ANTIBACTERIAL AGENTS TARGETING AMINOACYL-tRNA SYNTHETASES: UNDERSTANDING RESISTANCE TO KNOWN INHIBITORS, AND BIOLOGICAL CHARACTERISATION OF NOVEL INHIBITORS

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Intellectual property and publication statements

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 5 contains work based on jointly-authored publications. The publication is referenced below, together with a description of the contribution made by each author to the publication.

Gupta A, Monteferrante C, Rasina D, Leitis G, Randall CP, Tomlinson JH, Jirgensons A, Goessens WHF, Hays JP and O'Neill AJ. 2016. A polymorphism in *leuS* confers reduced susceptibility to GSK2251052 in a clinical isolate of *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy.* **60:** 3219-3221.

AG conceived the study, designed and carried out experiments and wrote the manuscript; CM carried out preliminary antibacterial susceptibility experiments; DR & LG synthesized GSK'052; JHT, WHFG & JPH provided intellectual input; CPR and AJO assisted in conceiving the study, designing experiments and in writing the manuscript.

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Abstract

Antibiotics have been the pillars of modern medicine as they have been used for the prevention and treatment of bacterial infectious diseases. However, the rise in antibiotic resistance amongst pathogenic bacteria and the lack of new antibacterial agents reaching the clinic is concerning. The major aim of the consortium, New Antibacterials with Inhibitory Activity on Aminoacyl-tRNA Synthetases (NABARSI), was to discover new antibacterial agents with inhibitory activity against the underexploited drug targets, the aminoacyl-tRNA synthetase (aaRS) enzymes. As a part of NABARSI, this thesis describes the biological characterisation of novel aminoacyl-tRNA synthetase (aaRS) inhibitors and studies to better undersatnd resistance in *Staphylococcus aureus* to known aaRS inhibitors.

From a starting panel of 100,000 compounds screened *in silico,* 7000 were selected and tested for target binding activity against purified isoleucyl-tRNA synthetase (IleRS) and leucyl-tRNA synthetase (LeuRS) from both *Staphylococcus aureus* and *Escherichia coli*. Five of these were found to be inhibitory against *E. coli*. Conditional mutants of *E. coli* were used to confirm target specificity of these novel aaRS inhibitors, and it was shown that the antibacterial activity was a direct consequence of inhibition of LeuRS. However, *E. coli* was found to have a high frequency of spontaneous resistance to the compounds *in vitro*. Characterisation of spontaneous mutants resistant to these candidate antibacterial agents led to the identification of a novel resistance mechanism to aaRS inhibitors.

To aid in assessing anti Gram-positive activity of the compounds developed within NABARSI, conditional mutants of *S. aureus* for *ileS, leuS, serS* and *thrS* were also generated and validated. The strains exhibited an increase in sensitivity (32-64 fold) to their cognate inhibitors compared to the parental strain, thus providing a platform to identify compounds with both whole cell and target specific activity.

To better understand the underlying reason(s) of resistance to aaRS inhibitors and provide useful information for the possible development of new derivatives, the molecular basis of resistance to mupirocin and GSK2251052 (GSK'052) in *S. aureus* was examined. The results presented in this thesis provide evidence that the mupirocin-resistance proteins MupA and MupB, are functional isoleucyl-tRNA synthetase enzymes. Bioinformatic analysis of these proteins suggests the presence of an extra domain, which likely contributes to the observed mupirocin-resistance. Assessment of the resistance liability of GSK'052 in *S. aureus* indicate that the compound is not a suitable anti-staphylococcal agent and that resistance to the compound pre-exists in the clinic.

The findings presented in this thesis highlights the importance of both the thorough biological characterisation of novel candidate antibacterial agents and understanding the molecular basis of resistance to antibacterial agents. Together they are able to provide useful information for developing new antibiotics or potent derivatives of existing ones.

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List of abbreviations

aa-tRNA	Aminoacyl-tRNA
aaRS	Aminoacyl-tRNA synthetase
AlaRS	Alanyl-tRNA synthetase
ATc	Anhydrotetracycline
BHIA	Brain Heart Infusion Agar
CDC	Centres for Disease Control and Prevention
CTAB	Hexadecyltrimethylammonium bromide
dH_2O	Distilled H_2O
ECDC	European Centre for Disease Control and Prevention
ESBL	Extended-spectrum β-lactamase
EU	European Union
GSK	GlaxoSmithKline
HTS	High-throughput screening
iChip	Isolation Chip
IleRS	Isoleucyl-tRNA synthetase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KPC	*Klebsiella pneumoniae* carbapenemases
LBB	Lysogeny Broth
LBA	Lysogeny Agar
LeuRS	Leucyl-tRNA synthetase
McC	Microcin C
MDR	Multi-drug resistant
MHB-II	Mueller Hinton Broth II
MHAII	Mueller Hinton Agar II
MF	Mutation Frequency
MIC	Minimum Inhibitory concentration
MMS	Macromolecular synthesis
MoA	Mode of action
MRSA	Meticillin-resistant *Staphylococcus aureus*
Mup^R	Mupirocin-resistant
Mup^S	Mupirocin-susceptible
NABARSI	New Antibacterials with Inhibitory Activity on Aminoacyl-tRNA Synthetase
NDM-1	New Delhi metallo- β-lactamase-1
PheRS	Phenyl-tRNA synthetase
ProRS	Prolyly-tRNA synthetase
SBDD	Structure based drug design
Ser hydro.	Serine hydroxamate
SLiCE	Seamless Ligation Cloning Extract
SNP	Single Nucleotide Polymorphism
TAE	Tri-acetate EDTA
TCA	Tri-chloroacetic acid
ThrRS	Threonyl-tRNA synthetase
TSB	Tryptone Soya Broth
TSA	Tryptone Soya Agar

RBC	Red Blood Cell
ValRS	Valyl-tRNA synthetase
VRE	Vancomycin Resistant Enterococci
VISA	Vancomycin intermediate *S. aureus*
WHO	World Health Organisation

Chapter 1

Introduction

Chapter 1: Introduction

The clinical use of antibiotics for the treatment and control of a range of infections make them an indispensable part of modern medicine. The use of antibiotics has resulted in an increased life expectancy of approximately 30 years (Walsh, 2003). For example, introduction of sulphonamides reduced the death rate due to puerperal fever from approximately four deaths per 1000 births to < 1 death per 1000 births (Finch *et al.,* 2010); furthermore, the Centres for Disease Control and Prevention (CDC) also documented a 30% decline in meticillin-resistant *Staphylococcus aureus* (MRSA) infections in the US between 2005 and 2011 (van Hal *et al.,* 2012). Despite the good control measures and appropriate use of antibiotics, antibiotic resistance poses a major threat to global health, as the loss of effective antibiotic therapy will make treatment of diseases more difficult. Currently bacterial infections account for nearly two million annual deaths globally (Haque *et al.,* 2014).

Treatment of infections caused by such antibiotic-resistant pathogens lead to extended hospital stays, which not only result in greater expenditure of resources but also increases the chance of spreading the infection within hospitals and communities. The economic burden in the UK due to infectious diseases is approximately £30 billion per year (Davies, 2011). The discovery of antibiotics not only revolutionised the field of medicine but also led to the improvement of the quality of life. However, the steady rise in antibiotic-resistant pathogens is a global health concern as exemplified by the recent death of a patient in Nevada, who was suffering from an infection untreatable by all available antibiotics in the US (Chen *et al.,* 2017). It has been predicted that by 2050, 10 million deaths annually will be a result of antibiotic resistance (O'Neill, 2016). This could be the start of a post-antibiotic era within the ever evolving resistance era; thus, it is apparent that there is a need for the identification, development and characterisation of new antibacterial candidates with novel

structures to counteract this situation. The need for new antibacterial compounds has also been emphasized by both the World Health Organisation (WHO) and the European Centre for Disease Control and Prevention (ECDC) (Norrby *et al.,* 2011).

1.1. Antibiotic resistance: a perennial concern

The development of antibiotic resistance in bacterial pathogens is a natural and evolutionary response to antibiotic selective pressure, enabling bacteria to evade the inhibitory effect of antibiotics (Wright, 2010). A major contributing factor to the rise in antibiotic resistance has been the regular use of antibiotics to treat infections (both human and animal) and in agriculture as growth promoters. This in turn has created an environment for the rapid transfer of resistance genes amongst bacterial populations. The mechanisms by which bacteria evade the inhibitory effect of antibiotics can be classified into four categories: alteration of target, alteration of drug, decreased accumulation of drug within the cell and target bypass/protection (Figure 1.1). These resistance mechanisms can either be intrinsic to the bacterium or be acquired exogenously (*e.g.* acquisition of a plasmid encoding a resistance determinant) or endogenously (*e.g.* mutations in target gene or those that affect gene expression). Bacteria may also develop resistance to multiple antibiotics by the acquisition of plasmids encoding multiple resistance genes, or by the expression of multi-drug efflux pumps (Nikaido, 2009). As such, resistance to antibiotics in the clinical setting has been observed since the time they were first introduced for the treatment of infectious diseases.

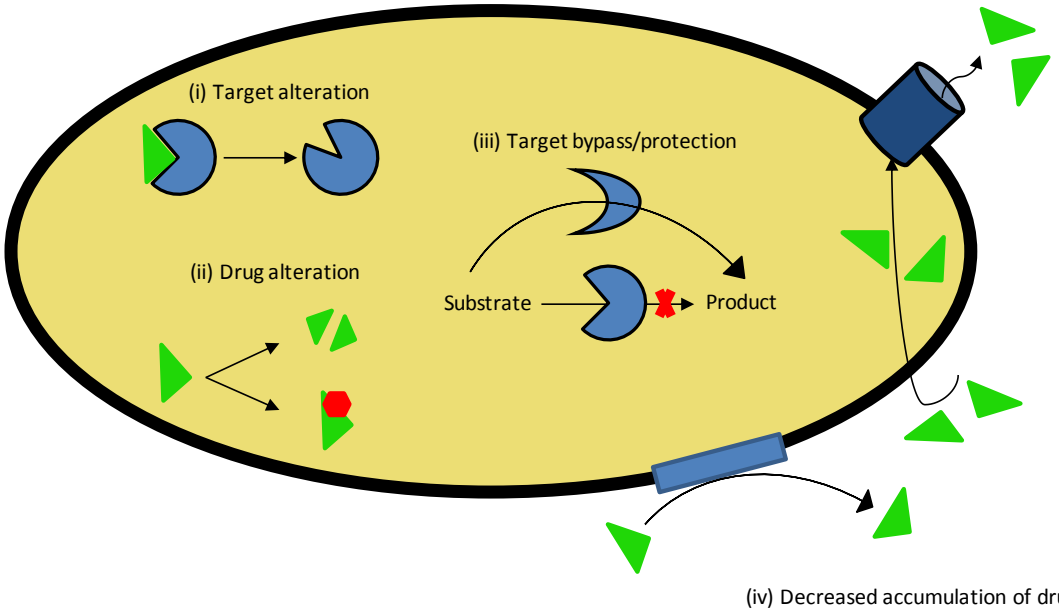

Figure 1.1: Schematic representation of bacterial resistance mechanisms to antibiotics (green). (i) represents the mechanism by which a target is modified to prevent antibiotic binding; (ii) represents the modification of the antibiotic due to antibiotic degrading or altering enzymes; (iii) represents bypassing the effect of an antibiotic by utilising a different protein and (iv) represents the use of efflux pumps (blue) or the presence of an impermeable outer membrane (dark blue) leading to decreased accumulation of the drug.

1.1.1. Staphylococci and antibiotic resistance

Amongst the Gram-positive organisms, resistant *Staphylococcus aureus* poses the greatest threat. *S. aureus* is a major cause of hospital and community acquired infections worldwide. Sixty percent of healthy adults are intermittent carriers, with 20% being persistent carriers (Kluytmans *et al.,* 1997, Fair and Tor, 2014). Traditionally referred to as an opportunistic pathogen, many strains of *S. aureus* are now referred to as aggressive pathogens (Walsh, 2003, Fair and Tor, 2014), as they have the propensity to gain resistance when challenged with antibiotics, making it a significant human pathogen. It has been estimated the MRSA kills more Americans each year than HIV/AIDS, Parkinson ’s disease and homicides combined. The following section provides a brief overview on resistance to antibiotics in this organism.

Clinical resistance to penicillin due to the production of β-lactamases, which inactivate the drug, was first recorded in 1942, the same year the drug was introduced into clinical use (Fuda *et al.,* 2005). However, this is unsurprising as resistance to this class of antibiotics was first observed in 1940 (Abraham and Chain, 1940). The burden of infections caused by β-lactamase producing strains was mitigated by the discovery and introduction of antibiotics such as the aminoglycosides, fluoroquinolones and semi-synthetic, β-lactamase resistant derivatives of penicillin such as meticillin. However, resistance to aminoglycosides and fluoroquinolones was reported within three years of their FDA approval (Bush, 2004) and meticillin-resistant *Staphylococcus aureus* (MRSA) strains were identified only a year after the introduction of meticillin into clinical practice in 1960 (Aeshlimann *et al.,* 1999). Much like the other antibiotics, clinical resistance to tetracycline was first recorded in 1953, a year after its introduction into clinical use (Chopra and Roberts, 2001).

Despite the side-effects associated with vancomycin, the glycopeptide antibiotic was reserved for treatment of infections caused by meticillin-resistant strains. However, the excessive use of vancomycin in the 1980's led to the emergence of vancomycin-resistant enterococci (VRE) in Europe and the USA in 1986 and 1987 (Murray, 2000), respectively and the first incidence of staphylococci exhibiting reduced susceptibility was reported in 1997 in Japan (Hiramatsu *et al.,* 1997). These strains, with reduced susceptibility denoted as vancomycin intermediate *S. aureus* resistant strains (VISA), have been associated with treatment failure (Moore *et al.,* 2003). The introduction of linezolid and daptomycin was a breakthrough at the time as both were active against VRE and VISA strains. Alarmingly, resistance to both antibiotics were reported within one year and two years of their introduction, respectively (Pillai *et al.,* 2002, Hayden *et al.,* 2005). The first recorded incidence of resistance to daptomycin was in 2005, when MRSA strains isolated from patients suffering from septicaemia and osteomyelitis exhibited reduced susceptibility to the antibiotic (Hayden *et al.,* 2005, Marty *et al.,* 2006).

The continued use of antibiotics in conjunction with the ability of bacteria to vertically and horizontally transfer genetic material facilitates the emergence of multi-drug resistant (MDR) bacteria (Levy, 2002; Nordmann *et al.,* 2011; Walsh, 2010), which exhibit resistance to at least one agent in three or more antibiotic classes (Magiorakos *et al.,* 2012). This phenomenon of multi-drug resistance was observed in hospital-associated MRSA (HA-MRSA), with specific strains exhibiting resistance to aminoglycosides and fluoroquinolones (Nikaido, 2009). Since the 1990s new clones of MRSA such as EMRSA-15 (CC22 SCC*mec*IV) and EMRSA-16 (CC30 SCC*mec*II) were identified. By 2000, both these strains accounted for 95.6% of bacteraemia cases in UK. However, the late 1990s saw a change in the epidemiology of MRSA with the emergence of community-associated MRSA (CA-MRSA) in USA (Kayaba *et al.,* 1997), which resulted in infections in healthy individuals. CA-MRSA strains were subsequently observed worldwide, with the first clone of CA-MRSA in Europe being isolated in Denmark in 1997 (Stegger *et al.,* 2014). CA-MRSA disseminate more rapidly compared with HA-MRSA and display enhanced virulence (Deurenberg *et al.,* 2007). These MRSA strains possess the Panton-Valentine leucocidin (PVL) toxin, which is responsible for the increased virulence. The toxin is encoded by *lukS* and *lukF,* found on bacteriophage Φ2, which is found in the *S. aureus* chromosome (McCarthy *et al.,* 2012, Stegger *et al.,* 2014).

Although infections caused by MRSA is of clinical concern, the issue has receded to a degree with good control measures such as active detection and isolation (ADI). This involves MRSA screening of all patients, proper hand hygiene in hospitals and use of mupirocin for nasal decolonisation. Depending on the site of infection and possible MRSA involvement, *S. aureus* infections are currently treated topically, orally or intravenously. Treatment involves β-lactamase resistant β-lactams such as dicloxacillin and flucloxacillin (Rayner and Munckhof, 2005). Combination therapies with aminoglycosides has also resulted in reduced rates of recurrent bacteraemia (Lemonovich *et al.,* 2011). The clinical management of MRSA involves treating patients with gylcopeptide antibiotics such as vancomycin or teicoplanin (Rayner

and Munckhof, 2005, Giersing *et al.,* 2016). Although resistance to vancomycin has been documented, no VRSA has been detected in the UK. In addition, mupirocin is used topically to eradicate *S. aureus*/MRSA carriage in healthcare workers and patients (van Rijen *et al.,* 2008).

1.1.1.1. S. aureus vaccines as an alternative treatment strategy?

The success of pneumococcal vaccines and the tetanus toxoid provide support for the development of vaccines that target capsular polysaccharides present in *S. aureus*. However, as *S. aureus* comprise normal human flora, it has developed/evolved ways to evade the host immune system, thus making development of vaccines challenging (Giersing *et al.,* 2016, Jansen *et al.,* 2013). All the vaccine candidates thus far have targeted individual cell surface components, such as polysaccharide capsule molecules (CP5 and CP8) or proteins associated with attachment (Giersing *et al.,* 2016). Although *S. aureus* vaccine candidates have shown pre-clinical efficacy, they have been unable to demonstrate efficacy in clinical trials (Giersing *et al.,* 2016, Jansen *et al.,* 2013). For example, a Phase III trial of the candidate vaccine, V710, which targets the scavenger protein IsdB was terminated as administration of the candidate vaccine led to increased mortality rate due to *S. aureus* infection (Giersing *et al.,* 2016, Jansen *et al.,* 2013). The other factor which affects development of vaccines, is the inconsistency in production of different vaccine lots (Giersing *et al.,* 2016, Jansen *et al.,* 2013). In addition, the antigenic variation encountered in *S. aureus* presents a significant obstacle in the design, and development of successful candidates (Proctor, 2012). Furthermore, the versatility of *S. aureus* implies that a candidate vaccine must be tested against a wide variety of strains (Proctor, 2012). Thus, as it currently stands, the use of vaccines could possibly be more suited for decreasing colonisation in an individual rather than a therapeutic option of infections caused by the organism.

