in *M. bovis* BCG.** Plates contained 60  $\mu$ M, 30  $\mu$ M, 15  $\mu$ M, 7.5  $\mu$ M and 0  $\mu$ M vanoxerine as the MIC used was determined in a previous study as 7.5  $\mu$ M (Christopher Burke, University of Birmingham).

### **Overexpression of CYP Genes Leads to an Increased Sensitivity to Vanoxerine in *M. smegmatis***

A number of plasmid samples prepared by Christopher Burke (University of Birmingham) were tested at the commencement of this study to ensure that the expected plasmids needed for this project were present (Figure 10.A). Preps labelled pTIC-2240 C, pTIC-2038 C and D, and pVV16-2240 C and D, were digested to remove the inserted gene, either MSMEG\_2240 or MSMEG\_2038. The letters C and D are used to indicate and identify plasmids from separate plasmid preps. DNA fragments were separated on an agarose gel. The gene length of MSMEG\_2240 is 1209 bp and MSMEG\_2038 is 1560 bp. Correct DNA fragments confirmed the presence of the expected plasmids (Figure 10.A, lanes 2, 4, 5 and 6). As seen in Figure 10.A, there was no DNA in the plasmid prep labelled pTIC2038 C (lane 3) so this was discarded.

Electrocompetent *M. smegmatis* and *M. smegmatis* pSNT3eGFP were transformed with the verified expression plasmids pTIC2240 C, pTIC2038 D and pVV16-2240 C and grown on selective agar plates. Successful transformants were cultured and the MIC99 against vanoxerine was determined for each. Liquid MIC99 tests revealed that over-expression of MSMEG\_240 and MSMEG\_2038 had the opposite effect to what was expected: the MIC99 decreased and the cells became more sensitive to vanoxerine (Figure 10. B, C, D, E).