1.1.2. Antibiotic resistance in Gram-negative organisms

The most serious current threat however, is posed by MDR Gram-negative pathogens such as *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneomoniae, Acinetobacter baumanii* and *Enterobacter* spp. (Fishbach and Walsh, 2009). These pathogens cause a variety of disease such as urinary tract, bloodstream, airway and healthcare-associated infections. Treatment of infections caused by Gram-negative pathogens has become challenging due to the presence of many resistance determinants, including extended-spectrum β-lactamases (ESBLs), which confer resistance to so called 3rd generation cephalosporins such as cefotaxime, ceftazidime and ceftriaxone. In addition, plasmid-mediated aminoglycoside resistance determinants confer resistance to aminoglycosides such as neomycin and tobramycin. The high incidence of CTX-M ESBLs reported in *Klebsiella* spp. led to the increased use of carbapenems (Wellington *et al.,* 2013), in turn leading to the selection of a novel carbapenemase, New Delhi metallo-β-lactamase-1 (NDM-1), capable of hydrolysing almost all β-lactams (Wellington *et al.,* 2013, Canton and Lumb, 2011, Walsh *et al.,* 2009). Since the identification of *Klebsiella pneumoniae* carbapenemases 1 and 2 (KPC) in the USA, five other variants of these resistance proteins have been isolated in strains in different countries, indicating that horizontal gene transfer between bacterial species help in the exchange of resistance determinants (Wellington *et al.,* 2013, Hawkey and Jones, 2009, Nordmann *et al.,* 2009). Alarmingly, plasmid-mediated resistance to colistin, which has been used as a drug of last resort to treat infections caused by MDR bacteria, was observed for the first time in 2015 in China (Liu *et al.,* 2016). Isolates carrying the plasmid-encoded *mcr-1* gene has also been reported in Europe shedding doubt on the usefyl lifespan of the compound (Hasman *et al.,* 2015).

1.2. Antibiotic discovery and the need for new antibiotics

Antibiotics have been an essential part of modern medicine since they were introduced into clinical practice for the treatment of infectious diseases. The identification of sulphanilamide, an inhibitor of folate biosynthesis, and its release into the market in 1932 was a result of screening of chemicals and natural dyes for the identification of compounds exhibiting antimicrobial activity (Silver, 2011). The accidental discovery of penicillin in 1929, facilitated successful treatment of staphylococcal and streptococcal infections (Fleming, 1929) and the isolation of streptomycin in 1943 enabled the control of *Mycobacterium tuberculosis*, a causative agent of tuberculosis (Comroe*,* 1978). These two breakthrough discoveries paved the way for the "Golden Age" of antibiotics, a period during which most of the known antibacterial classes were discovered and introduced into clinical practice (Lewis, 2013, Pelaez, 2006, Schatz *et al.,* 1944). The "Golden Age" of drug discovery involved the screening of natural products from microorganisms for the identification of compounds possessing antimicrobial properties.

With time the discovery of novel compounds possessing potent antibacterial activity decreased, as screening of natural products frequently led to the re-discovery of existing antibiotics (Silver, 2011). In 1977, a year after the identification of clavulanic acid, Cohen suggested that the focus of drug discovery should be on screening inhibitors against metabolically essential bacterial targets which are absent in humans (Cohen, 1977). This prompted the screening of large chemical libraries in an attempt to identify the next antibiotic. This was also the start of the era of high-throughput screening (HTS) platforms, as the major pharmaceuticals were competing against each other to reach the market first. However, this approach of *in vitro* screening of chemical libraries against essential targets was unsuccessful, as it was unable to yield a candidate antibacterial agent which could be pursued as a lead (Silver, 2011, Payne *et al.,* 2007).

To combat the rise in antibiotic resistance, the pharmaceutical industry focussed on designing synthetic compounds based on bacterial genomics, combinatorial chemistry and rational drug design. The switch in the drug discovery approach was prompted by the successful sequence determination of the entire *Haemophilus influenzae* genome in the mid-1990's (Payne *et al.,* 2007). Use of genomics and bioinformatic approaches led to the identification of 160 essential bacterial targets and thus offered potential novel targets for new antibacterial agents (Payne *et al.,* 2007). The analysis of these essential proteins in conjunction with the use of structure-based drug design (SBDD) programs provided a platform to virtually assess the binding affinity of a compound to its corresponding target and then test the inhibitor against the protein target *in vitro* (Simmons *et al.,* 2010). Although inhibitors of targets were readily identified, most lacked whole cell activity and/or were toxic as they showed non-specific activity when within the cell. The discovery and lead optimization can be hindered by the following issues, (a) the candidate agent has *in vitro* activity against purified protein but no antibacterial activity, (b) inhibitory activity is detected but cannot be linked to inhibition of intended target and, (c) the candidate compound rapidly selects for resistance (Silver, 2011).

The last 18 years have only seen the introduction of only four classes of antibiotic with novel scaffolds - the oxazolidone linezolid (Ford *et al.,* 2001), the lipopeptide daptomycin (Carpenter and Chambers, 2004), the pleuromutilin retapamulin (Parish and Parish, 2008) and the macrocycle fidaxomicin (Cornely *et al.,* 2012). However, all these classes of antibiotics had been identified in 1978, 1952, 1987 and 1975, respectively, implying that no new class of antibiotics suitable for clinical use has been discovered since the 1980's (Silver, 2011).

The sharp decline in the characterisation and identification of novel inhibitors has been described as a discovery void partly due to the following reasons. It has been estimated that

it takes approximately 12 years for a lead compound (identified from screening projects) to be launched as an antibacterial agent (Payne *et al.,* 2007). Additionally, the entire process of identifying a lead compound, its pre-clinical evaluation and subsequent clinical trials costs in excess of US$500 million (Projan and Youngman, 2002). The time taken to identify and launch a new antibiotic, coupled with high financial investments has resulted in most pharmaceutical companies withdrawing from research and development in the area of antibacterials. Furthermore, it has been difficult to identify a lead compound through screening projects in the first instance, as both natural product and high-throughput screening projects have resulted in very few promising lead compounds. For example, 70 high-throughput screening (HTS) projects conducted by GlaxoSmithKline (GSK) at a cost of $1 million per project over a period of seven years resulted in only five hits, none of which have made it to the clinic (Payne *et al.,* 2007). AstraZeneca also published findings from their antibacterial discovery projects in 2015. The same issue was faced, where they were unable to convert hit compounds to viable leads that could be pursued as viable antibacterial agents (Tommasi *et al.,* 2015).

However, both reviews suggest that pursuing validated bacterial targets rather than just essential targets is a more robust strategy for developing novel antibacterial agents. According to Payne *et al.,* it is easier to identify a compound with whole-cell antibacterial activity than to modify the structure of the inhibitor to improve cell permeability. As such, certain targets are more worthwhile pursuing since known inhibitors against them pre-exist providing a reference and rationale for the development of new inhibitors (Payne *et al.,* 2007, Tommasi *et al.,* 2015). This bypasses the issue of identifying a novel target which may or may not be druggable despite being metabolically essential (Payne *et al.,* 2007) and also means that the cellular target is accessible.

In addition to developing a new agent with potent antibacterial activity, one must also ensure that the emergence of spontaneous resistance against the candidate agent occurs at a low frequency. Compounds with a single enzyme target are susceptible to resistance as a consequence of mutations within the target; however, a compound with multiple cellular targets is less likely to be affected as it would require simultaneous mutation of multiple targets. This hypothesis is exemplified by antibacterial drugs (such as macrolides, tetracyclines, oxazolidinones etc.) which target the bacterial ribosome (Silver, 2011). Bacteria contain multiple copies of rRNA genes, thus minimising the development of high-level target based resistance due to mutations in the encoding genes (Silver, 2011). In addition to these antibacterial agents, the β-lactams and fluoroquinolones, which inhibit bacterial cell wall and DNA synthesis respectively, also provide support for the multi-target hypothesis. Therefore, the ideal candidate antibacterial agent would be one with a novel scaffold that hits multiple targets.

1.3. The EU consortium: NABARSI and the search for new antibacterial agents

To address the lack of novel, clinically available antibiotics and increase the antibiotic pipeline, the EU consortium, New Antibacterials with Inhibitory Activity on Aminoacyl-tRNA Synthetase (NABARSI) aimed to design and develop inhibitors with whole cell activity against the underexploited bacterial target, aminoacyl-tRNA synthetase (aaRS) enzymes, in both Gram-positive and Gram-negative organisms. The partners in the consortium included Erasmus MC (Rotterdam, The Netherlands), InhibOx Ltd. (London, UK), Omia Molecular Ltd. (Barcelona, Spain), Latvian Institute of Organic Synthesis (LIOS) (Riga, Latvia) and the O'Neill group (University of Leeds, Leeds, UK).

NABARSI, was dedicated to identify and develop novel bacterial aaRS inhibitors specifically targeting IleRS, LeuRS and ValRS. Figure 1.2 illustrates the workflow followed within NABARSI, with the bulk of work of this study contributing to work package 5 (WP 5).

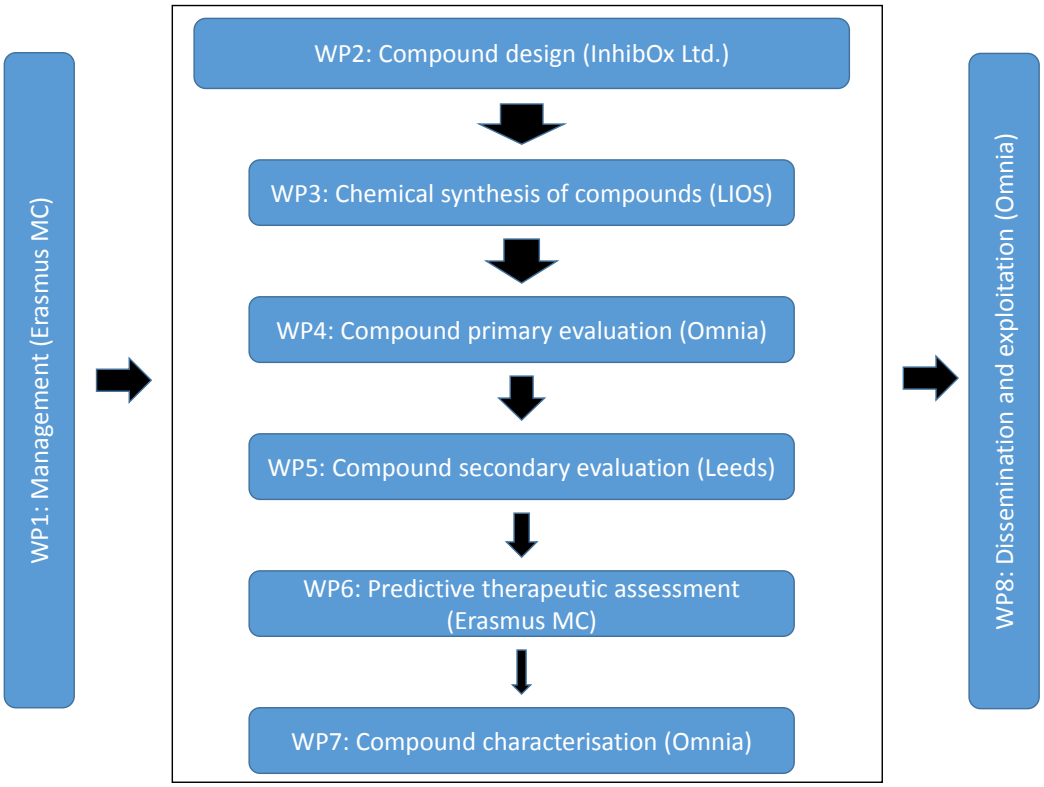

Figure 1.2: Workflow and interdependency of work packages (WP) within the NABARSI consortium

Initial compound design and docking studies against the available protein structures of *Staphylococcus aureus* isoleucyl-tRNA synthetase (IleRS, PDB ID: 1ffy), *Escherichia coli* leucyl-tRNA synthetase (LeuRS, PDB ID: 3zgz) and *Thermus thermophilus* valyl-tRNA synthetase (ValRS, PDB ID: 1IVS) was conducted at InhibOx Ltd. The sequence conservation of these proteins were analysed and the crystal structures were overlaid to provide a common coordinate framework to aid in comparison. Based on structure comparison, key protein-ligand interactions (pharmacophore points) were identified and used in compound selecetion. The most promising molecules were then synthesised at LIOS. The consortium

hoped to utilise the In-Omnia (*in vivo*) assay and the Biothema (*in vitro*) assay (Saint-Leger and de Pouplana, 2017) to provide both molecular and biochemical platforms for the initial screening of compounds to identify prospective hit candidates. This primary evaluation was done by our partners at Omnia. The following sections discusses why aaRS enzymes present themselves as viable targets for generating multi-targeting antibacterial agents, the known inhibitors that have previously been discovered and/or are in clinical use and the approach taken within NABARSI.

1.3.1. Aminoacyl tRNA-synthetases as drug targets

The aaRS enzymes are considered essential for cell viability in all organisms (Ibba and Soll, 2000). These enzymes catalyse charging of a cognate tRNA with its corresponding amino acid which not only leads to activation of that particular amino acid for peptide bond formation but aids in transport of amino acids to the ribosome for translation. Both of these criteria are essential in the initiation of protein synthesis (Nelson and Cox, 2004). The formation of aa-tRNA is a two-step reaction (Figure 1.2), where ATP reacts with an amino acid to yield an

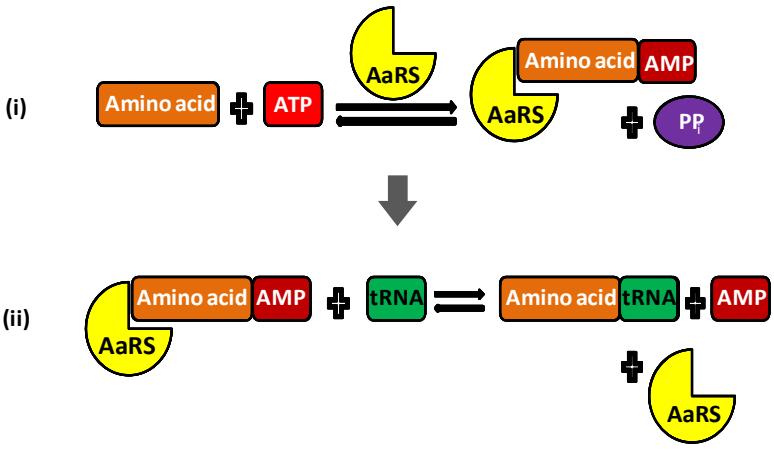

Figure 1.3: The aminoacylation reaction. (i) The synthetase binds ATP and amino acid to form the aminoacyl-adenylate with the release of pyrophosphate; (ii) the amino acid is then transferred to cognate tRNA yielding aminoacyl-tRNA

aminoacyl-adenylate intermediate with the release of pyrophosphate in the first step. This is followed by transfer of the amino acid moiety to its corresponding tRNA to form an aa-tRNA derivative (Ibba and Soll, 2000). Each of the 20 amino acids have a corresponding aaRS and although these enzymes catalyse the same fundamental reaction, they are categorized into two classes, each comprising 10 aaRS, that are based on differences in structure and their respective reaction mechanisms. The classification is based on the similarity observed within the active site of aaRS and how the enzymes bind tRNA (Table 1.1).

Table 1.1: Classification of aminoacyl tRNA synthetase enzymes

Characteristic	Class I aaRS	Class II aaRS
Active site	Rossman fold	Antiparallel β sheet
Motifs	HIGH and KMSKS	Motif 1, 2 and 3
Subclasses	***Subclass Ia***	***Subclass IIa***
	MetRS	ThrRS
	ArgRS	GlyRS
	IleRS	ProRS
	LeuRS	HisRS
	ValRS	SerRS
	CysRS	
	LysRS	
	Subclass Ib	***Subclass IIb***
	GluRS	AspRS
	GlnRS	AsnRS
		LysRS II
	Subclass Ic	***Subclass IIc***
	TyrRS	AlaRS
	TrpRS	PheRS
		GlyRS

The active site of class I enzymes consist of a Rossmann fold, whereas class II enzymes contain an anti-parallel β-sheet surrounded by α-helices (Figure 1.3, Ibba and Soll, 2000). Class I enzymes consists of HIGH and KMSKS conserved regions within their active site (Cusack, 1995, Arnez and Moras, 1997, Hurdle *et al.,* 2005); class II consists of three different motifs made of α-helices and β-strands. Motifs 2 and 3 form the catalytic site and consists of a conserved arginine residue (Arnez and Moras, 1997, Ibba and Soll, 2000). Both class I and class II enzymes are further divided into three subclasses based on the type of amino acids to which they bind (Cusack, 1995).

Accurate translation requires the aaRS to distinguish between chemically and structurally similar amino acids when charging tRNAs. The active site of a particular aaRS cannot accommodate amino acids larger than its actual substrate and hydrolyses activated/charged species that are smaller than its particular amino acid (Berg *et al.,* 2002). As such, some aaRS enzymes like isoleucyl-tRNA synthetase (IleRS), valyl-tRNA synthetase (ValRS), leucyl-tRNA synthetase (LeuRS), threonyl-tRNA synthetase (ThrRS), prolyl-tRNA synthetase (ProRS), alanyl-tRNA synthetase (AlaRS) and phenyl-tRNA synthetase (PheRS), also contain an editing site in addition to the presence of an active site. This editing site maintains the fidelity of tRNA charging; the aminoacyl-tRNA bond is hydrolysed if the incorrect tRNA is added. This two check-point system thus helps in ensuring fidelity of the aminoacylation reaction. Inhibition of aaRS leads to the accumulation of uncharged tRNA, which binds to the ribosome resulting in inhibition of protein synthesis and eventual arrest of bacterial growth (Cassels *et al.,* 1995), making them biologically essential enzymes and prime targets for development of novel antibacterial agents capable of inhibiting one or more enzymes belonging to same class of enzymes (Table 1.1).

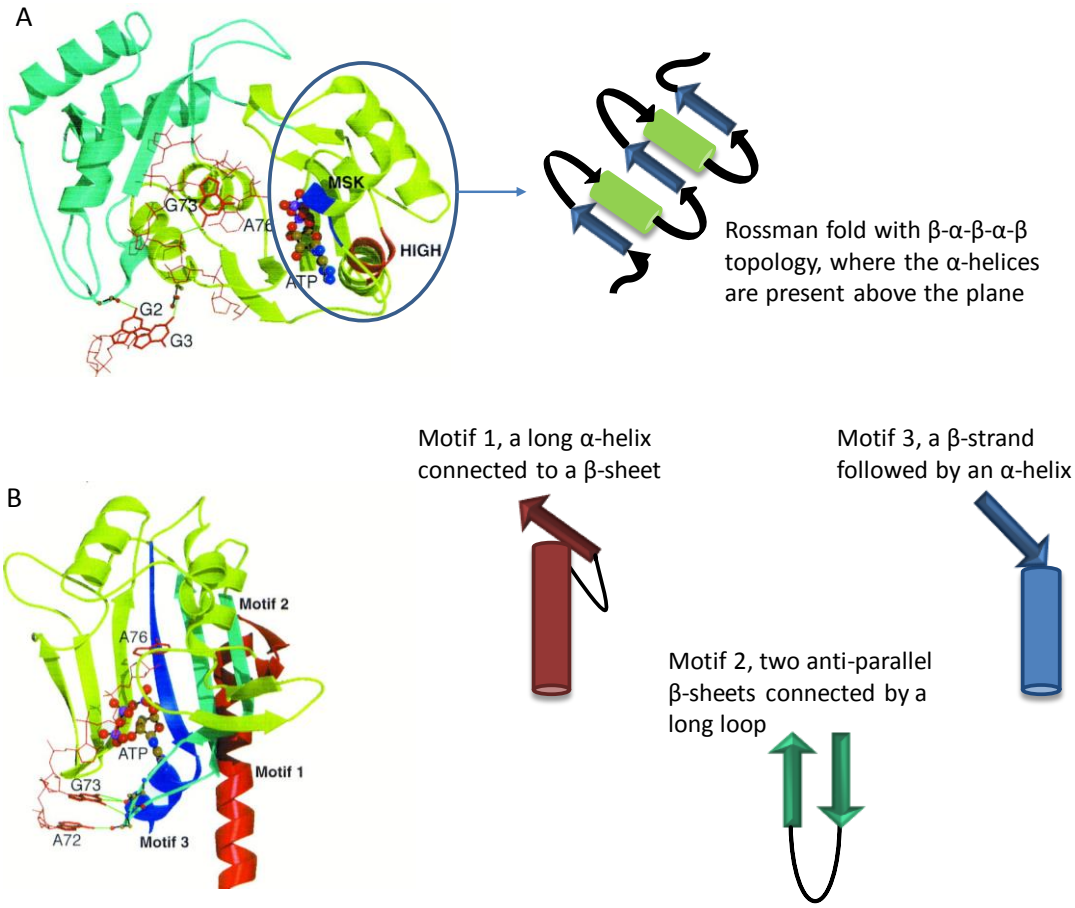

Figure 1.4: Schematic representation of the active site domain of the different classes of aminoacyl-tRNA synthetase (aaRS) enzymes. (A) Class I aaRS active site domain (Ibba and Soll, 2000) and a cartoon diagram of the conserved Rossman fold. Blue solid arrows represent sheets and green cylinders represent helices; (B) Class II aaRS active site domain (Ibba and Soll, 2000) and cartoon diagrams of the commonly found motifs in the catalytic site. Arrow represent sheets and cylinders represent helices.

The stalling of peptide chain elongation due to uncharged tRNA also induces the synthesis of ppGpp and (p)ppGpp from ATP and GTP, respectively, two nucleotides which play a key role in the bacterial stringent response (Hurdle *et al.,* 2005). The nucleotide, ppGpp binds to RNA polymerase (RNAP), leading to differential promoter activity. In *E. coli,* (p)ppGpp is synthesized by either RelA as a result bound uncharged tRNAs to the ribosome or by SpoT in response to other stress signals (Wolz *et al.,* 2010). In combination with the cofactor protein DksA, (p)ppGpp binds to RNAP which results in the inhibition of *rrn* operons (Wolz *et al.,*

2010) and transcriptional regulation of gene-specific promoters (Srivatsan and Wang, 2008). In *S. aureus*, the alarmone is synthesised by RSH, a synthase/hydrolase enzyme. It is also the only enzyme responsible for the synthesis of (p)ppGpp upon amino acid starvation or mupirocin treatment (Geiger *et al.,* 2012). Characterisation of an RSH_{mutant} in *S. aureus* showed that regulation of amino acid metabolism genes occurs as a result of (p)ppGpp induced de-repression of the CodY regulon (Geiger *et al.,* 2012). Thus, the initiation of the stringent response as a knock-on effect of treatment with an aaRS inhibitor results in a rapid reduction of mRNA synthesis of metabolically important genes. Indeed, several compounds active against aaRS have been discovered, which are discussed in the following sections.

1.3.2. Inhibitors of aaRS enzymes

Further underscoring the essentiality and potential of these enzymes to be good antibacterial targets, is the discovery of several natural and synthetic compounds over the last six decades. The following sections sheds light on some of the previously discovered compounds, both natural and synthetic, and the scope to develop new aaRS inhibitors. Amongst these, mupirocin and GSK2251052 are the only inhibitors to reach the clinic and clinical trials, respectively. Both of these are described in more detail in 1.3.2.2 and 1.3.2.3.

Discovered in 1960, indolmycin is an analogue of tryptophan and inhibits TrpRS by competitive inhibition (Hurdle *et al.,* 2005). Although, it exhibits potent anti-staphylococcal activity with an MIC range of 0.125 mg/L – 2 mg/L (Hurdle *et al.,* 2004c), it is inactive against most members of Enterobacteriaceae, streptococci and enterococci (Werner, 1980, Kanamaru *et al.,* 2001). While indolmycin does possess effective antibacterial activity, it has been shown that indolmycin binds to eukaryotic tryptophan pyrolase and carboxylase (Werne and Reuter, 1976).

Table 1.2: Inhibitors of aaRS enzymes; based on Hurdle and O'Neill (2005) and Fang *et al.,* (2015)

Antibiotic	Producing organism	Year discovered	Target aaRS	Target specificity
Indolmycin	*Streptomyces griseus*	1960	Tryptophanyl	P
Chuangxinmycin	*Actinoplanes tsinanensis*	1977	Tryptophanyl	P
Borrelidin	*Sterptomyces parvulus*	1969	Threonyl	P/E
Ochratoxin A	*Aspergillus ochraceus*	1977	Phenylalanyl	P/E
Granaticin	*Streptomyces violaceoruber*	1975	Leucyl	P/E
Furanomycin	*Streptomyces* L-803	1969	Isoleucyl	P/E
Mupirocin	*Pseudomonas fluorescens*	1971	Isoleucyl	P
Albomycin	*Actinomyces subtropicus*	1951	Seryl	P
Cispentacin	*Bacillus cereus*	1989	Prolyl	P/E
GSK2251052	Synthetic	2013	Leucyl	P
ZCL039	Synthetic	2013	Leucyl	P

where P = prokaryotic and E = eukaryotic

These two enzymes play a crucial role in typrophan catabolism and the binding of indolmycin therefore affects tryptophan metabolism, implying that systemic use of the compound might result in toxicity in patients.

Borrelidin, an inhibitor of ThrRS has not been pursued as a candidate antibacterial agent primarily because it binds to both the prokaryotic and eukaryotic enzymes (Bhikshapathi *et al.,* 2010). It has also been suggested that borrelidin leads to hepatotoxicity in rats. However, recent research has shown that semi-synthetic derivatives of borrelidin possess anti-parasitic activity without having an effect on eukaryotic ThrRS (Novoa *et al.,* 2014).

Other natural product inhibitors of aaRS include ochratoxin A and granaticin, which inhibit PheRS and LeuRS, respectively (Hurdle *et al.,* 2005, Ogilvie *et al.,* 1975). Ochratoxin A exhibits

several toxicological effects such as, nephrotoxicity, neurotoxicity, hepatotoxicity and immunotoxicity (Hurdle *et al.,* 2005), whilst granaticin, although possessing potent antibacterial activity, inhibits RNA synthesis in mammalian cells at concentrations required for therapy (Heinstein, 1982). The naturally occurring nucleoside, dealanylascamycin also inhibits aaRS and is active against both Gram-positive and Gram-negative organisms. In spite of its broad spectrum activity, interest in the compound for use as an antibacterial agent subsided after it was shown to be toxic in mice (Osada and Isono, 1985).

1.3.2.1. Structural analogues of aminoacyl-adenylates as aaRS inhibitors

In order to expand the arsenal of aaRS inhibitors, structural analogues of both naturally occurring inhibitors and the reaction intermediate (amino-acyl AMP) in the aminoacylation process have been designed and characterised. The intermediate, amino-acyl AMP, is a mixed anhydride and known to bind more tightly to the corresponding aaRS compared to the tRNA or amino acid (Lee *et al.,* 1999, Kim *et al.,* 2003).

Non-hydrolysable analogues of the reaction intermediates have been shown to exhibit antibacterial activity. An analogue of prolyl adenylate was synthesised by replacing the phosphate ester of the adenylate with the more stable sulfamoyl linkage resulting in L- and D- prolyl-sulfamoyladenylates (Heacock *et al.,* 1996). However, both compounds reportedly bound to *E. coli* and human ProRS, highlighting the challenge in designing pathogen-specific potent inhibitors. Although, these analogues show non-specific activity with more binding affinity for the human enzyme, these non-hydrolysable analogues can serve as potential avenues for discovering novel inhibitors. The 5'-*O*-[*N*-(L-seryl)-sulfamoyl] adenosine analogue of seryl adenylate inhibited *E. coli* SerRS in the nanomolar range and Cubist Pharmaceuticals were the first to design target selective adenylate analogues active against

IleRS. One compound in particular, CB-432, exhibited ~600 fold higher specificity for bacterial IleRS (0.5 – 8 nM) compared to the human counterpart (570 nM) (Schimmel *et al.,* 1998). The target specific activity of CB-432 was further confirmed by showing that the addition of excess isoleucine resulted in a loss of antibacterial activity (Schimmel *et al.,* 1998). However, extremely high doses were required to clear *S. pyogenes* infection in mice; this was attributed to the high serum binding nature of CB-432, thus making the compound unsuitable for use systemically (Schimmel *et al.,* 1998). Adenylate analogues targeting other aaRS enzymes such as MetRS, GlnRS and TyrRS have also been assessed for their antibacterial activity. Most of these have not been pursued either due to lack of specific activity, inability to cross the bacterial cell membrane or toxicity in higher eukaryotic cells. Chemical modifictions of inhibitors based on the enzyme structures can facilitate refinement of biological activity.

Although the natural product and synthetic inhibitors are either inactive against bacteria or exhibit promiscuous activity *i.e.* they are not target specific or inhibit a eukaryotic homologue, there are two exceptions in mupirocin and GSK2251052, which exhibit selective toxicity against bacteria and are discussed below.

1.3.2.2. Mupirocin

Mupirocin (Figure 1.4), a natural product isolated from *Pseuodomonas fluorescens,* is the only aaRS inhibitor used clinically for the treatment of bacterial infections (Vondenhoff and Aerschot, 2011). It is structurally dissimilar to other clinically used antibiotics and exhibits its antibacterial activity by competitive inhibition of isoleucyl tRNA-synthetase enzyme (IleRS).

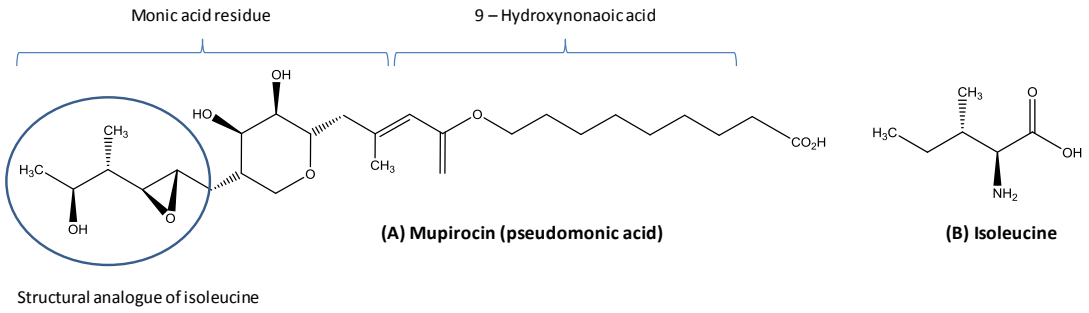

Figure 1.5: Chemical structures of mupirocin (A) and isoleucine (B). The structural similarity of mupirocin to isoleucine is encircled and both the monic acid and 9-hydroxynonaoic acid residue are demarcated

It has a narrow spectrum of activity, primarily active against staphylococci (including MRSA) and streptococci, with an MIC range of 0.06 mg/L – 0.5 mg/L (Sutherland *et al.,* 1985). It is inactive against Gram-negative bacilli such as *E. coli, Klebsiella pneumoniae, Enterobacter* spp. and *Proteus* spp., which is associated with poor penetration of the molecule across the outer membrane of these organisms (Sutherland *et al.,* 1985). Although inactive against Gram-negative bacilli, mupirocin is active against Gram-negative organisms such as *Haemophilus influenzae* and *Nessieria gonorrhoeae* (MIC = 0.05 – 0.12 mg/L) (Sutherland *et al.,* 1985). In addition to possessing effective antibacterial activity, mupirocin is target specific and has no deleterious effects on eukaryotic IleRS and is inactive against skin commensals such as *Micrococcus luteus* and *Propionibacterium* spp (Sutherland *et al.,* 1985), both of which are essential qualities of a desirable antibacterial agent (Sutherland *et al.,* 1985). Although a potent anti-staphylococcal agent, mupirocin can only be used topically, owing to its rapid degradation to monic acid which lacks antibacterial activity. The degradation of mupirocin in addition to high serum binding results in poor bioavailability, which has restricted the use of mupirocin to topical application, and hence it is only used as a 2% (w/v) ointment or cream for application on skin and nares (Cookson, 1998).

1.3.2.3. Resistance to mupirocin

Although a potent antistaphylococcal agent, mupirocin selects for spontaneous resistant mutants at a frequency of 10^{-8} both *in vitro* and *in vivo*. Currently, two principal resistance genotypes are known (Gilbart *et al.,* 1993, Hurdle *et al.,* 2005). One leads to low level resistance (MIC: 8-256 µg/ml) due to single nucleotide polymorphisms *ileS*, which encodes IleRS (Antonio *et al.,* 2002, Hurdle *et al.,* 2004a, Hurdle *et al.,* 2004b). The amino acid substitutions $V_{588}F$ and $V_{631}F$ in staphylococcal IleRS, lead to the disruption of a hydrophobic pocket, essential for the binding of mupirocin within the Rossman fold, thus giving rise to low level resistance (Hurdle *et al.,* 2004a, Hurdle *et al.,* 2004b). As a result, the enzyme undergoes a conformational change reducing the ability of mupirocin to bind its target. It has been shown that $V_{588}F$ mutation does not result in any significant fitness cost in mupirocin resistant strains of *S. aureus* compared to the susceptible strain (Hurdle *et al.,* 2004b), indicating that these strains are capable of persisting in the absence of selective antibiotic pressure. The other confers high level resistance to mupirocin (MIC: >256 µg/ml) and is a result of the acquisition of plasmid-borne *mupA* or *mupB* which encode MupA and MupB, respectively (Seah *et al.,* 2012).

1.3.2.4. GSK'052

GSK'052 (Figure 1.5), a derivative of the antifungal agent Tavaborole, is an experimental inhibitor of bacterial LeuRS exhibiting broad spectrum antibacterial activity (Hernandez *et al.,* 2013). The essential feature of this compound is the presence of a boron atom, which is part of an oxaborole ring and can bind to either the 2'OH or 3'OH of the ribose moiety present in tRNA leading to the formation of a $tRNA^{leu}$-AN2690 adduct (Rock *et al.,* 2007). The

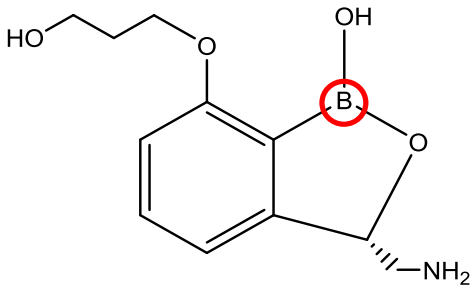

Figure 1.6: Chemical structure of GSK'052. Boron atom encircled in red forms the oxaborole ring with aminoacyl-tRNA

formation of the adduct traps charged tRNA in the editing site of LeuRS, blocking further aminoacylation activity of the enzyme (Rock *et al.,* 2007). GSK'052 exhibited activity against 19 isolates of strains belonging to the Enterobactericeae and against other multi-drug resistant organisms (Hernandez *et al.,* 2013). It also exhibited activity against Gram-positive cocci; however, the compound was primarily being pursued as a potential treatment for infections caused by Gram-negative organisms due to the current dearth of antibacterial agents available for such infections.

1.3.2.5. Resistance to GSK'052

Unfortunately, much like mupirocin, the resistance liability of GSK'052 is high, making it unsuitable for clinical use as a mono-therapeutic agent. GSK'052 selects for spontaneous *E. coli* resistant mutants at a frequency of 10^{-8} *in vitro* (Hernandez *et al.,* 2013), a fundamental reason for the failure of its Phase II clinical trials for complicated urinary tract infections (Hernanadez *et al.,* 2013). Although, the use of GSK'052 resulted in therapeutic responses, *i.e.* clearance of pathogen load in the urine, the trial was stopped following the emergence of resistance in three out of 14 patients within two days of administration (Hernandez *et al.,*

2013, O'Dwyer *et al.,* 2015). Analysis of spontaneous GSK'052 resistant mutants showed that the mutations resided within the editing domain of LeuRS; the amino acid polymorphisms identified were found in strains isolated both, *in vivo* and *in vitro* (O'Dwyer *et al.,* 2015). However, whether this compound will be pursued as a topical agent remains to be seen. Concentrations used in formulations is likely to negate the resistance liability as it is in excess of the concentration required to prevent insusceptibility in a pathogen due to mutational resistance.