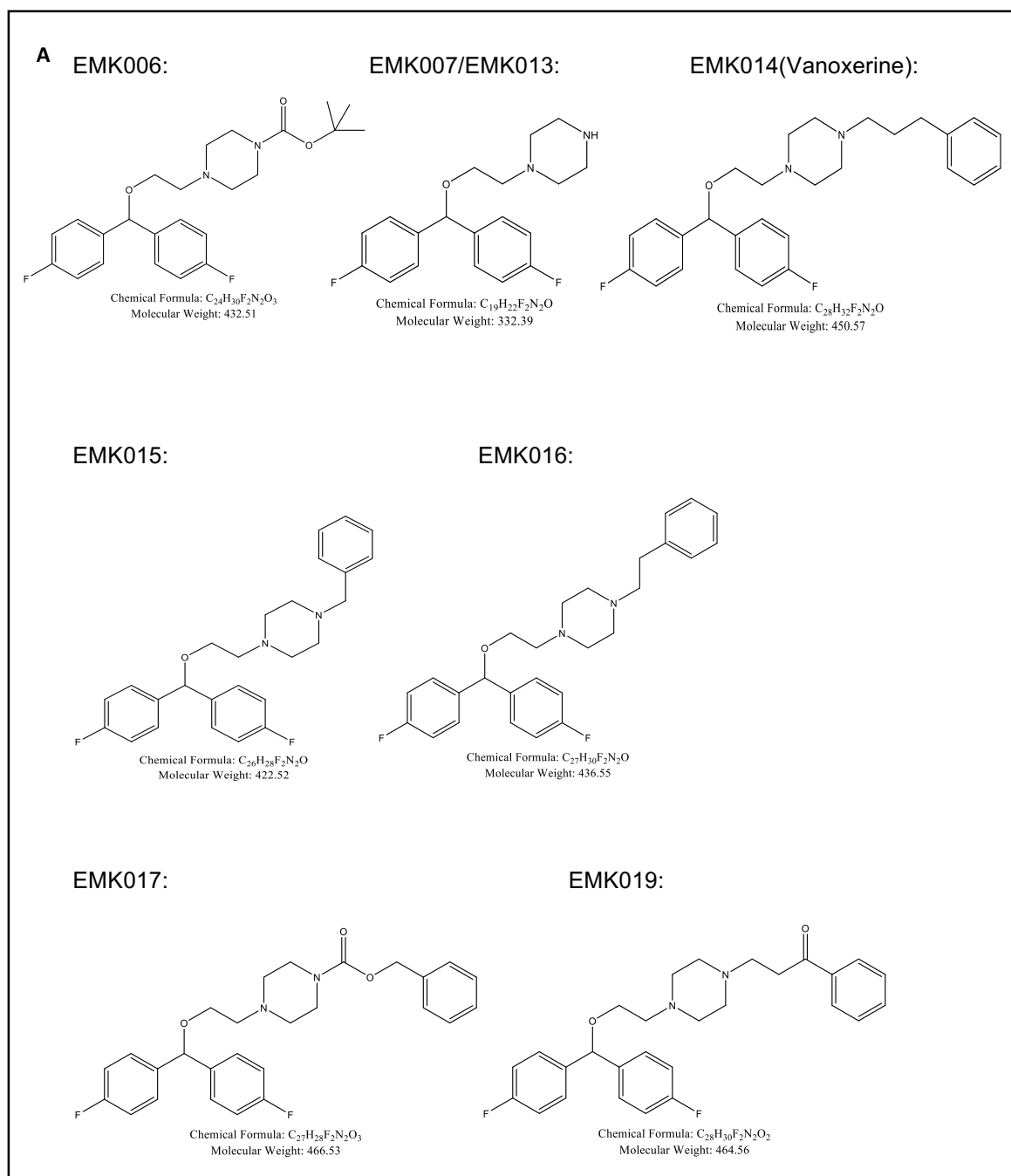
**Figure 10.** Expression plasmids were verified by removing the gene fragment with a double digest and separating the results on a gel (A). The plasmids were prepared by Christopher Burke (University of Birmingham) using different methods, and the letter at the end of each plasmid name corresponds to the method used. The plasmid prep from lane 3 did not contain DNA. Correct plasmids were transformed into *M. smegmatis* pSNT3eGFP and successful transformants were used to determine the MIC for vanoxerine in a liquid MIC test. The MIC of *M. smegmatis* pSNT3eGFP was 120.4  $\mu\text{M}$  (B) and the MIC of the expression strains containing pTIC6a-2240, pTIC6a-2038 and pVV16-2240 was 48.33  $\mu\text{M}$ , 52.44  $\mu\text{M}$  and 52.3  $\mu\text{M}$  respectively (C, D, E).

## **Vanoxerine Analogues Exhibit Altered Activity on *M. smegmatis***

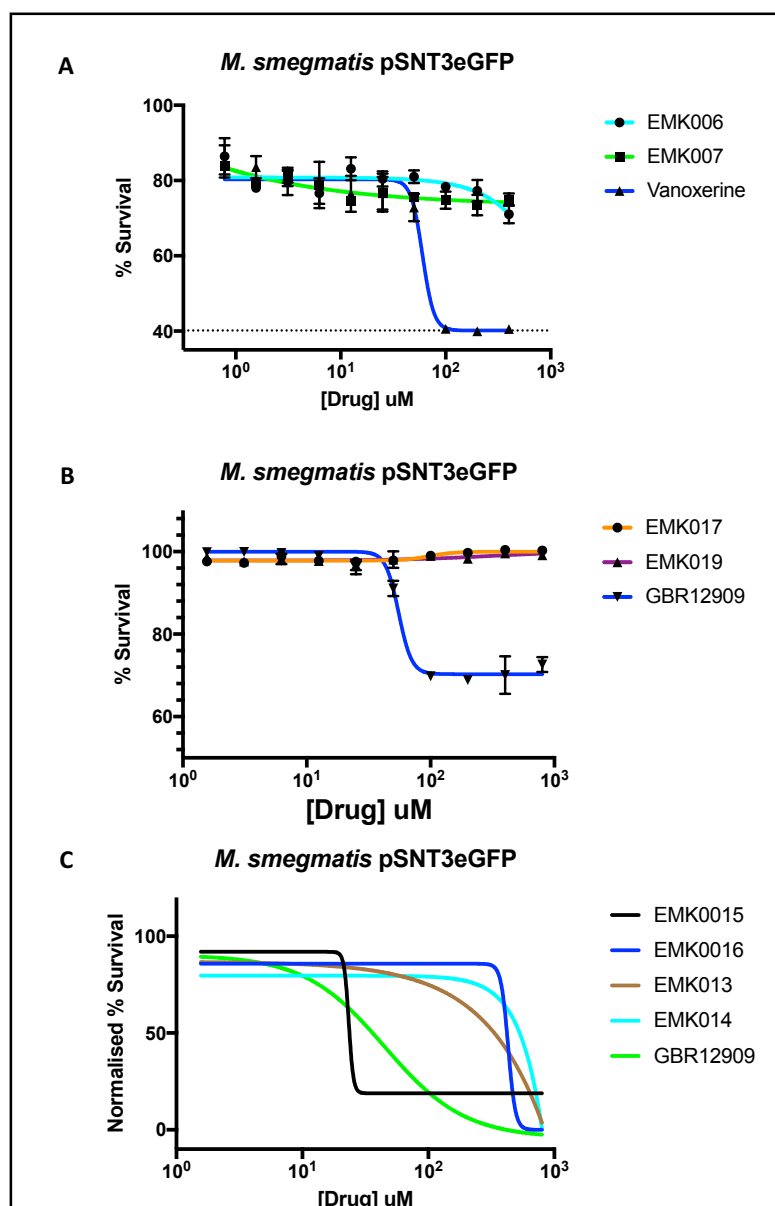
Due to the promising effects of vanoxerine on *M. smegmatis* and *M. bovis* BCG, vanoxerine analogues were chemically synthesised through a collaboration with another laboratory, and these were then tested for their antimicrobial properties (Figure 11).

Figure 12.A shows that EMK007 and EMK006 have no antimicrobial properties. Similarly, EMK017 and EMK019 did not inhibit cell survival (Figure 12.B). The curves for vanoxerine (GBR12909) in Figures A and B have different aspects because of background noise, likely present because the turnover of GFP proteins produced by the cells is too slow. The GFP protein is used in order to measure and quantify the population of cells using fluorescence.

EMK compounds that displayed activity against *M. smegmatis* pSNT3eGFP can be found in Figure 12. C. In order to compare the relative activities of these EMK compounds the results were normalised separately for each compound using Graphpad Prism so that 0% was defined as the smallest mean in each EMK compound data set, and 100% was the largest mean (Figure 12.C). Normalisation was carried out because there was a wide range in values for % survival at non-inhibitory and fully inhibitory concentrations between the EMK compound assays, including those of the vanoxerine controls, due to varying background levels of GFP. Therefore, the data sets have been presented in a manner that makes them comparable. Figure 12.C shows that EMK013, EMK014 and EMK016 only impacted growth under the highest concentrations. Interestingly, EMK015 appears far more potent at inhibiting cell growth than vanoxerine. Vanoxerine (GBR12909) was chemically synthesised in the lab and labelled EMK014. However, this did not have the same MIC99 as the industrially-made chemical.



**Figure 11. Chemical structures of vanoxerine analogues.** Also referred to as "EMK compounds".



**Figure 12. A, B, C: Liquid MIC tests against these analogues with vanoxerine (also referred to as GBR12909) as a control.** Vanoxerine synthesized in this study is referred to as EMK014. Figure 12. C shows a normalised % survival for EMK compounds 015, 016, 013 and 014 with vanoxerine (GBR12909) as the control. The curves appear different as all data has been normalised to aid comparison. Normalisation was carried out because there was a wide range in % survival at non-inhibitory and completely inhibitory concentrations across the EMK data sets. To overcome this each data set was normalised to fit a scale of 0 to 100, with 0 being the lowest value of % survival and 100, the highest. The normalised % survival shows that EMK0015 has a lower MIC than that of vanoxerine and is therefore more potent.