1.3.2.6. Scope to develop candidate aaRS inhibitors

Given the success of mupirocin clinically and the structural similarities of aaRS enzymes, approaches can be taken to design compounds with multi-targeting activity. NABARSI set out to design and generate candidate inhibitors with multi-targeting activity against IleRS, LeuRS and ValRS. NABARSI aimed to utilise ligand-based virtual screening to build an in-house database of scaffolds, of which the most promising compounds based on structure activity relationship would be used for optimization. However, all drug discovery programs require a thorough pre-clinical evaluation platform. The biological characterisation of candidate aaRS inhibitors was a major work undertaken in this thesis and the following sections discusses the importance of pre-clinical evaluation of candidate antibacterial agents.

1.4. Importance of pre-clinical evaluation in antibiotic discovery

Similar to currently used antibiotics, new antibacterial agents must inhibit essential bacterial pathways, have a low resistance potential, show target specificity and target selectivity (Hurdle *et al.,* 2005, Vondenhoff and Aerschot, 2011). Target specificity and selectivity are

key attributes of an antibacterial agent, as inhibitors exhibiting off-target activity hinder refinement of its chemical structure due to lack of information on its interaction with the target and frequently results in toxicity in mammalian cells. Therefore, a crucial part of the biological evaluation of novel antibacterial drug candidates involves confirming their antibacterial activity and that they demonstrate on-target activity (*i.e.* that the antibacterial effect results specifically from activity of the agent upon the intended biochemical target), and ensure there is no evidence for off-target activity, as pursuing such compounds would be futile and a waste of resources. Non-specific perturbation of the membrane represents the primary type of off-target effect encountered with candidate antibacterial agents. It is thus prudent to de-select compounds which display this property since their non-specific mechanism of bacterial killing usually reflects a lack of prokaryotic specificity and is predictive of toxicity in mammals. In addition, characterisation of the mode of action (MoA) of a candidate antibacterial agent may also guide further modification of the compound (O'Neill and Chopra, 2004).

1.4.1. Determination of antibacterial activity

Prior to extensive MoA studies, the antibacterial activity of a candidate agent should be determined against a panel of both Gram-positive and Gram-negative organisms (O'Neill and Chopra, 2004). This helps identify the activity spectrum of the compound and classifies pathogens according to their susceptibility to the candidate agent. The antibacterial activity of a candidate antibacterial agent can be established by determining its minimum inhibitory concentration (MIC) against a panel of strains. The MIC is defined as the lowest concentration of drug able to inhibit visible growth of bacteria (Andrews, 2004). It has also been suggested that to be considered for systemic use for treating infections, an antibacterial agent should

ideally exhibit an MIC of ≤1 mg/L; however, those with an MIC up to the range 16 – 32 mg/L may also be considered (O'Neill & Chopra, 2004).

1.4.2. Determination of on-target activity

Following the determination of antibacterial activity of candidate antibacterial agents, it is important to link the observed inhibitory activity to target specificity at a cellular level. The target specificity of a candidate agent can be determined by assessing the effect it has on specific bacterial synthesis pathways, characterisation of resistant mutants or by overproduction/under-production of the intended target. The rationale for all three methods have been explained in the following sub-sections.

1.4.2.1. Inhibition of macromolecular synthesis pathways

This method helps assess the MoA of a candidate antibacterial agent by monitoring the inhibition of DNA, RNA, protein or fatty acid synthesis (macromolecular synthesis or MMS), when bacteria are treated with the compound. The MMS assay monitors the incorporation of radiolabeled precursors to macromolecules upon exposure of bacteria to an inhibitor over a defined period of time, relative to a drug-free control. The assay provides information regarding the biosynthesis pathway targeted by the candidate agent; inhibition of more than one pathway would indicate non-specific activity such as membrane damage.

1.4.2.2. Resistance development

Test organisms are exposed to candidate agent to select for resistant mutants, which are characterised further (eg: genome sequencing), to provide information regarding the underlying reason for the reduction in susceptibility to a candidate agent. This has also been a conventional way of determining the target of a novel antibacterial agent (Silver 2011, O'Neill and Chopra, 2004). Assessment of the frequency of resistance development in bacteria against a candidate antibacterial agent forms a crucial part in its pre-clinical evaluation as it provides information regarding the clinical efficacy of the compound (Gwynn *et al.,* 2010).

1.4.2.3. Overproduction and underproduction of intended target

Specific tools which aid in target titration at a cellular level can be useful in determining both the antibacterial activity and target specificity of a candidate antibacterial agent. Bacterial systems where expression of the target gene can be modulated to manipulate cellular levels of encoded protein can minimize screening times of HTS projects. Modulation of gene expression can either result in overproduction or underproduction of target protein (O'Neill and Chopra, 2004, Payne *et al.,* 2007). The alteration in target gene expression under dose dependent induction is likely to result in a change in the concentration of inhibitor required to cause inhibition. Strains overproducing the intended/predicted target for a candidate agent should show a reduction in susceptibility to the agent. Whereas, underproduction of the target should result in a strain with increased susceptibility to a candidate agent. Thus, use of the aforementioned strains help provide an immediate indication on both the antibacterial activity and specificity of the candidate antibacterial agent, considerably reducing the screening time (O'Neill and Chopra, 2004, Payne *et al.,* 2007, Silver 2011).

1.4.3. Characterisation of membrane damage and prokaryotic specificity

Candidate antibacterial agents identified in HTS programs often exhibit non-specific activity, which is most commonly associated with perturbation of the bacterial membrane. Membrane damage is an undesirable characteristic for a candidate antibacterial agent as it translates to toxicity in humans. Hence, compounds exhibiting antibacterial activity by deleterious effects on the membrane have no chemotherapeutic potential. The effect a compound has on the membrane can be assessed using the *Bac*Light™ assay, DiSC(3)5 assay and assays which measure the loss of metabolites such as K^+ and ATP. Determination of antibacterial activity of a compound against a lower eukaryote (*e.g. Candida* spp.) can also provide information about the specificity of a test compound. Ideally the activity of a candidate agent against yeast should be at least 10-fold higher compared with its antibacterial activity.

1.5. Aims of the study

The careful biological characterisation of a prospective antibacterial candidate is crucial in providing information regarding its potential clinical efficacy. Thus, it is imperative to undertake a thorough pre-clinical evaluation of the novel bacterial aaRS inhibitors being generated within NABARSI. In addition, determination of target specificity of novel antibacterial candidates under whole-cell conditions is likely to provide insight on necessary structural refinement required for improved antibacterial activity and could also aid in detecting potential problems related to the drug candidate.

The aim of this study was to generate and validate tools where target gene expression could be artificially regulated, to help characterise the antibacterial activity and specificity of the candidate aaRS inhibitors and carry out the pre-clinical evaluation of candidate aaRS inhibitors. As mentioned in 1.4.2.2, characterisation of resistance to a candidate antibacterial agent is a crucial part of its pre-clinical evaluation. However, it is also important to investigate pre-existing resistance mechanisms (known or unknown) to inhibitors against a validated bacterial target (see 1.3.2.3). With this in mind, the study also looks into the molecular basis of mupirocin resistance in MupA and MupB, two proteins known to confer high-level mupirocin resistance; and the resistance liability of GSK'052 in *S. aureus,* both *in vitro* and whether resistance to this compound pre-exists in the clinic.

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1. Bacteria and plasmids

The bacterial strains and plasmids used and generated in this study are given in Table 2.1 and 2.2 respectively.

Table 2.1: Bacteria generated and used in this study

Strains	Description	Source/ Reference
Escherichia coli		
DH5-α	*glnX44, λ⁻, deoR481, rfbC1, gyrA96* (NalR), *recA1, end A1, thiE1, hsdR17*	Life Technologies
SA08B	DC10B; CC8-2 *hsdMS*	Monk *et al.,* (2015)
W0153	AB1157; *asmB1* Δ*tolC*::*neoR*	Ling *et al.,* (2015)
W0159	AB1157; *asmB1* Δ*rfaC*::*neoR*	Ling *et al.,* (2015)
BW25113	Δ*(araD–B)567*, Δ*lacZ4787*::*rrnB-3, λ⁻, rph-1* Δ*(rhaD–B) 568*, *hsdR514*	Datsenko and Wanner, (2000); Baba *et al.,* (2006)
BW25113 (Δ*tolC*)	BW25113 deficient in TolC (Δ*tolC*::*neoR*)	Baba *et al.,* (2006)
BL21 (pCA24N)	BL21 with empty backbone of IPTG inducible expression vector, pCA24N	Kitagawa *et al.,* (2005)
BL21 (pCA24N:*leuS*)	Strain expressing *E. coli leuS* gene under control of the P_{spac} promoter	Kitagawa *et al.,* (2005)
BL21 Δ*tolC*	BL21 deficient in TolC (Δ*tolC*::*neoR*)	Gift from Daniel Wilson
Staphylococcus aureus		
RN4220	Restriction deficient, modification proficient, cloning host	Fairweather *et al.,* (1983)
SH1000	*rsbU*⁺ derivative of 8325-4	Horsburgh *et al.,* (2002)
USA300	Community associated-MRSA	Diep *et al.,* (2006)
SH1000 (Δ*ileS*::*mupA*)	SH1000, with complete replacement of native staphylococcal *ileS* with *mupA*	This study
SH1000 (Δ*ileS*::*mupB*)	SH1000, with complete replacement of native staphylococcal *ileS* with *mupB*	This study
***S. aureus* strains over-expressing aaRS genes**		
SH1000 (pEPSA5:*ileS*)	Strain with copy of staphylococcal *ileS* under the control of the pXyl-*xylR* promoter, for ectopic expression	This study
SH1000 (pLOW:*ileS*)	Strain with copy of staphylococcal *ileS* under the control of P_{spac}, for ectopic expression	This study

Table 2.1 contd.

Strains	Description	Source/ Reference
SH1000 (pSK5487:*ileS*)	Strain with copy of staphylococcal *ileS* under the control of the P_{qacR} promoter, for ectopic expression	This study
SH1000 (pSK5487:*mupA*)	Strain with *mupA* under the control of P_{qacR}, for ectopic expression	This study
SH1000 (pSK5487:*mupB*)	Strain with *mupB* under the control of P_{qacR}, for ectopic expression	This study
SH1000 (pLOW:$leuS_{SH1000}$)	Strain with $leuS_{SH1000}$ under the control of P_{spac}, for ectopic expression	This study
SH1000 (pLOW:$leuS_{1372}$)	Strain with $leuS_{1372}$ under the control of P_{spac}, for ectopic expression	This study
***S. aureus* conditional mutants for aaRS genes**		
SH1000 (↓*ileS*)	Conditional mutants of *ileS* and *leuS* in SH1000; complete replacement of *ileS* and *leuS*, with copy of native gene under the control of P_{spac} either on chromosome (pAR89:*ileS*) or plasmid (pLOW:*leuS*); repression of P_{spac} mediated by expression of *lacI* on pGL485	This study
SH1000 (↓*leuS*)		
SH1000 (↓*serS*)	Conditional mutants of *serS* and *thrS* in SH1000; native staphylococcal *serS* and *thrS* under control of P_{spac} on pMUTIN4 (Table 2.2); repression of P_{spac} mediated by expression of *lacI* on pGL485	This study
SH1000 (↓*thrS*)		

Table 2.2: Plasmids used and generated in this study

Plasmids	Description	Source/Reference
pEPSA5	Xylose-inducible staphylococcal expression vector	Forsyth *et al.,* (2002)
pLOW	IPTG-inducible staphylococcal expression vector	Liew *et al.,* (2011)
pSK5487	Constitutive staphylococcal expression vector under the control of P_{qacR}	Unpublished data, O'Neill group
pIMAY	Used for allelic replacement of desired chromosomal *S. aureus* gene	Monk *et al.,* (2012)
pLL39	Integrative vector, for integrating into Φ11 *attB* site on the chromosome, consists of three transcriptional terminators	Luong and Lee, (2007)
pAR89	pLL39:P_{spac}	This study
pLL2787	Expresses the Φ11 *int* gene	Luong and Lee, (2007)
pMUTIN4	Suicide plasmid for disruption or IPTG-dependent control of target genes in *S. aureus*	Vagner *et al.,* (1998)
pGL485	Source of LacI for repression of P_{spac}	Liew *et al.,* (2011)
pCA24N	Expression of N-terminal His tagged and C-terminal GFP fused protein, IPTG inducible	Kitagawa *et al.,* (2005)

2.2. Bacterial growth media, reagents and chemicals

Mueller Hinton Broth II and Agar II (MHB-II, MHA-II), Luria Bertini Broth and Agar (LBB, LBA), Brain Heart Infusion Agar (BHIA) and Tryptone Soya Broth and Agar (TSB and TSA) were from Oxoid (Basingstoke, Hampshire, UK). Chemicals were from Sigma Aldrich (Dorset, United Kingdom) unless specified otherwise. Xylose and glycerol were from Fischer Scientific UK (Leicestershire, United Kingdom). The sources of antibacterial agents used in this work, and the solvents used to dissolve them, are shown in Table 2.3. All experimental antibacterial agents were synthesized by collaborators at the Latvian Institute of Organic Synthesis (LIOS) and dissolved in 100% DMSO.

2.3. Microbiological methods

2.3.1. Routine growth conditions

Bacterial cultures were grown in broth or on agar at 37^{o}C for 18 h, with aeration in the case of broth cultures.

2.3.2. Determination of minimum inhibitory concentrations (MIC)

The MIC of antibacterial agents used in this work was determined by broth micro-dilution in MHB-II (Wayne, 2012). Bacterial suspensions (5.56 X 10^5 cfu/mL) of either *S. aureus* or *E. coli* were added to antibiotic dilutions in 96 well micro-titre plates (VWR, United Kingdom). The MIC was defined as the lowest concentration of antibiotic which inhibited visible bacterial growth after an 18 h overnight incubation at 37^{o}C with shaking.

Table 2.3: List of antibacterial agents used in this study

Antibacterial agent	Solvent	Manufacturer
Albomycin	100% DMSO	Genaxxon bioscience (Ulm, Germany)
Ampicillin	Distilled water (dH_2O)	Sigma Aldrich (Dorset, United Kingdom)
Borrelidin	100% DMSO	LKT Laboratories (Minnesota, USA)
Chloramphenicol	50% ethanol	Sigma Aldrich
Ciprofloxacin	20 mM HCl	Sigma Aldrich
Daptomycin	dH_2O supplemented with $CaCl_2$	Cubist Pharmaceuticals (Massachusetts, USA)
Erythromycin	50% ethanol	Sigma Aldrich
Fusidic acid	50% ethanol	Sigma Aldrich
Gentamicin	dH_2O	Sigma Aldrich
GSK2251052 (GSK'052)	dH_2O	Latvian Institute of Organic Synthesis (LIOS), Riga, Latvia
Kanamycin	dH_2O	Sigma Aldrich
Mupirocin	50% ethanol	Sigma Aldrich
Linezolid	dH_2O	Cambridge Bioscience (Cambridge, United Kingdom
Penicillin G	dH_2O	Sigma Aldrich
Rifampicin	100% DMSO	Sigma Aldrich
Spectinomycin	dH_2O	Fisher Scientific UK (Leicestershire, United Kingdom)
Tetracycline	dH_2O	Sigma Aldrich

2.3.3. Bacterial growth curves

Overnight cultures were diluted 1:100 in MHB-II. The initial 1:100 diluted cultures were grown to an OD_{600} of 0.2, pelleted by centrifugation at 4000 x *g* for 15 min and washed twice with equal volumes of pre-warmed MHB-II. Cultures were then re-suspended in pre-warmed MHB-II and were grown for five hours with OD_{600} being recorded every hour for 5 h.

2.3.4. Recovery of spontaneous antibacterial-resistant mutants, and determination of mutation frequencies to antibacterial resistance

Spontaneous resistant mutants were selected by plating saturated cultures onto MHA-II containing 4 X MIC of antibacterial agent. Plates were incubated for 48 hours at 37^{o}C, and MICs were determined for randomly selected colonies to confirm reduced susceptibility to the antibacterial agent compared with the parental strain. Cultures were also plated onto non-selective MHA-II to measure colony forming units. The mutation frequency (MF) was calculated as the number of resistant bacteria per number of viable bacteria (O'Neill *et al.,* 2001).

2.3.5. Competitive fitness

Pair-wise competition assays were performed to determine the competitive fitness (*W*) between strains as previously described (Lenski 1988, Hurdle *et al.,* 2004). Briefly, cultures of both parental and mutant strains were grown to saturation in MHB-II. Mutant cultures were diluted 1:100 in fresh MHB-II, and an aliquot (9 µL) was mixed with equal volumes of undiluted parental culture in fresh MHB-II. The mixture was plated out onto selective (containing 4 X MIC of parental strain) and non-selective MHA-II; plates were incubated at 37^{o}C for 24 hours.

The competitive fitness (*W*) was calculated using the following equation:

$$W = \frac{\ln[\frac{Nr(24)}{Nr(0)}]}{\ln[\frac{Ns(24)}{Ns(0)}]}$$

Where, Nr(t) and Ns(t) represent the number of antibiotic-resistant and antibiotic-sensitive colonies respectively at time t (0 and 24 h).

2.3.6. Bacteriophage transduction

Overnight cultures of donor strain with gene of interest/putative integrate was grown in phage broth [Oxoid nutrient broth No.2 (20 g/L)]. Cultures were diluted 1:100 in 2 mL of phage broth supplemented with 10 mM $CaCl_2$ and aliquots of 300 µL were made. To each aliquot, 200 µL of appropriate phage ϕ11 dilution was added and left at room temperature for 30 min. Following the half-hour incubation, 10 mL of molten top agar (phage broth plus 3.5 g/L of Oxoid agar No.1) supplemented with 10 mM $CaCl_2$ was added to each dilution and immediately poured equally over two base plates (phage broth plus 7 g/L of Oxoid agar No.1). Plates were then incubated at 37^{o}C in a sealed bag overnight. Phage were harvested by scraping the layer of top agar off the plate with the highest dilution exhibiting confluent lysis and subsequent centrifugation at 15000 x *g* for 10 min at 4^{o}C. The supernatant was removed and filtered twice through 0.45 µM filters and stored at 4^{o}C for further applications (Foster, 1998).