### 3.5. DISCUSSION

#### **The FDA Compound Screen Revealed Drugs that Increase the Potency of DCS in Mycobacteria**

Due to the efficacy of and lack of cross-resistance, DCS is an effective second-line drug for MDR-TB treatment, yet 19-55% of patients stop chemotherapy due to its highly toxic nature <sup>69</sup>. Prescribing the lowest dose of DCS that will cure tuberculosis is therefore a necessity, and finding compounds that enable a lower dose to be administered would both improve the patients' quality of life and reduce treatment discontinuation <sup>99</sup>. The aim of this study was to find compounds that reduce cell survival when a non-inhibitory concentration of DCS is also present, thus showing synergy with DCS.

Current literature states the MIC of DCS as 15 µg/ml in *M. tuberculosis* <sup>100</sup>. In *M. bovis BCG* the MIC99 was determined as 12.66 µg/ml, but is nearly 18-fold higher in *M. smegmatis* emphasising the greater clinical relevance of *M. bovis BCG* as a model. At 11.66 µg/ml the IC50 was too similar to the MIC99 to use as a sub-inhibitory concentration for testing with *M. bovis BCG* against the FDA library, instead 6.25 µg/ml DCS was used.

During this study 50 hits were identified; around 4% of the FDA library. Most results were already known antibiotics, such as the sulphonamides, moxifloxacin, gentamicine and thiamphenicol. The first-line TB drugs rifampicin and rifabutin had promising synergistic effects with DCS. Rifampicin is used in combination with ethambutol, isoniazid and pyrazinamide for treatment against drug-susceptible tuberculosis<sup>64</sup>. Rifabutin is used in the place of rifampicin for HIV/AIDS patients undergoing first-line drug treatment, due to the complications that arise when used in combination with anti-retroviral therapy (ART)<sup>64</sup>. Briefly, rifampicins induce hepatic enzymes such as CYP3A which in turn lower serum concentrations of protease inhibitors used in ART. Rifabutin has a much lower effect on CYP3A induction than rifampicin and so is used



as a substitute for treating drug-susceptible tuberculosis in HIV/AIDS patients<sup>64</sup>. However, clinical use of rifamycins in combination with DCS would be unlikely, as DCS is used only in cases where TB is already resistant to two first-line drugs. Other first-line drugs such as isoniazid and pyrazinamide did not lower the cell survival from ~100 % when combined with sub-inhibitory concentrations of DCS. Clavulanate potassium salt also shows synergistic effects with DCS. Also known as clavulanic acid, it is commonly used with penicillins as it is a beta-lactamase inhibitor and can re-sensitise beta-lactam resistant bacteria<sup>89</sup>. A synergistic effect with clavulanic acid has been shown in other drug-repurposing studies with cephalosporins<sup>75</sup>. There were a number of antibiotics that had no effect on cell survival when in the presence or absence of DCS. This is likely because *M. bovis* BCG is resistant to both the drugs and DCS at the concentrations used.

The most promising result is auranofin, an antirheumatic gold-containing compound. With 40  $\mu$ M auranofin present the survival of *M. bovis* BCG was 21.35 % which was then reduced to 4.9 % in the presence of 6.25  $\mu$ g/ml of DCS. This study has demonstrated that auranofin works synergistically with DCS as cell survival was reduced to values close to known TB drugs such as rifampicin and rifabutin. Auranofin is currently used as an antirheumatic drug, with a plasma half-life of 15-25 days and a bioavailability of 40%<sup>73,101</sup>. As mycobacteria exist in the body in different metabolic states - actively growing cells double only every 24 h and persistors remain dormant - the long half-life is promising for TB therapy because the longer the drug remains active in the body the more likely a successful outcome.<sup>64</sup>

There were a couple of cases where the cell survival was greater in the screen containing DCS, suggesting that potential drug interactions are antagonistic. This was most dramatically seen with methiothepin maleate, where the inhibitory properties were perturbed completely in the presence of DCS. Methiothepin maleate is an antipsychotic drug and serotonin receptor antagonist<sup>97</sup>. It is hard to speculate why this occurs without knowing the target of methiothepin maleate in the mycobacterial

cell. Vancomycin also produced a B/A ratio greater than 1, and this may be easier to explain as both vancomycin and D-cycloserine target the bacterial cell wall. D-cycloserine targets the D-Alanine-D-Alanine Ligase Ddl in *Mycobacterium tuberculosis*, and Vancomycin targets terminal D-Ala-D-Ala dipeptides in peptidoglycan precursors<sup>102,103</sup>. Therefore, with sub-inhibitory concentrations of DCS present perhaps fewer D-Ala-D-Ala dipeptides are synthesised and the antibacterial effects of vancomycin are reduced.