For transduction, recipient bacteria were grown in 20 mL of TSB and cells were harvested by centrifugation at 5000 x *g* for 10 min followed by resuspension in 1 mL of TSB. Aliquots (500 µL) of phage were mixed with 500 µL of cells and 1 ml of LBB supplemented with 10 mM $CaCl_2$ was added to make it a final volume of 2 mL. The remaining 500 µL of cells served as

the control. The mixtures were incubated statically for 30 min at 37°C followed by a 15 min incubation at 37°C in an orbital shaker set at 200 rpm. The mixtures were subsequently placed on ice and 1 mL of cold 0.02 M sodium citrate was added. Cells were then harvested by centrifugation at 5000 x *g* for 10 min, resuspended in 1 mL 0.02 M sodium citrate and incubated on ice for two hours. Aliquots (100 µL) aliquots were then plated onto TSA containing selective antibiotic and sodium citrate (Foster, 1998).

2.4. Antibacterial mode of action studies

2.4.1. *Bac*Light™ assay

The extent of membrane damage caused by antibacterial agents was assessed using the LIVE/DEAD *Bac*Light™ bacterial viability kit (Invitrogen, California, USA), essentially as described by Hilliard *et al* (1999). *S. aureus* SH1000 or *E. coli* BW25113 was grown to an OD_{600} of 0.5 or 0.6, respectively. Aliquots (500 µL) of culture were centrifuged at 16000 x *g* for 3 min and washed with 1 ml of sterile distilled H_2O (dH_2O). Cell pellets were re-suspended in 900 µL dH_2O, to which 100 µL of 40 X MIC of hit or control antibacterial agents were added to achieve a final concentration of 4 X MIC. Samples were incubated at 37°C on a shaking platform for 10 min. Cells were then pelleted by centrifugation and washed twice with 1 mL dH_2O, re-suspended in 1 mL dH_2O and 50 µL transferred to the wells of a black-bottomed 96-well plate. An aliquot (150 µL) of 1:300 *Bac*Light™ reagent [containing red (propidium iodide) and green (SYTO(R) 9) at a 1:1 ratio] was added to each sample and incubated at room temperature for 15 min in the dark. Fluorescence was then measured (excitation: 485 nm, red fluorescence emission: 645 nm and green fluorescence emission: 530 nm) on a FLUOstar Optima spectrometer (BMG Labtech). Percent membrane damage was expressed as the ratio of the green : red fluorescence relative to a drug-free control.

2.4.2. $DiSC_3(5)$ assay

Membrane potential of cells re-suspended in HEPES and glucose buffer (pH 7.2) was determined following exposure to 4 X MIC of antibacterial agents over 1 h at 37°C (Higgins *et al.,* 2005, Hobbs *et al.,* 2008). Cultures of SH1000 were grown to OD_{600} of 0.2, following which cells were incubated with 0.1 M KCl and 2 µM $DiSC_3(5)$ for 30 min at 37°C. Cultures were then exposed to control and test compounds at 4 X MIC for 1 h at 37°C. Subsequently, cells were pelleted and 1 mL of supernatant mixed with 1 mL DMSO; the pellet was lysed in DMSO for 10 min and added to equal volumes of HEPES and glucose buffer. Extracellular and intracellular fluorescence was measured on a LS 45 luminescence spectrometer (PerkinElmer) at an excitation and emission of 622 nm and 670 nm respectively. Membrane potential was calculated using the Nernst equation (Silverman *et al.,* 2001) and expressed as a percentage of the initial value.

$$\Delta\vartheta = -\frac{RT}{F}\ln\left(\frac{DiSC3(5)inside}{DiSC3(5)outside}\right)$$

where, $\Delta\vartheta$ = membrane potential, R = gas constant and F = Faraday constant

2.4.3. Macromolecular synthesis (MMS) assay

Inhibition of major macromolecular biosynthesis pathways (DNA, RNA and protein) was assessed following exposure of mid-exponential phase cultures of SH1000 or BW25113 to 4 X MIC of antibacterial agents for 10 min at 37°C. Cells were exposed to antibacterial agents and incorporation of radio-labelled precursors [methyl-^{3}H thymidine (DNA), [5,6-^{3}H] uridine (RNA), and L-[G-^{3}H] glutamine (protein) at 1 mCi/mL, was measured as previously described (Wilson *et al.,* 1995, Hobbs *et al.,* 2008). Cells were grown to an OD_{600} of 0.2 and exposed to appropriate radiolabel at a ratio of 1:1000 at 37°C for 10 min with vigorous shaking. Following

10-minute incubation, 100 µL of culture was added to 100 µL tri-chloroacetic acid (TCA) pre-chilled in a 96 well plate and placed back on ice. Aliquots (180 µL) were then added to 20 µL of 40 X MIC of control agents or inhibitors and incubated at 37^{o}C with vigorous shaking for 10 min; 100 µL of cultures was added to 100 µL pre-chilled TCA in a 96 well plate and incubated on ice for 30 min. Samples were then processed and radioactivity was measured using a scintillation counter (Plate Chameleon, Hidex) as previously described (Wilson *et al.,* 1995).

2.4.4. Haemolysis assay

This assay was adapted from Fernandez-Lopez *et al.* (2001), Lee & Oh (2000) and Hilliard *et al.* (1999). Briefly, whole blood (25 mL) was centrifuged at 1000 X *g* for 10 min at 4^{o}C. Supernatant was removed and erythrocyte pellets were washed three times and resuspended to 5% v/v in 10 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl. Erythrocytes were then diluted 25-fold in buffer and incubated for 15 min at 37^{o}C. Aliquots (180 µL) of pre-warmed erythrocytes was exposed to 4 X MIC of control antibiotics or test compound in a 96-well conical plate. The plate was then incubated for an hour at 37^{o}C; followed by centrifugation at 2000 X *g* for 10 min. The supernatant was transferred to a flat bottomed 96-well plate and absorbance was measured at OD_{540} on a FLUOstar Optima spectrometer (BMG Labtech). Heamolysis was expressed as a percentage relative to 5% SDS (which corresponds to 100% damage).

2.5. Molecular biology techniques

2.5.1. Extraction of genomic DNA

Overnight cultures (3 mL) of *E. coli* or *S. aureus* were pelleted at 16000 x *g* for three min and subsequently washed with 1 mL TE Buffer (pH 8.0) at 16000 x *g* for 5 min. The cell pellet was the re-suspended in 400 µL of spheroplast buffer. Extraction of genomic DNA was conducted using the PurElute™ Bacterial Genomic Kit (EdgeBio, Gaithersburg, USA) according to the manufacturer's protocol. Extraction of genomic DNA from *S. aureus* used the same protocol with the exception that lysostaphin was added to the cell suspension in spheroplast buffer to a final concentration of 100 µg/mL and incubated at 37°C for 40 min. The DNA pellet obtained was dissolved in 100 µL TE buffer (pH 8.0) and stored at -20°C.

2.5.2. Purification of plasmid DNA and PCR products

Plasmid DNA from *E. coli* DH5-α and SA08B and *S. aureus* RN4220 was isolated using the Plasmid Mini Kit (VWR, Leicestershire, United Kingdom). When purifying plasmid DNA from *S. aureus* RN4220, lysostaphin at a final concentration of 100 µg/mL was added after the addition of P1 (re-suspension) buffer and incubated at 37°C for 40 min; the succeeding steps for extraction of plasmid DNA followed the manufacturer's protocol. Plasmid DNA was stored at -20°C.

PCR products were purified using the Cycle Pure Kit (VWR, Leicestershire, United Kingdom) according to the manufacturer's instructions. The QIAquick Gel Extraction Kit (Qiagen, Manchester, United Kingdom) was also used according to the manufacturer's instructions to purify PCR products and restriction enzyme digested products (section 2.6.8).

2.5.3. Quantification of nucleic acids

To determine the concentration of DNA after purification, absorbance was measured at 260 nm. The ratio of absorbance obtained at 260 nm : 280 nm and 260 nm : 230 nm was measured to gauge purity of the samples (Sambrook *et al.,* 2001); ratios between 1.8 and 2 were indicative of pure samples.

2.5.4. Concentrating nucleic acids

Nucleic acid samples were concentrated using Pellet Paint NF Co-Precipitant (Novagen, London, United Kingdom) following the manufacturer's protocol. Briefly, 2 µL of Pellet Paint followed by 0.1 volume of 3 M sodium acetate and two volumes of ethanol were added to nucleic acid sample. Samples were incubated at room temperature for two min prior to pelleting at 15000 x *g* for 5 min at 4^{o}C. The supernatant was removed and nucleic acid pellet was washed with 500 µL of 70% ethanol followed by rinsing with 500 µL 100% ethanol. The supernatant was removed and pellet dried at 70^{o}C for five min followed by dissolving the pellet in TE buffer (pH 8.0) to achieve a final concentration of 1 µg/µL.

2.5.5. Polymerase chain reaction (PCR)

Amplification of genomic or plasmid DNA was carried out using Phusion High Fidelity Polymerase (New England Biolabs, Hertfordshire, UK), according to the manufacturer's protocol. Oligonucleotide primers (refer to appendix) used for amplification were synthesized by Eurofins MWG Operon (Ebersburg, Germany). The annealing temperature was based on the melting temperatures (Tm) of a primer pair, calculated using the Phusion Tm calculator at https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator

(last accessed 23/11/2016). PCR products were visualised using agarose gel electrophoresis (section 2.6.5).

Diagnostic colony PCR was performed with MyTaq ™ Red Mix (Bioline Reagents Ltd., London, UK) according to the manufacturer's protocol to identify positive clones after transformation (section 2.6.11). A single colony was homogenised in 20 µL nuclease-free water, of which 1.5 µL was used as template in a total reaction volume of 25 µL.

2.5.6. Agarose gel electrophoresis

PCR products, plasmids (circular and linearized) and restriction digest products were visualised by agarose gel electrophoresis. Agarose was prepared at a final concentration of 0.8% by dissolving powdered agarose in Tris-acetate-EDTA (TAE) buffer (40mM Tris acetate, 1mM EDTA, pH 8) and was subsequently mixed with SYBRsafe DNA gel stain (Invitrogen, Paisley, UK) while preparing gels. Hyperladder 1 kb (Bioline Reagents Ltd., London, UK) was used as a DNA marker alongside nucleic acid samples to aid in the determination of fragment size of the PCR products. A potential difference of 90 V was applied to the gel for 30 min and the gel was subsequently exposed to blue light (450 nm – 495 nm) to allow visualisation of DNA bands.

2.5.7. DNA sequence determination

DNA sequence determination of plasmids was performed by Beckman Coulter Genomics Inc., Takeley, United Kingdom. DNA sequence data were aligned to native sequences of cloned or amplified genes and analysed using Sequencher 4.8 (Gene Codes, Michigan, USA).

Whole genome sequencing of both *S. aureus* and *E. coli* strains and subsequent analysis to generate single nucleotide polymorphisms (SNPs), insertions and deletions (INDELs) was carried out at Leeds Institute of Molecular Medicine, Leeds, UK.

2.5.8. Restriction enzyme digests

Restriction enzyme digests of vectors and PCR products used during the course of this work were performed in a total reaction volume of 35 µL. Each reaction consisted of approximately 2 µg of DNA, 3.5 µL CutSmart buffer (New England Biolabs, Hertfordshire, UK), 0.35 µL restriction enzyme and nuclease-free water to a total volume of 35 µL. Reactions were incubated at 37^{o}C or 65^{o}C for 4 hours and enzymes were heat inactivated at appropriate temperature if required. Digested products were subsequently gel purified (refer to section 2.5.2).

2.5.9. Ligation of DNA fragments

2.5.9.1. Ligation with T4 DNA ligase

Standard ligation reactions were performed using T4 DNA Ligase (New England Biolabs, Hertfordshire, UK). Reactions contained 30 ng digested plasmid, PCR product at a 3:1 ratio to the plasmid, 1 µL T4 ligase buffer, 0.5 µL T4 DNA ligase and nuclease-free water to a final volume of 10 µL. Reactions were incubated at 22.5^{o}C for 30 min followed by heat inactivation of the enzyme at 65^{O}C for 10 min.

2.5.9.2. Ligation with Seamless ligation cloning extract (SLiCE)

Seamless ligation cloning extract (SLiCE) was also used to join DNA fragments (Zhang *et al.,* 2012). The SLiCE buffer and extract required for the experiment was prepared as described previously (Zhang *et al.,* 2012). Each reaction consisted of 100 ng of linearized vector DNA, a 3:1 molecular ratio of insert to vector, 1 µL SLiCE buffer, 1 µL SLiCE extract and nuclease free water to a final volume of 10 µL. The reaction was incubated at 37^{o}C for 1 h. Chemically competent *E. coli* DH5-α/SA08B cells were then transformed (section 2.5.11.1) with 6 µL of the SLiCE reaction.

2.5.9.3. Ligation with Gibson assembly

DNA fragments were also joined using the Gibson Assembly Master Mix (NEB, Hertfordshire, UK) following the manufacturer's protocol. Briefly, the reaction comprised of 50 ng of linearized vector DNA, a 3:1 molecular ratio of insert to vector, 10 µL Gibson assembly master mix and nuclease-free water to a volume of 20 µL. The reaction was incubated at 50^{o}C for one hour, following which chemically competent *E. coli* DH5-α/SA08B was transformed (section 2.5.11.1) with 10 µL of the reaction.

2.5.10. Site directed mutagenesis

Mutagenesis of desired genes (*mupA, mupB, ileS* and *leuS*) were achieved using oligonucleotide primers listed (appendix) and by following the manufacture's protocol for the Q5 mutagenesis kit (NEB).

2.5.11. Transformation of strains

2.5.11.1. Transformation of E. coli

Chemically competent cells were prepared according to the method described by Green and Rogers (2013). Cells were thawed on ice for 20 min and 100 µL of cells was dispensed into sterile micro-centrifuge tubes. Approximately 50 ng of DNA or entire ligation reaction was added and allowed to incubate on ice for 30 min. Following incubation on ice, cells were incubated at 42^{o}C for 30 seconds and immediately placed on ice for 2 min. Cells were then transferred to 900 µL LBB and incubated for 1 h at 37^{o}C with aeration. Aliquots (100 µL) of cells were spread onto LBA containing selective antibiotic and incubated at 37^{o}C overnight.

2.5.11.2 Transformation of S. aureus

Electrocompetent cells were prepared as described previously (Monk *et al.,* 2012). Plasmid DNA (~ 3 µg) was added to the cells and transferred to a 1 mm cuvette. The cells were pulsed at 2100 V/cm, 100 ohm and 25 µF in a Genepulser XCell electroporator (Biorad Ltd, Hemel Hempstead, Hertfordshire, UK). Following electroporation, cells were incubated in 1 mL TSB with sucrose at a concentration of 500 mM and incubated at 28^{o}C or 37^{o}C before plating onto TSA containing selective antibiotic at appropriate concentrations and incubated at 28^{o}C or 37^{o}C overnight.

2.5.12. Deletion of chromosomal aaRS genes in *S. aureus*

To generate deletion constructs for *ileS,* synthetic DNA fragments consisting of 250 bp regions both upstream and downstream of the gene were synthesized by Life Technologies (Paisley, UK). The fragments were ligated into pIMAY using the Gibson assembly reaction (section 2.5.9.3), generating the plasmid pMIAY:*ileS*KO and pIMAY:*leuS*KO. Plasmid DNA was

subsequently extracted, concentrated using Pellet Paint NF Co-Precipitant (Novagen, London, United Kingdom) as described in 2.5.4 and introduced by electroporation into SH1000 cells as described in 2.5.11.2, with the exception that cells were recovered and plated onto TSA with chloramphenicol at a final concentration of 10 µg/mL (Cm^{10}) at 28°C to maintain plasmid replication. In the case of *leuS,* a 1 kb sequence upstream and downstream were amplified independent of each other using appropriate oligonucleotide primers (Table A1).

To integrate pIMAY constructs into the SH1000 chromosome, a single colony of the transformant was homogenised in 200 µL of TSB. The suspension was diluted to 10^{-3} dilution and 100 µL of each dilution was plated onto BHIA plus Cm^{10} and incubated at 37°C overnight. A selection of large colonies recovered were then streaked onto BHIA plus Cm^{10} and plates were incubated at 37°C for another 24 h. Integration of the pIMAY construct was confirmed by colony PCR to check for absence of extrachromosomal DNA using pIMAY MCS primers (appendix).

Excision of chromosomal aaRS gene was performed over 48 h. First, an overnight culture of the above integrant was grown at 28°C in TSB without selection. The culture was then diluted 1:100 in pre-warmed TSB plus anhydro-tetracycline (ATc at 1 mg/L) and grown for 8 h at 28°C with aeration. The culture was then diluted 10-fold to 10^{-5} and 100 µL of the 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated onto BHIA plus ATc at 1 mg/L. The plates were incubated at 28°C for 48 h. Diagnostic colony PCR was performed using appropriate oligonucleotide primers to confirm deletion of the desired gene. Subsequently, the entire region was amplified by PCR and its DNA sequence determined to confirm successful deletion.

2.5.13. Chromosomal integration of pMUTIN4:*serS* and pMUTIN4:*thrS* conferring P_{spac} control of staphylococcal *serS* and *thrS* genes

To mediate the control of staphylococcal *serS* and *thrS*, constructs in pMUTIN4 to allow site-specific integration were generated according to the method described by Vagner *et al.,* 1998 and Blake *et al.,* 2010. The *serS* RBS and subsequent 600 bp were amplified and ligated into pMUTIN4 to generate pMUTIN4:*serS*. Plasmid DNA was extracted from SA08B cells, concentrated as in 2.5.4 and introduced into RN4220 by electroporation. The integration of the plasmids was confirmed by PCR using oligonucleotide primers. The PCR product was also subjected to sequence determination. Following the confirmation of integration, phage ϕ11 was propagated in putative integrates and was subsequently used to transduce SH1000 as described in 2.5.12. The same steps were followed for the generation and integration of pMUTIN4:*thrS*.