The experiment was shown to be both reliable and repeatable as compounds that have no effect on *M. bovis* BCG had values similar to each other and to the positive control. The assay is of good quality as the determined Z-prime values are extremely high. Continuation of this project should focus on determining the mode-of-action of auranofin and other hits that are not already known for being antimicrobials. Where possible their chemical scaffold should be used to develop more potent analogues.

### **No Mutants were Raised against vanoxerine in *M. bovis* BCG**

*M. bovis* BCG is a more clinically relevant model than *M. smegmatis*. During this study, several attempts were made to raise mutants against vanoxerine for mode-of-action studies. Despite numerous attempts with different numbers of cells no mutants were raised, and there was no growth at all on either the 5X and 10X MIC99 plate. Bacterial films formed on plates, which allowed growth on higher concentrations of vanoxerine by reducing the concentration at the interface. It is likely that due to the low mutation frequency of *Mycobacteria*, particularly in the slow-growing strains, mutants could not be raised<sup>104,105</sup>. In the future, more attempts should be made to raise mutants against high concentrations of vanoxerine, then their genomes should be sequenced and any genes containing non-synonymous mutations would highlight potential targets of vanoxerine.

### **Solid and Liquid MIC Tests Give Different Results**

The *M. smegmatis* pSNT3eGFP MIC of vanoxerine, determined by liquid and solid MIC tests, is 120.4  $\mu$ M and 10 $\mu$ M respectively. Such a large difference is unexpected but may be due to a preferred medium for cell growth (liquid over solid) giving faster growth and a higher MIC.

### **Vanoxerine is a Prodrug Metabolised and Activated by Cytochrome P450s**

Vanoxerine is a high affinity inhibitor of dopamine uptake, it binds strongly to dopamine transporters and dissociates slowly making it a strong contender as a cocaine antagonist. In humans, it is metabolised by cytochrome p450s (CYPs), a broad family of enzymes which catalyse the oxidation of their substrate and are often involved in drug biotransformation<sup>106,107</sup>. The genes involved in this study, MSMEG\_2240 and MSMEG\_2038 encode a CYP and a flavin-binding mono-oxygenase respectively. Both enzyme families have very similar activity profiles; they catalyse oxidative metabolism of their substrates.

As shown, overexpression of the genes MSMEG\_2240 and MSMEG\_2038 result in a significant decrease in the MIC<sub>99</sub> towards vanoxerine. This suggests that instead of targeting these proteins, vanoxerine is actually a prodrug which is metabolised to an active form in the cell by CYPs, much like isoniazid<sup>108</sup>. This study provides strong evidence that, with respect to vanoxerine, either these two enzymes are oxidising the molecule separately or are distinct steps in the same metabolic process.

These genes were highlighted as potential targets because they were mutated in two vanoxerine-resistant strains. These results show that mutations in these genes confer resistance not because they are the target, but because they are the activators. The overexpression of

these genes in *M. smegmatis* is less clinically relevant than *M. bovis* BCG but is useful for mode-of-action studies.

As vanoxerine is metabolised by human cytochrome p450s including CYP3A4, clinical use with rifampicin may have unforeseen effects as the front-line drug induces CYP3A4 activity<sup>64</sup>. Therefore, metabolism-based pharmacokinetic studies and drug-drug interactions are important before the drugs are used concomitantly<sup>106</sup>. For example, inducing CYP3A4 with rifampicin may increase the presence of active vanoxerine, but this could have negative effects on the patient and would complicate calculating the correct dosage.

### Comparing Vanoxerine Analogues

As it is likely that vanoxerine is being oxidized, the chemical scaffold was used to design and create a number of similarly-related chemicals, as seen in Figure 11. A, in order to compare MICs and determine where the oxidation reaction is occurring on the compound. These mode-of actions studies were unfortunately impeded by the fact that the EMK compounds were not soluble in 40% DMSO, and so the negative results could be due to a lack of solubility and not because of the altered structure. It was necessary to dissolve the compounds in 40% DMSO, not 100% DMSO, as a starting volume of 16 µl or 32 µl 10mM stock solution would mean that an inhibitory concentration of DMSO is also used. In future, these vanoxerine analogues should be remade with a salt present to aid solubility. Vanoxerine purchased from Sigma Aldrich was a salt containing dihydrochloride, and dissolved easily.

Despite problems with solubility, initial experiments revealed that EMK015 is far more potent than vanoxerine at inhibiting the growth of *M. smegmatis*. This result is promising, and once a soluble salt version is available further testing is needed against *M. bovis* BCG to see if it has potential as an antimycobacterial agent. The chemical scaffold of

vanoxerine should continue to be used to generate analogues, not only to find the site of oxidation, but also to create new compounds that may have a higher inhibitory effect than vanoxerine, much like EMK015.

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Luke Alderwick for his guidance during this project, as well as Stephen Bethnell for all his help with *M. smegmatis*, Chris Burke for creating the plasmids used in this study and everyone who helped with this project. I would also like to thank Eleni for producing the vanoxerine analogues during our collaboration.

## 4. DISCUSSION: PERSPECTIVE FOR THE NEXT THREE YEARS OF EACH PROJECT

Antimicrobials and Antimicrobial resistance is a rapidly emerging global crisis. Resistance to almost every antibiotic developed has now been reported and the spread of resistance leading to global outbreaks of disease is well documented<sup>30,109–111</sup>. The efficacy of current antibiotics is now in decline, yet the production of new compounds is also waning due to a lack of discoveries, economic pressures and increased regulatory measures making it harder to get novel antibiotics to the market. A world-wide effort to implement new policies to control resistance development, and restart research into discovery and development of new compounds is greatly needed<sup>13</sup>.

The studies undertaken in this thesis were designed to address specific antimicrobial and antimicrobial resistance themes. The AMR umbrella covers a broad range of research areas. The first project focused on finding new drug targets by targeting the gram-negative cell envelope, surface and secreted antigens, and proteins involved in host interactions. Targeting AaaA was an unconventional approach to eradicating bacterial pathogens, as inhibition of this would not be bactericidal or bacteriostatic but would perturb virulence and make *P. aeruginosa* more susceptible to antibiotic clearance<sup>38</sup>. The second project tackled research into discovery of novel antitubercular agents, also using an unconventional approach as work involved repurposing drugs from an FDA library of small-molecule compounds licenced for alternate uses in the USA.

There were a number of limitations for the project “Investigating the regulation of arginine-specific protease AaaA in *P. aeruginosa* and creating a soluble truncated version for cell free inhibitor screens”. The main limitation being the strict time constraint of 16 weeks for the entire project. Such time constraints often limit what can be achieved during a project, and cloning in particular takes a long time due to the need for

optimisation of each step in the process. The overall aim for the project was to clone and express the histidine-tagged truncated version of AaaA, which contained the N-terminal signal domain and the central passenger/catalytic domain but did not include the C-terminal  $\beta$ -barrell domain. Previous versions of the truncated AaaA protein created by Daniella Spencer (University of Nottingham) were unsuccessful due to large inclusion bodies forming in the cytoplasm, hence the need for an N-terminal signal to direct the protein to the periplasm where it should be folded and active but less toxic to the cell. The truncated gene was successfully cloned into pGEM-T-Easy vector, the final step before using restriction digest and ligation to move the DNA fragment into the expression vector, pET21a. Should this project be continued for an additional 3 years as a PhD project, there will be far more time available to complete the cloning and expression of the truncated AaaA protein.