2.5.14. Single stranded oligonucleotide recombination

A previously described method by Sawitzke *et al.* (2011) was used to modify genes of interest in *E. coli* by introducing point mutations at the region of interest. First, plasmid pSIM19 was introduced into the strain of interest following the protocol described in section 2.5.11.1, with the exception that the strains were recovered at 30°C as opposed to 37°C. This was done to prevent the loss of plasmid due to a temperature shift. Overnight cultures of desired strain was diluted 1:100 in 30 mL LBB and grown till an OD_{600} of 0.5 at 30°C. The culture was then incubated at 42°C for 15 min to induce the λ-red system and subsequently made electrocompetent by washing twice in ice-cold sterile dH_2O and resuspending in a final volume of 200 µL ice-cold sterile dH_2O. 50 µL of cells was mixed with 5 pmol of oligo in a pre-chilled 10 mm electroporation cuvette and pulsed in a Genepulser XCell electroporator at

1800V/cm, 200 ohm and 25 µF. Following electroporation, 1 mL of LBB was immediately added to the cuvette and the contents transferred to a 50 mL Falcon tube. The culture was then incubated at 30^{o}C with aeration for 45 min, following which 100 µL aliquots were plated onto LBA with no selection. 9 mL of fresh LBB was then added to the remaining culture and cells were incubated at 30^{o}C till an OD_{600} of 0.5 was attained and the same procedure as above was repeated for a further 6 cycles. At the end of seven cycles, 20 colonies were selected and MICs determined to confirm resistant phenotype. The presence of gene modification was then confirmed by PCR amplification and sequence determination.

Chapter 3

Development and validation of regulated hypo- and hypermorph strains to aid discovery and characterisation of novel aaRS inhibitors

Chapter 3: Development and validation of regulated hypo- and hypermorph strains to aid discovery and characterisation of novel aaRS inhibitors

3.1. Abstract

Sensitisation of a bacterial strain to an antibacterial agent, by tightly regulating expression of the bacterial protein target can be used as a screening tool for the identification of novel antibacterial agents. This approach directly helps in establishing target specificity of candidate antibacterial agents. In this chapter, both hypermorph (overexpressing) and hypomorph (under-expressing) strains were generated and validated. While overexpression of staphylococcal LeuRS led to a strain with reduced susceptibility to GSK'052 (MIC 64 mg/L) compared to SH1000 (MIC 4 mg/L), overexpression of IleRS did not lead to a reduction in mupirocin susceptibility. Overexpression of *E. coli* IleRS and LeuRS resulted in strains with a four-fold and 16-fold decrease in susceptibility to mupirocin and GSK'052, respectively. In addition to the hypermorphs, four *S. aureus* hypomorph strains were generated which allowed conditional expression of *ileS, leuS, serS* and *thrS*, respectively. The dependence of these strains on the inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG), for growth, confirmed the essentiality of these genes in *S. aureus*. Hyper-susceptibility of these strains to the respective cognate inhibitors was confirmed by determining the effect of regulated gene expression on MIC of the inhibitors in the presence of varied concentrations of IPTG. On an average, the strains showed a 32-fold increase in susceptibility to their cognate inhibitors at the lowest concentration of IPTG capable of sustaining growth.

3.2. Introduction

The two major challenges that face antibacterial drug discovery include the identification of a compound exhibiting whole-cell and target-specific activity, and the conversion of such hits to lead compounds suitable for clinical trials (Tommasi *et al.,* 2015, Payne *et al.,* 2007). The utilization of bacterial strains, where expression of the molecular target can be artificially regulated can not only aid in discovery but also in ensuring that the candidate antibacterial agent remains on-target. In addition, elucidating the mode of action (MoA) of an antibacterial agent has always been a crucial part of its pre-clinical evaluation (O'Neill and Chopra, 2004). Inhibitors are likely to interact with cognate targets at a set ratio, usually 1:1, suggesting that alteration of concentration of target would result in a change in susceptibility to the inhibitor (O'Neill and Chopra, 2004). The overproduction of target protein should result in a strain that is less susceptible to the candidate antibacterial agent, whilst the converse should result in a strain that is more susceptible to the inhibitor. Thus, the artificial regulation of gene expression in bacterial strains, leading to either the overproduction (hypermorph) or under-production (hypomorph) of encoded target protein, can be used to aid in the identification of a candidate antibacterial agent with whole-cell and target-specific activity (Payne *et al.,* 2007, O'Neill and Chopra, 2004). In cases where the target is not known, this approach provides the means to identify and confirm the corresponding cellular target of an inhibitor (Payne *et al.,* 2007).

The downregulation of gene expression can be achieved by different methods (O'Neill and Chopra, 2004, Payne *et al.,* 2007). For instance, the use of inducible antisense mRNA to down-regulate essential genes in *S. aureus* has been successfully used to identify the natural products, platensimycin and platencin, both of which inhibit fatty acid synthesis (Wang *et al.,* 2006) and kibdelomycin which inhibits DNA synthesis (Wang *et al.,* 2006, Phillips *et al.,* 2011). This system involves the overexpression of cognate antisense mRNA, which forms

degradable complexes with target protein or complexes which reduce translation, consequently leading to reduction in target protein, thereby sensitising the strain to an inhibitor of that molecular target. The above system has also been successfully used to identify two structurally related synthetic inhibitors of the SAV1754 gene that enhance β-lactam activity in Mu50, a MRSA strain with resistance to vancomycin (Huber *et al.,* 2009). More recently, the CRISPR-cas9 system has been used in *Bacillus subtilis* for regulation of essential gene expression, which can also be exploited for screening compound libraries to assess their antibacterial activity and target specificity (Peters *et al*., 2016). These two approaches provide the opportunity for generating target screening libraries and are beneficial for identifying the target of an inhibitor with an unknown MoA. Drug hyper-susceptibility in bacterial strains can also be achieved by regulated ectopic expression of a gene that has been deleted from the chromosome or by replacing the native promoter of a gene with an inducible promoter. This approach is useful when screening a library of compounds designed against a particular target. Conditional strains allowing artificial regulation of target genes generated by the above approach have been used as a tool in *E. coli* for identification of novel inhibitors in synthetic screening libraries (DeVito *et al.,* 2002). They generated an array of *E. coli* strains deficient in the chromosomal version of the target gene, but were supplemented with a copy of the gene as a result of regulated expression from a plasmid and was used for compound identification (DeVito *et al.,* 2002)**.**

Work presented in this chapter utilised the validated principle of artificial gene regulation to develop strains specifically sensitised for aaRS proteins. In this chapter, hypomorph *S. aureus* strains for *ileS, leuS, serS* and *thrS* and hypermorph strains for *S. aureus ileS* and *leuS* were generated. In addition to the aforementioned staphylococcal strains, *E. coli* strains overexpressing *E. coli ileS* and *leuS* were acquired from the ASKA library (Kitagawa *et al*., 2005) for determining target specificity of candidate aaRS inhibtors. The main purpose for

the generation and use of these strains was to develop rapid tools for confirming on-target activity of the novel candidate antibacterial agents generated within NABARSI.

3.3. Results and discussion

3.3.1. Effect of overexpression of *ileS* and *leuS* on susceptibility to aaRS inhibitors in *E. coli* and *S. aureus*

The aim of NABARSI was to identify and generate an inhibitor with antibacterial activity against both *S. aureus* and *E. coli* or either of them. The use of both hyper- and hypomorph strains are useful in a screening platform to confirm target specificity of candidate antibacterial agents. In the first instance hypermorph strains were evaluated, as *E. coli* strains overexpressing *E. coli ileS* and *leuS* were readily available from the ASKA library – a complete set of *E. coli* ORF clones (Kitagawa *et al.,* 2005). On-target activity is measured as the decrease in susceptibility of the strain to a candidate compound, when the cognate target is overexpressed off an inducible plasmid. These strains carry a copy of either *ileS* or *leuS* on the IPTG-inducible expression vector, pCA24N, which leads to overproduction of either IleRS or LeuRS. To validate these strains as useful screening tools, the antibacterial susceptibilities of *E. coli* (pCA24N:*ileS*) and *E. coli* (pCA24N:*leuS*) were determined against mupirocin and GSK'052. Overexpression of *ileS* and *leuS* in the presence of IPTG in *E. coli* (pCA24N:*ileS)* and *E. coli* (pCA24N:*leuS*) led to a four-fold and 16-fold reduction in susceptibility to both mupirocin (MIC = 128 mg/L) and GSK'052 (MIC = 4 mg/L) respectively, compared to *E. coli* (pCA24N) (MICs = 32 mg/L and 0.25 mg/L, respectively). The strains were specific for their cognate inhibitors and no cross resistance was observed, thus making them useful screening tools to determine target specificity of candidate aaRS inhibitors generated within NABARSI.

As mentioned above, in addition to determining the specificity of the candidate inhibitors in *E. coli,* the on-target activity of the compounds also needed to be evaluated against the corresponding staphylococcal enzymes. Cloning of staphylococcal *leuS* in pLOW, for ectopic expression in SH1000, resulted in the strain SH1000 (pLOW:*leuS*), which exhibited a four-fold reduction in susceptibility to GSK'052 (MIC = 16 mg/L) compared with SH1000 (pLOW) (GSK'052 MIC = 4 mg/L). However, cloning of *ileS* in three different vectors, pEPSA5, pLOW and pSK5487, possessing the pXyl-*xylR*, P_{spac} and P_{qacR} promoters respectively, did not result in reduced susceptibility to mupirocin, with the MIC being 0.125 mg/L in all cases, equivalent to wild-type SH1000 (MIC = 0.125 mg/L).

In the simplest model, overexpression of an antibacterial target should be directly proportional to decrease in susceptibility to a cognate inhibitor, implying that an increase in target requires a higher concentration of cognate inhibitor to achieve inhibition of growth. However, in some circumstances, like in the case of ciprofloxacin, overexpression of *gyrA* leads to increased susceptibility to the inhibitor (Palmer and Kishony, 2014). Ciprofloxacin recognises DNA-Gyrase complexes and allows cleavage of DNA but prevents re-ligation of DNA by Gyrase, thus generating double-stranded breaks. Therefore, the overexpression of *gyrA* is likely to lead to the increased susceptibility to the antibiotic as more DNA-Gyrase complexes will be present within the cell (Palmer and Kishony, 2014). Results obtained in this chapter regarding the increased production of staphylococcal IleRS suggest that overexpression of *ileS* does not have an impact on the anti-staphylococcal activity of mupirocin. Expression of *ileS* from two different inducible (pEPSA5 and pLOW) and a constitutive (pSK5487) expression vector suggest that the lack of an effect on mupirocin susceptibility is unlikely to be related to the expression system. The lack of any impact on the inhibitory activity of mupirocin could possibly be a result of elevated levels of synthetase within the cell, leading to mischarging of $tRNA^{ile}$ or depletion of tRNA pool as a consequence of mis-charging non-cognate tRNAs. This was postulated by Bedouelle and colleagues when

they showed that overexpression of tyrosyl-tRNA synthetase is toxic in *E. coli* (Bedouelle *et al.,* 1990); however, it was not investigated any further in this thesis.

As the *S. aureus* strain overexpressing *ileS* was unable to exhibit reduced susceptibility to mupirocin, I decided to generate *S. aureus* strains where the expression of *ileS* and *leuS* could be downregulated. The aim was to use these hypomorphs in conjunction with the *E. coli* hypermorphs as rapid tools to determine target specificity of the candidate inhibitors. In addition to *S. aureus* hypomorphs for *ileS* and *leuS,* I also generated hypomorphs for staphylococcal *serS* and *thrS*. These are class II aaRS enzymes and together with the other hypomorphs serve as a proof of principle that, downregulation of the target gene can also aid in determining the target specificity of antibacterial agents. The generation and validation of these hypomorphs are discussed in the following sections.

3.3.2. Generation of *S. aureus* conditional mutants for *ileS, leuS, serS* and *thrS*

As *serS* and *thrS* do not reside within monocistronic operons in *S. aureus*, the integrative vector pMUTIN4 was successfully used to place the genes under the control of the P_{spac} promoter. The constructs, pMUTIN4:*serS* and pMUTIN4:*thrS* were generated in *E. coli* and confirmed by sequence determination (Figure 3.1). The constructs were integrated independently into the RN4220 chromosome and then introduced into *S. aureus* SH1000 by transduction to yield SH1000 (pMUTIN4:*serS*) and SH1000 (pMUTIN4:*thrS*), where expression of the staphylococcal *serS* and *thrS* genes was now under the control of the P_{spac} promoter (Figure 3.1). To achieve titrability of *serS* and *thrS* in these strains, the plasmid pGL485 expressing *lacI* was introduced to generate the strains, SH1000 (↓*serS*) and SH1000 (↓*thrS*), respectively. The presence of an additional copy of *lacI* being expressed off pGL485, allows for the complete repression of P_{spac}, resulting in better regulation of gene expression.

However, pMUTIN4 could not be used for regulation of staphylococcal *ileS* and *leuS* as they lie within an operon. To avoid affecting expression of downstream genes within the operons, a different strategy was required; this has been described in the following section. Briefly, the allelic replacement vector, pIMAY, was used to delete the native chromosomal aaRS gene (*ileS* or *leuS*), while a functional copy of the gene was placed under an inducible promoter either at a neutral site on the chromosome or for ectopic expression from a staphylococcal expression vector.

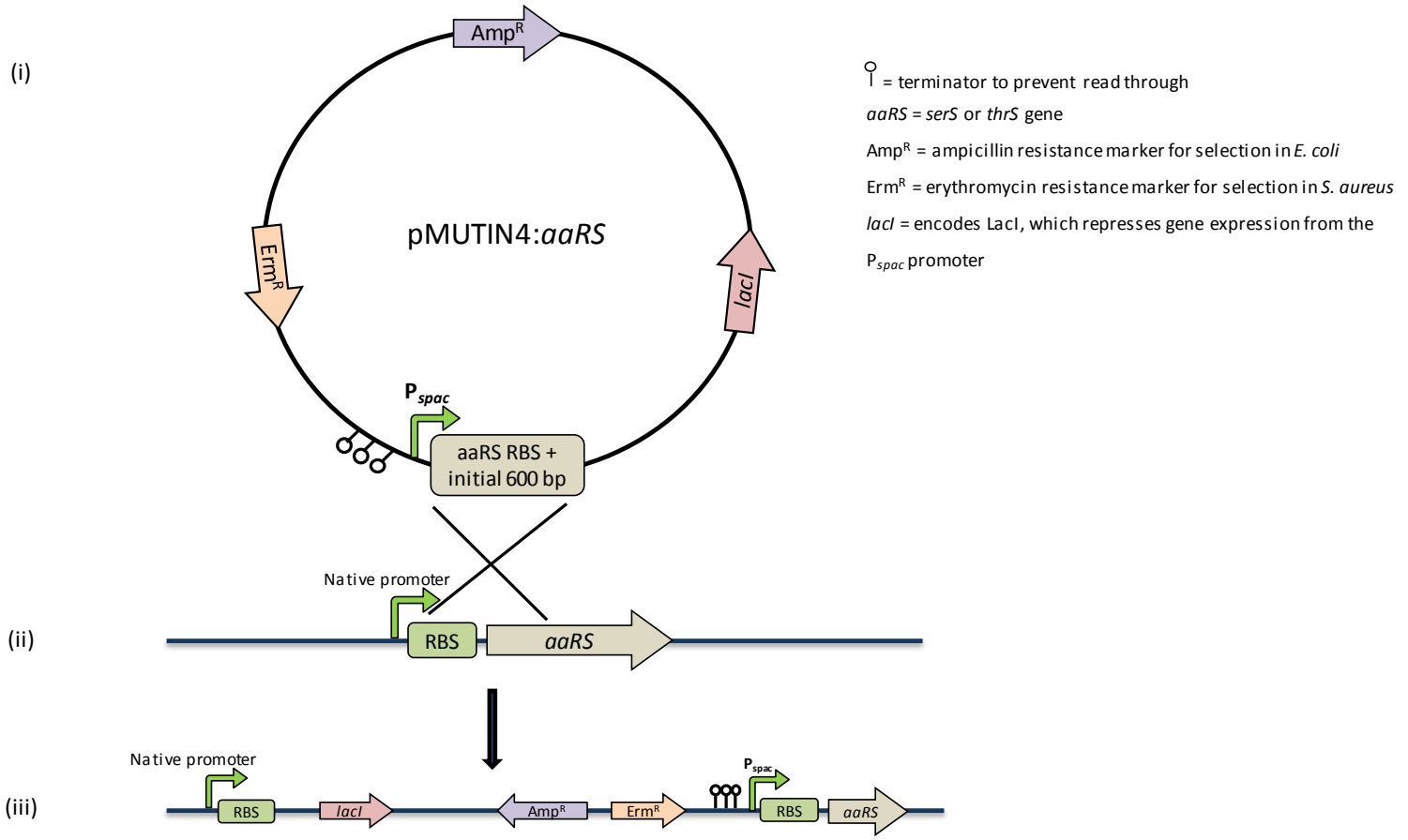

Figure 3.1: Construction and integration of pMUTIN4:*aaRS*, generating strains SH1000 (pMUTIN4:*serS*) and SH1000 (pMUTIN4:*thrS*). (i) The RBS and intial 600 bp of *serS* or *thrS* were amplified and cloned into pMUTIN4, generating pMUTIN4:*aaRS*, where *aaRS* stands for *serS* or *thrS;* (ii) this is followed by a single cross over event resulting in the chromosomal integration of pMUTIN4:*aaRS* in *S. aureus* SH1000 and thereby putting the *serS* and *thrS* under control of the IPTG-inducible, P_{spac} promoter (iii). Arrows indicate the direction of transcription

3.3.2.1. Modifying an integrative vector for S. aureus to enable regulation of gene expression

Several integrative vectors for *S. aureus* have been generated previously, such as pCL84, pCL85, pCL25, pLL29 and pLL39. Amongst these, pLL39 seemed the obvious choice as it consists of five repeats of the *T1* terminator of the *E. coli rrnB* operon, preventing read through into the cloning sites of pLL39 (Luong *et al.,* 2007), thus avoiding any effect on the expression of downstream genes. It integrates at the ϕ11 *attB* site on the *S. aureus* chromosome, a site specific for phage integration, thus avoiding disruption of gene function and can be used for regulated expression of genes on the chromosome. However, it lacks an inducible promoter, which is required for artificially regulating the expression of a target gene. To make it an inducible integrative vector like pMUTIN4 (Vagner *et al.,* 1998), the P_{spac} promoter was introduced at the *Sma*I site of pLL39. Integration of the resultant plasmid, pAR89, in *S. aureus* is facilitated by the presence of the helper plasmid pLL2787 which expresses the ϕ11 integrase. Therefore, for the purpose of the work described in this chapter, the pAR89 constructs were first integrated in *S. aureus* RN4220 carrying the helper plasmid, pLL2787, and was then introduced into a clean background of *S. aureus* SH1000 by transduction, where further manipulations were carried out to finally yield the hyper-sensitive strains.

Being essential genes, *ileS* and *leuS* cannot be deleted without complementation from a different source. Hence, before the chromosomal copy of both *ileS* and *leuS* was deleted in SH1000, a copy of the same needed to be engineered into a different site on the chromosome. The plasmid, pAR89, was used to place a copy of *ileS* or *leuS* at the ϕ11 *attB* site in SH1000. This led to the construction of SH1000 (pAR89:*ileS*) and SH1000 (pAR89:*leuS*), strains with two copies of *ileS* and *leuS,* respectively; with one copy under the control of the plasmid encoded P_{spac} promoter. Both these strains served as the genotypic background for deletion of the staphylococcal *ileS* and *leuS* genes, respectively.

Marker-less deletions of these two essential genes was achieved using the allelic replacement vector, pIMAY. The constructs pIMAY:*ileS*KO and pIMAY:*leuS*KO were generated in *E. coli*, their identity confirmed by PCR and DNA sequence determination (data not shown) and these were subsequently used to delete the native *ileS* and *leuS* genes in SH1000 (pAR89:*ileS*) and SH1000 (pAR89:*leuS*), as described in section 2.5.13. In the case of *ileS,* the chromosomal copy was successfully deleted, generating strain AG01 (Figure 3.2). The integrity of the deletion, to ensure that the deletion was in-frame was confirmed by PCR and sequence determination (data not shown). In case of *leuS,* screening of ~450 colonies (section 2.5.13) did not yield a strain with the desired deletion. It was hypothesized that, a single copy of *leuS* under control of a non-native promoter might not be resulting in sufficient amounts of LeuRS within the cell. To examine whether it was an effect of gene dosage, the overexpression strain, SH1000 (pLOW:*leuS*), generated in section 3.3.1, was used as the genotypic background for deletion of the *leuS* gene. The construct pIMAY:*leuS*KO was introduced in SH1000 (pLOW:*leuS*) by electroporation for deletion of *leuS*, leading to strain AG02. As in the case of *ileS*, the integrity of the deletion of *leuS* was confirmed by PCR and sequence determination (data not shown). The ability to delete native staphylococcal *leuS* in the SH1000 (pLOW:*leuS*) background suggest that it was indeed gene dosage that disallowed deletion of *leuS* in SH1000 (pAR89:*leuS*).

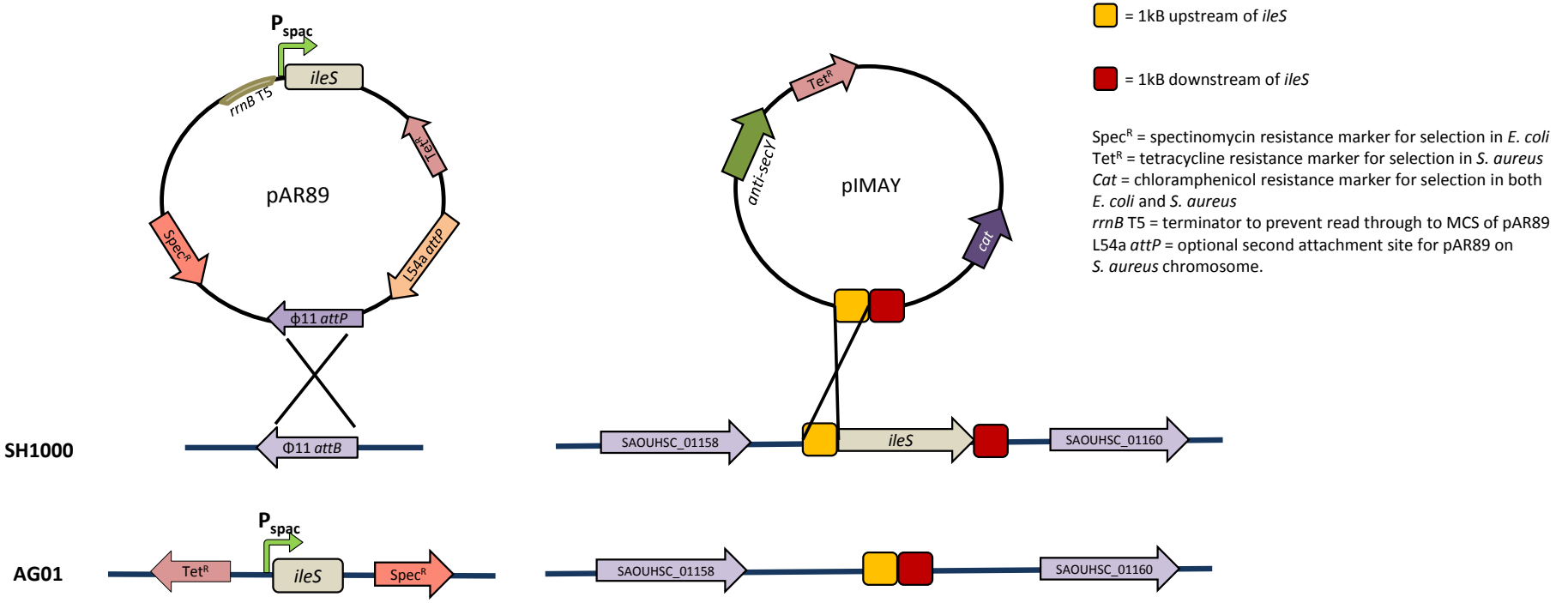

Figure 3.2: **Schematic of generating *S. aureus* hypomorph for *ileS*.** (i) The construct pAR89:*ileS* integrates at the ϕ11 *attB* site resulting in two copies of the *ileS* gene in the same strain. (ii) The allelic replacement vector, pIMAY is then used to knockout the native staphylococcal *ileS* gene on the chromosome, thereby leading to the generation of strain, AG01. Single-crossover integration of pIMAY construct in SH1000 was facilitated by growth at 37°C and excision of native *ileS* was achieved by growing positive integrants at 37°C in the presence of anhydro-tetracycline (1 µg/mL)

Following successful deletion of chromosomal versions of *ileS* and *leuS,* plasmid pGL485 expressing *lacI*, was introduced in the strains, AG01 and AG02, resulting in the hypomorphs, SH1000 (↓*ileS*) and SH1000 (↓*leuS*), respectively. The *lacI* gene encodes the protein, LacI, which facilitates complete repression of downstream genes from the P_{spac} promoter. Thus, the introduction of pGL485 makes these four strains dependent on IPTG for growth, making them conditional mutants for the respective genes and also useful tools for the identification of novel aaRS inhibitors.

3.3.3. IPTG dependent growth of SH1000 (↓*ileS*), SH1000 (↓*leuS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*)

To assess if the conditional mutants generated in section 3.3.2 and 3.3.2.1 are dependent on IPTG for viability, the *S. aureus* hypomorph strains were streaked onto MHA-II plates supplemented with or without IPTG. Observation of growth on these plates indicated that these strains required IPTG in the media for growth (data not shown). To establish a better representation of the dependence on IPTG of these strains, growth experiments over a five-hour period were performed. Growth curves were determined as described in 2.3.2 with the following differences. Following re-suspension of cultures in pre-warmed MHB-II, the cultures were split into two equal volumes, one supplemented with 0.1 mM IPTG. Cultures were then grown for five hours with OD_{600} being recorded every hour for five hours. Figure 3.3 shows the need for IPTG in terms of growth and survival of these conditional mutant strains. The removal of IPTG after the cultures reached an OD_{600} of 0.2 drastically impacts growth in the case of SH1000 (↓*ileS*) and SH1000 (↓*serS*) [Figure 3.3 (A) and (B)]. Restoration of growth was not observed during a five-hour period in the absence of IPTG,

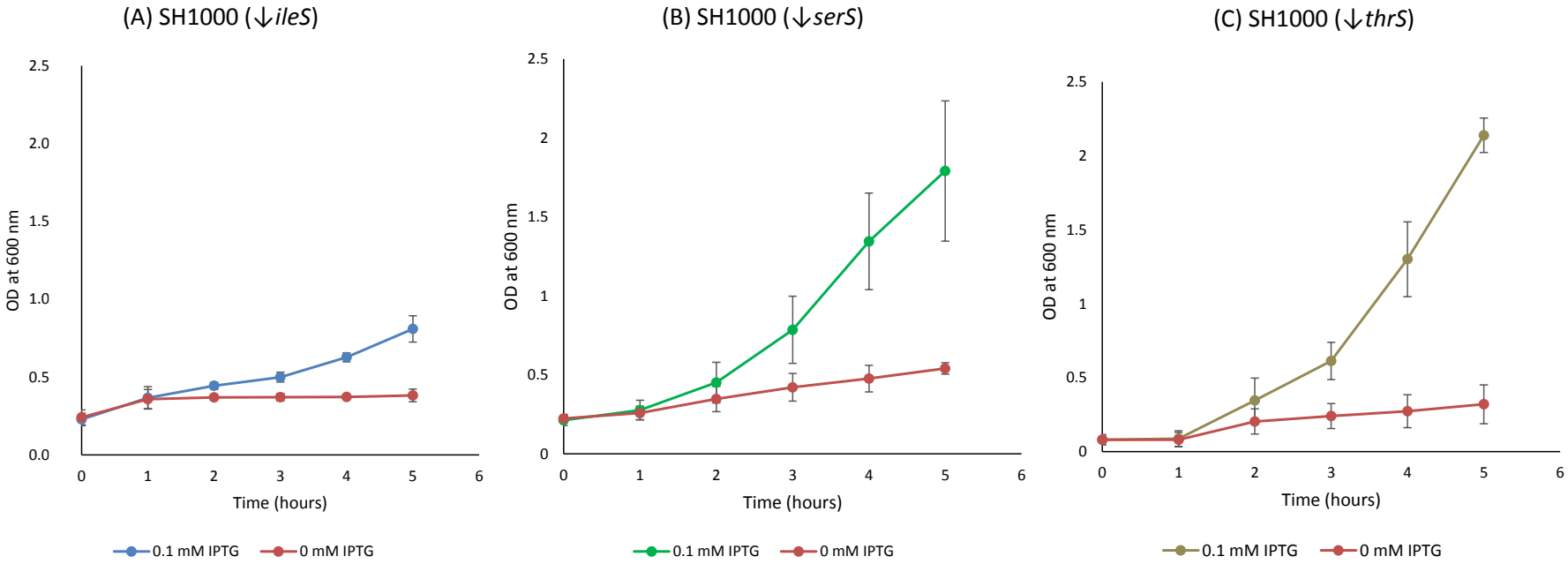

Figure 3.3: Dependence on IPTG for growth in SH1000 (↓*ileS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*). (A), (B) and (C) show growth of SH1000 (↓*ileS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*) in the presence and absence of IPTG over a five hour period, with OD_{600} being monitored every hour. IPTG in the case of (A) and (B) was removed after cultures reached an OD_{600} of 0.2; in case of (C), IPTG was removed at OD_{600} 0.1 (see page 57 for explanation). Graphs are a result of triplicate experiments (n=3) and error bars represent standard deviation (SD).

contrary to what was observed in the presence of 0.1 mM IPTG, confirming that growth is dependent on IPTG. Although it appears that there is not much difference in SH1000 (↓*ileS*) under the two conditions, the same strain when used in Chapter 5 demostrates that it is indeed dependent on IPTG for growth (Figure 5.1, pg – 99). However, removal of IPTG at OD_{600} 0.2 in the case of SH1000 (↓*thrS*) did not show a similar dependency on IPTG for growth, suggesting that the amount on protein produced till OD_{600} 0.2 is possibly sufficient to sustain growth of SH1000 (↓*thrS*) in the absence of IPTG. To test if this was indeed the case and provide better resolution between the two differently treated cultures, IPTG was removed after cultures of SH1000 (↓*thrS*) reached OD_{600} 0.1 [Figure 3.3 (C)]. Removal of IPTG at OD_{600} 0.1 considerably affects the growth of SH1000 (↓*thrS*) and restoration of growth was not observed in the absence of IPTG; a profile similar to what is observed for SH1000 (↓*ileS*) and SH1000 (↓*serS*).

However the same profile was not observed for SH1000 (↓*leuS*) when growth was monitored after removal of IPTG at OD_{600} of 0.2 [Figure 3.4 (A)]. The complementing copy of *leuS* in SH1000 (↓*leuS*) is expressed from the multi-copy plasmid, pLOW (10 copies per cell). Therefore, the inability to completely switch off growth in this assay is likely related to gene dosage. With the issue of plasmid copy number in mind, I sought to determine whether removal of IPTG at OD_{600} 0.1 [like in the case of SH1000 (↓*thrS*)] had any effect on growth pattern. Removal of IPTG from the growth medium before the cells reached OD_{600} 0.2 did have an effect on growth [Figure 3.4 (B)], albeit only slightly. The modest effect on growth suggested that there was sufficient amount of LeuRS produced till OD_{600} 0.1 to sustain growth in the absence of IPTG. Based on these observations I sought to determine if removal of IPTG before OD_{600} 0.1 made a difference to the resolution between the two different conditions. The removal of IPTG after SH1000 (↓*leuS*) cultures reached OD_{600} 0.05, shows that growth can be switched off over a period of 8 hours [Figure 3.5 (C)] corroborating what is observed when the strain is streaked on MHA-II with or without IPTG (not shown).

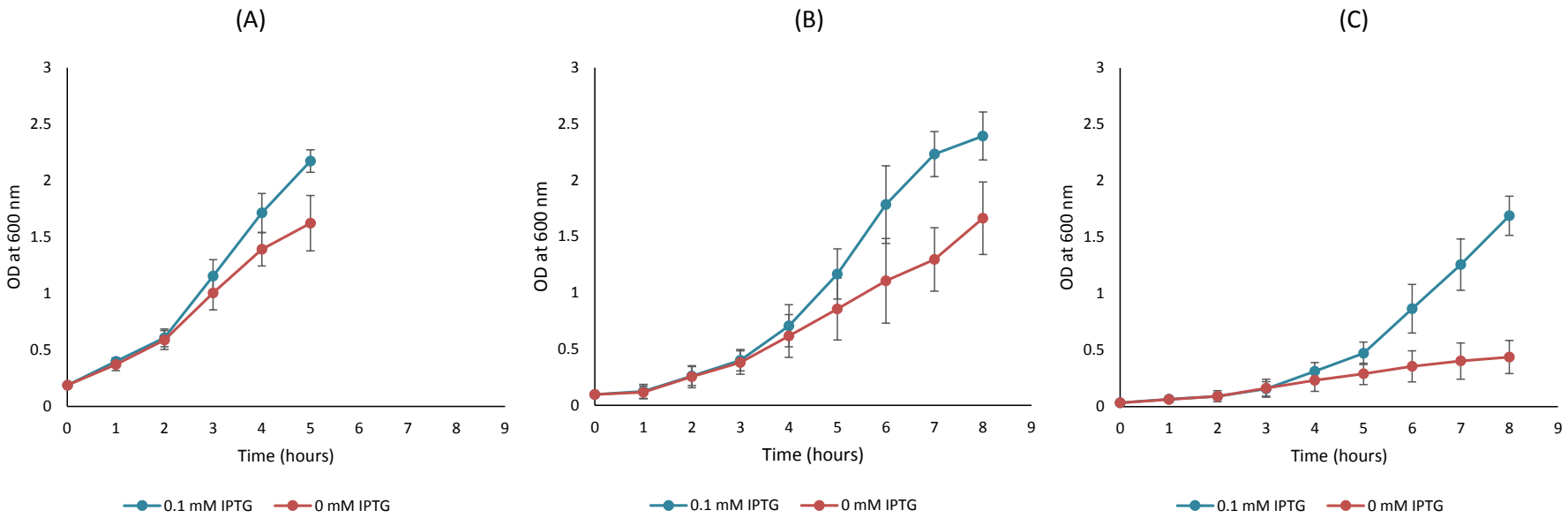

Figure 3.4: Dependence on IPTG for growth in SH1000 (↓*leuS*). Graphical representation of IPTG-dependent growth of SH1000 (↓*leuS*) after the removal of IPTG from cultures growing at 37°C, once an OD_{600} of 0.2 (represented in A), 0.1 (represented in B) and 0.05 (represented in C) was obtained. Graphs are a result of triplicate experiments, where error bars represent standard deviation (SD).

Thus, from these data it canbe proposed that the use of conditional mutant strains where gene supplementation occurs on the chromosome rather than controlled expression from a plasmid is advantageous as it eliminates the variability of plasmid copy number within a cell.

To date, there is no evidence to directly show that aaRS enzymes are essential in staphylococci. The use of global transposon mutagenesis has suggested that these enzymes are essential (Chaudhuri *et al.,* 2009). The method however depends on the inability to generate a random mutant to confirm essentiality of a gene, implying that it is indirect evidence (Chaudhuri *et al.,* 2009). The dependence of these *S. aureus* hypomorph strains for *ileS, leuS, serS* and *thrS,* on IPTG for growth, further support essentiality of aaRS enzymes in *S. aureus*.