If expression of taaaA3 is toxic to the cell, there are a number of alternate methods that can be used to obtain a soluble truncated AaaA protein. The polyhistidine tag could be moved to the N-terminus after the signal peptide, as currently it is cloned into the C-terminus. Additionally, the current truncated version could be expressed in a pseudomonas vector so that if tAaaA3 is toxic to *E. coli* cells, then cloning could be done in *E. coli* but ultimately the protein will be expressed and purified in *P. aeruginosa*. Alternatively, purification of both the truncated version and for the soluble and active form of the full AaaA version could be optimised.

Progression of *P. aeruginosa* infection to the chronic state in the CF lung is met with a number of physiologic and metabolic changes in order for the bacteria to adapt to its environment and survive<sup>112</sup>. Through progression of the disease *P. aeruginosa* faces aerobic, microaerobic and anaerobic conditions and must adapt its metabolism accordingly<sup>19</sup>. Oxygen is preferentially used as a terminal electron acceptor for substrate catabolism, but under anaerobic conditions *P. aeruginosa* can use available nitrogen from denitrification or fermentation of arginine

instead, as an alternate electron acceptor<sup>113–115</sup>. Denitrification occurs when nitrogen oxides are available in the environment, but in some conditions the only available source of nitrogen is from the fermentation of arginine to ornithine<sup>114</sup>. Even under these conditions *P. aeruginosa* grows and forms biofilms. AaaA is an arginine-specific aminopeptidase and cleaves amino terminal arginine from peptides providing an alternate nitrogen source<sup>38</sup>. It is needed for establishment of chronic but not acute infections, so expressing the truncated version of AaaA in conditions mimicking that of a chronic infection should increase the tolerance to tAaaA by the *P. aeruginosa* cell, and higher levels could be purified. Following purification, structural analysis will be performed to investigate inhibitor binding sites.

Understanding when AaaA is most useful for *P. aeruginosa* should be one of the aims of the PhD project. A high-throughput screen, such as a 96 well pin-lid assay, can be used to screen biofilm growth under a number of conditions mimicking those found in the CF lung<sup>116</sup>. These approaches could be done in parallel with protein purification. The pin-lid assay can also be used to screen for novel inhibitors of the AaaA protein. A wild-type, *aaaA* mutant and complemented strain can be assessed for biofilm formation in the presence of novel inhibitory molecules designed to target AaaA.

The other part of this project was investigating the transcriptional regulation of *aaaA*. Two separate approaches and a very wide range of conditions were tested during optimisation of site-directed mutagenesis but unfortunately neither yielded the correctly mutated IHF site in the MiniCTX:*aaaA*:lux plasmid. Due to the large size of the plasmid in question (13Kb) SDM should be far more successful if the promoter region was mutated in a smaller vector and then transferred back into the MiniCTX::lux plasmid. This process has more steps but SDM should be successful in a smaller plasmid. To back up SDM, gel mobility shift assays could be carried out in parallel. These have been used to show IHF binding to the *phtD* operon in *P. syringae*<sup>47</sup>. If successful, SDM should be carried out in other regulatory binding sites in the promoter of



*aaaA*, including NarL, ArgR and RpoN. NarL is part of the two-component system NarX-NarL, which is responsible for sensing and responding to concentrations of nitrate and nitrite<sup>117,118</sup>. It is unsurprising, therefore that its binding site can be found in the *aaaA* promoter. RpoN negatively regulates the expression from the *aaaA* promoter and again is involved in transcriptional control of a number of genes for nitrogen utilization<sup>46</sup>. Interestingly, most isolates found in the CF lung infections have *rpoN* mutations not found in strains collected from other habitats<sup>119</sup>. ArgR regulates arginine, and has been shown to positively regulate transcription from *PaaaA*<sup>46</sup>.

Once all four binding sites have been mutated, the bioluminescent reporters can be used to determine *aaaA* transcription compared with the wild-type. Bioluminescence can be quantified so that levels of expression can be analysed. In addition, transcription of the mutated reporters can be compared in different strain backgrounds, including and ArgR and RpoN mutant. Mutated transcriptional reporters can also be tested for expression and biofilm defects using the 96 pin-lid assay in different growth conditions. These experiments should help identify regulators that directly interact with the promoter of *aaaA* and the conditions in which they are needed.

The second project undertaken involved assessing FDA-approved drugs for their ability to act as antitubercular agents. Additionally, the mode of action of the drug vanoxerine was investigated in *M. smegmatis*. Vanoxerine has been trialled for depression, parkinsonism, and cocaine dependency and showed potential as an antimycobacterial agent in a previous screen of the FDA library<sup>77</sup>. Collaboration with another laboratory allowed the testing of newly synthesised compounds based on the chemical-scaffold of vanoxerine (EMK compounds) in order to find a version with a higher activity. Again, time constraints meant that these compounds could not be fully investigated.

If this were to be carried on as a PhD project, then the EMK compounds should all be retested for their efficacy once soluble salt versions are available. This should be done using a similar method as before, with 96-well microtiter plates for MIC99 broth dilution tests. Comparison of IC50 and MIC99 with those determined for vanoxerine should point to more potent vanoxerine-based compounds. Repurposing chemical scaffolds has been used before to develop new antitubercular drugs. Analogues of Lobenzarit, an anti-arthritis drug, were generated in a targeted approach to drug discovery and tested for their ability to inhibit the *M. tuberculosis* enzyme anthranilate phosphoribosyl-transferase and this was met with success<sup>76</sup>. The changes in MIC99 with each change of the chemical scaffold can also be used to determine which areas of the molecule are essential for oxidation by CYP proteins in mycobacteria.

The target of vanoxerine and mode of action also needs to be identified in *M. smegmatis* and *M. bovis BCG*. I suggest the way forward is through computational methods. There are two *in silico* approaches for identification of drug-target interaction: ligand-based and target-based virtual screening. As we know the chemical structure of vanoxerine but not the target, a receptor-based approach should be applied. Target –based screening is also known as reverse ligand-protein docking and involves one ligand being screened for its ability to bind to an array of clinically relevant macromolecular targets<sup>120,121</sup>. The binding ability is then scored and ranked with the highest the most likely to bind. However, this method would be limited to the 3D-structures stored in databases such as protein drug target database (PDTD) and Target Fishing Dock (TarFisDock) and would not be consigned solely to *M. tuberculosis* proteins<sup>120,121</sup>. Another approach is through genomic screens of resistant mutants. Mutants to vanoxerine should be raised in high concentrations of up to 10 X the MIC99 in both *M. smegmatis* and *M. bovis BCG*. Their genomes should be sequenced and genes with non-synonymous mutations should be identified in each mutant. Genes which are mutated across all strains indicate a potential vanoxerine

target and should be investigated further. This could be done through overexpression in wild-type strains, the method employed during this study. Alternatively, isothermal titration calorimetry (ITC) could be used to measure biochemical interactions between vanoxerine and a number of proteins in a crude mixture<sup>122</sup>. ITC can be used with gel-filtration chromatography to “fish” for target proteins within a complex sample of macromolecules<sup>122</sup>. By separating out proteins with gel-filtration chromatography, each fraction can then be tested for ligand-binding using ITC. Those that bind can be identified using N-terminal amino acid sequencing. Once the target of vanoxerine and its derivatives has been identified, structural analysis can be performed along with *in silico* ligand-binding screens for additional inhibitor scaffolds.

## CONCLUDING REMARKS

Drug discovery and development is becoming increasingly harder due to scientific bottlenecks, increased regulation from approval bodies and economic circumstance. Using unusual approaches to drug discovery can fast-track the production of new antimicrobials, as is the case with drug repurposing. There is a strong need for antimicrobial agents that bacteria can't develop resistance to and the best way to address this is by developing drugs that do not create a strong selection for resistance. Antivirulence drugs are ideal in this respect as they do not target essential biological processes. It is naïve, however, to assume that bacteria will never develop resistance to any of the antibiotics they are presented with. Careful control over the use of antibiotics should be employed to prevent the development and spread of resistance if we are to avoid the upcoming antibiotic apocalypse.

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