3.3.4. Antibacterial susceptibility of SH1000 (↓*ileS*), SH1000 (↓*leuS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*)

To validate these constructs as tools for characterisation and identification of candidate aaRS inhibitors, the antibiotic susceptibilities of these strains to their cognate inhibitors needed to be established. To determine whether SH1000 (↓*ileS*), SH1000 (↓*leuS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*) were hyper-susceptible to mupirocin, GSK'052, albomycin and borrelidin, which target staphylococcal IleRS, LeuRS, SerRS and ThrRS respectively, MIC determinations were made in the presence of a range of IPTG concentrations. The use of different IPTG concentrations should result in target titration, which in turn should alter the susceptibility of the strains to its cognate inhibitor. Figures 3.5.1 and 3.5.2 illustrate the sensitised phenotype of SH1000 (↓*ileS*), SH1000 (↓*leuS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*) to the corresponding antibiotics.

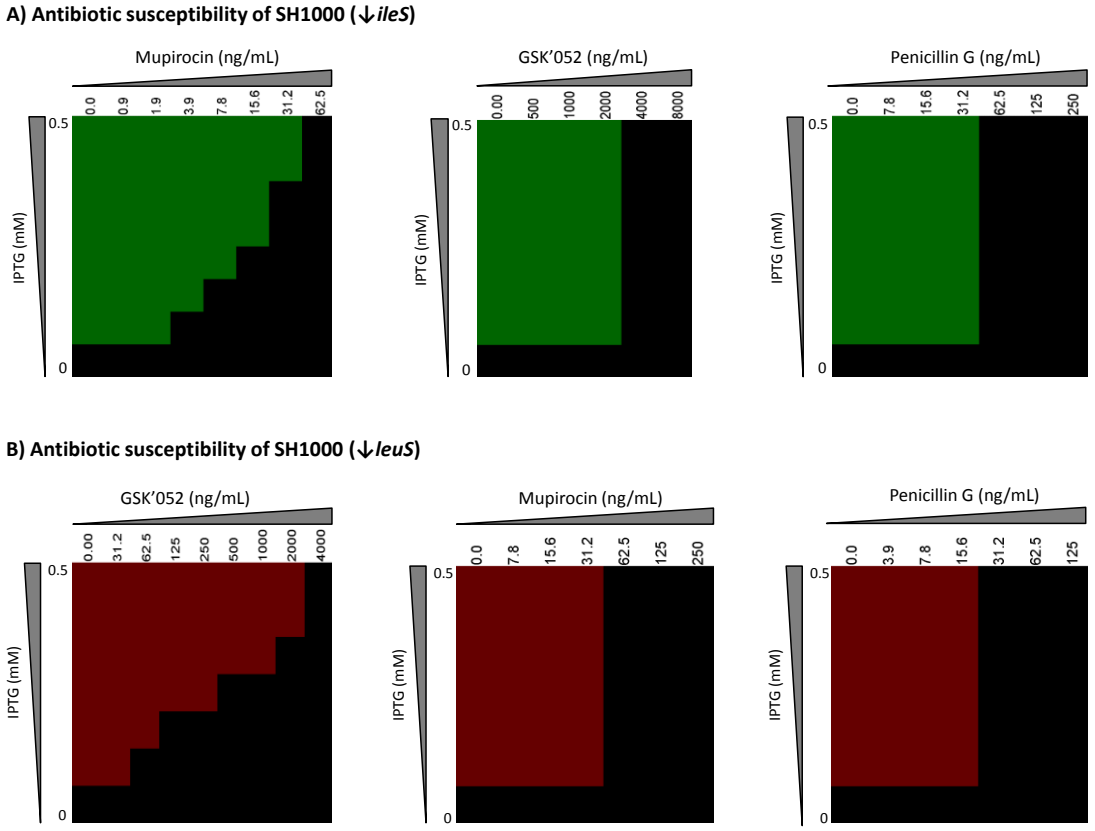

Figure 3.5.1: Checkerboard assays showing the susceptibilities of SH1000 (↓*ileS*) and SH1000 (↓*leuS*) against mupirocin, GSK'052 and penicillin G, respectively, at varying concentrations of IPTG, showing the specificity of the conditional mutants. Panel A and B show the susceptibility of SH1000 (↓*ileS*) [green] and SH1000 (↓*leuS*) [brown] to mupirocin, GSK'052 and Penicillin G, respectively. Data were quantitatively displayed with colour using JavaTreeview.

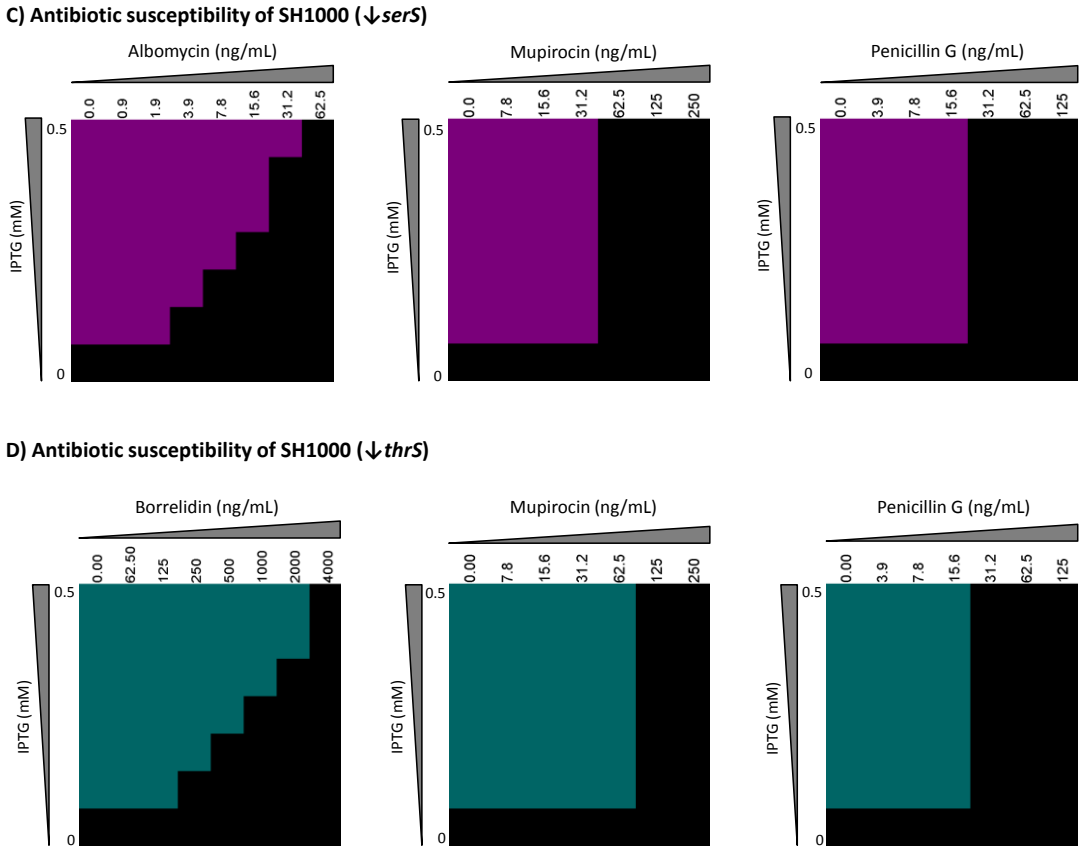

Figure 3.5.2: Checkerboard assays showing the susceptibilities of SH1000 (↓*serS*) and SH1000 (↓*thrS*) against albomycin, borrelidin, mupirocin and penicillin G, respectively, at different concentrations of IPTG and therefore showing the specificity of the conditional mutants. Panel C shows the susceptibility of SH1000 (↓*serS*) [purple] to albomycin, mupirocin and Penicllin G and panel D shows the susceptibility of SH1000 (↓*thrS*) [teal] to borrelidin, mupirocin and Penicillin G, respectively. Data were quantitatively displayed with colour using JavaTreeview.

The artificially regulated expression of *ileS, leuS, serS* and *thrS* in the preceding strains had a drastic phenotypic effect on antibiotic susceptibility compared to wild-type SH1000. The strains exhibited a dramatic increase in sensitivity to the cognate inhibitors; a 32-fold, 64-fold, 32-fold and 64-fold drop was seen in the cases of SH1000 (↓*ileS*), SH1000 (↓*leuS*), SH1000 (↓*serS*) and SH1000 (↓*thrS*), respectively. In order to confirm that the sensitisation of the strains were specific to their antibacterial inhibitors, susceptibility of the strains to a cell wall synthesis inhibitor and an alternate aaRS inhibitor was determined (Figure 3.5.1 and 3.5.2). In addition, the strains exhibited the same degree of susceptibility to the other antibiotics as SH1000 (Table 3.1), and no change was observed with varying concentrations of IPTG, implying that the conditional mutants are specific for their cognate inhibitors. Thus, these strains can be used to directly confirm on-target activity and selectivity of candidate aaRS inhibitors.

Table 3.1: Antibacterial susceptibility of SH1000 and SH1000 hypomorphs against different antibiotic classes

Antibiotic	SH1000	SH1000 (↓*ileS*)	SH1000 (↓*leuS*)	SH1000 (↓*serS*)	SH1000 (↓*thrS*)
Ciprofloxacin	0.25	0.25	0.25	0.25	0.25
Gentamicin	0.062	0.062	0.125	0.125	0.125
Fusidic acid	0.125	0.25	0.125	0.25	0.125
Daptomycin	1	1	2	2	2
Rifampicin	0.015	0.015	0.007	0.007	0.007
Linezolid	2	4	2	2	2

3.3.5. Utilisation of SH1000 (↓*ileS*) and SH1000 (↓*serS*) to elucidate MoA of two compounds with weak antibacterial activity

Validation of these strains to confirm that they are specific for their inhibitors, imply that these hypomorphs can also be used to help elucidate the target of a candidate aaRS inhibitor lacking potent antibacterial activity, thus aiding in the structural improvement of the candidate inhibitor.

GL-1931, a structural analogue of the Ile-Amp intermediate, designed and generated by Cubist Pharmaceuticals (now part of AstraZeneca), does not exhibit potent antibacterial activity against SH1000. Figure 3.6 (A) and (B) shows the effect *ileS* downregulation has on GL-1931 activity against SH1000 (↓*ileS*) and SH1000. Artificial regulation of staphylococcal *ileS* expression resulted in a titratable effect on GL-1931 inhibitory activity. This further exemplifies the feature of these strains as useful tools for confirming on-target activity.

The results presented in Figure 3.6 (C) show that serine hydroxamate, an analogue of serine which is used to induce stringent response in *E. coli* and *S. aureus* is specific for SerRS. It has been shown previously that serine hydroxamate inhibits the functionality of SerRS in *E. coli* (Tosa and Pizer, 1971). This is the first *in vivo* evidence to demonstrate that the compound is indeed specific for *S. aureus* SerRS as well, although it lacks potent antibacterial activity. These results provide further evidence that the generated hypomorphs are valuable tools in antibacterial drug discovery.

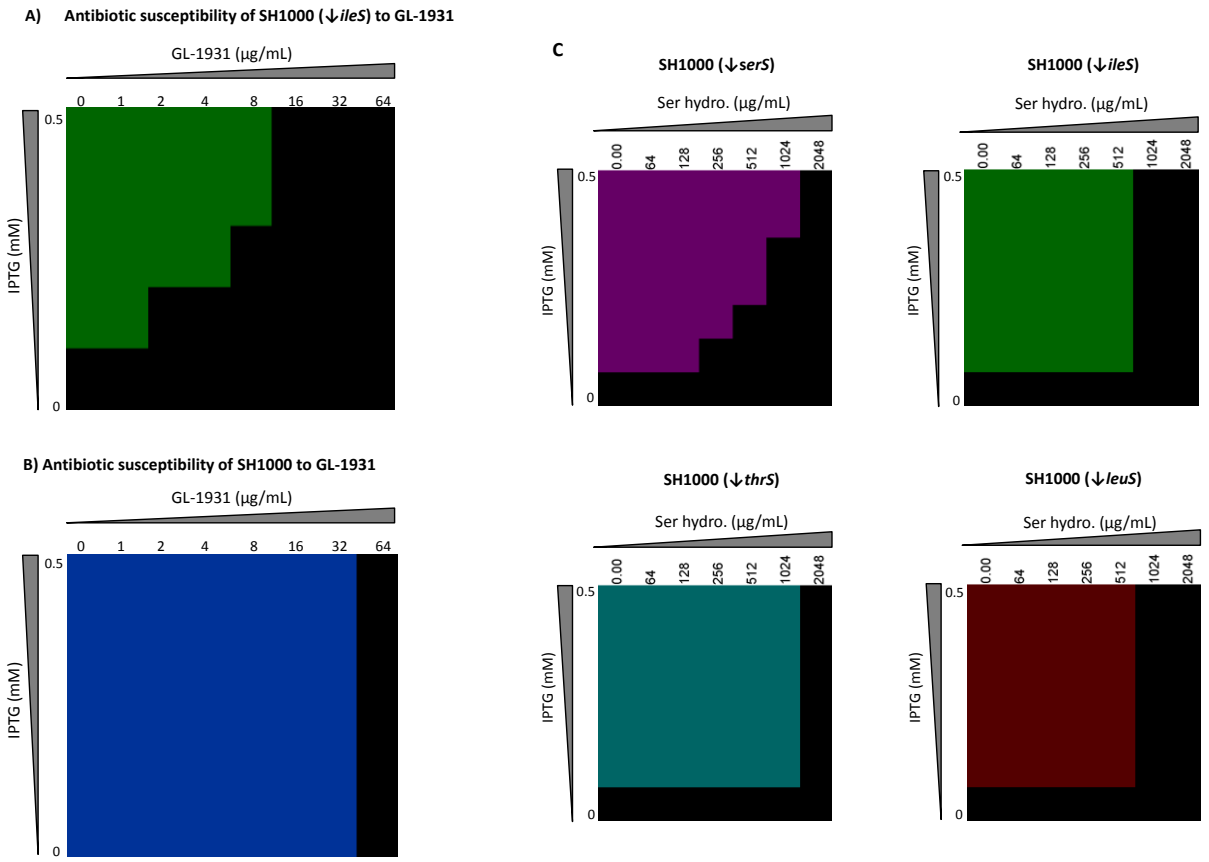

Figure 3.6: Utilisation of *S. aureus* conditional mutants to illustrate target specificity of compounds lacking potent antibacterial activity. Panels (A) and (B) show the antibacterial activity of GL-1931 against SH1000 (↓*ileS*) [green] and SH1000 [blue], respectively. Panel (C) shows the activity of serine hydroxymate (ser hydro.) against SH1000 (↓*serS*) [purple], SH1000 (↓*ileS*) [green], SH1000 (↓*leuS*) [brown] and SH1000 (↓*thrS*) [teal].

3.4. Conclusions

Reduction of intracellular levels of a drug target in bacteria leads to the sensitisation of the organism to the specific antibacterial agent inhibiting that target (Moir, 1999). Generation of bacterial strains where desired target gene expression can be artificially regulated will help find hit/lead compounds from a compound library based on their whole cell activity and target specificity. In summary, these staphylococcal conditional mutants represent tools that can aid drug discovery for the following advantages – (1) the same hypothesis of artificial regulation of gene expression can be applied to other targets as shown here and other studies, (2) the compounds identified by screening against these sets of strains will exhibit both whole cell and target specific activity, (3) the conditions for screening remain the same *i.e.* the same inducer is used in all cases. In general, the strains generated in this study also avoid the issue of polar effects on the expression of downstream genes which may arise while using anti-sense RNA strategies (Xu *et al.,* 2010). Although promoter replacement has been used previously to generate conditional mutants and identify essential genes in *S. aureus* (Xu *et al.,* 2010), the approach undertaken here is specific for the target gene irrespective of it being in an operon. This prevents the expression of the entire operon to be altered as a result of promoter replacement, since the native chromosomal copy of the gene (when in an operon) is deleted in its entirety with a copy of the same being placed at a different locus on the chromosome or on a plasmid. Together with the strains overexpressing desired molecular targets, these strains represent a robust strategy to identify compounds with not only whole cell activity but also ones which exhibit target specificity and selectivity; which are essential to the drug discovery process. The *E. coli* hypermorphs validated in this chapter have been used in Chapter 4 for illustrating the antibacterial activity of candidate aaRS inhibitors generated within NABARSI during the course of this work. The staphylococcal

conditional mutant for *ileS,* SH1000 (↓*ileS*), has been used in Chapter 5 for further study of the mupirocin resistance proteins, MupA and MupB.

Chapter 4

Biological characterisation of novel aaRS inhibitors

Chapter 4: Biological characterisation of novel aaRS inhibitors

4.1. Abstract

Work presented in this chapter was undertaken to evaluate the antibacterial activity, on-target specificity and resistance liabilities of novel aaRS inhibitors developed within the NABARSI consortium. The aim was to design and characterise a compound with whole cell inhibitory activity against one or more bacterial aaRS enzymes and exhibiting low resistance liability. To identify a novel aaRS inhibitor, approximately 100,000 compounds were screened virtually (*in silico*) against IleRS and LeuRS from both *E. coli* and *S. aureus*. Of these compounds, approximately 7000 were selected and tested for target binding activity in the first instance, yielding 65 compounds of interest that selectively inhibited bacterial IleRS or LeuRS compared with eukaryotic counterparts and were subsequently evaluated in this chapter. Of the 65, fifty were inactive against both *E. coli* and *S. aureus* (MIC = >128 mg/L). Ten compounds exhibited antibacterial activity specifically against *S. aureus* SH1000, but this was found to be a consequence of off-target activity *i.e.* it was not mediated by inhibition of protein synthesis and/or IleRS/LeuRS. The remaining five exhibited antibacterial activity against *E. coli* (MIC = 16 – 32 mg/L), but were inactive against *S. aureus* at the highest concentration tested (MIC > 128 mg/L). Over-expression of *E. coli leuS* resulted in a strain less susceptible to the hit compounds (MIC = 64 – 128 mg/L), implying that the hits are specific inhibitors of *E. coli* LeuRS. Resistant mutants of *E. coli* to the hits were selected at a frequency of 10^{-8}. However, no mutation was identified in *leuS* from the recovered mutants; instead a mutation ($G_{-38}T$) was identified in the *yhhY* locus, which encodes a putative acetyltransferase. This mutation was subsequently shown to be responsible for reduced susceptibility to the hits.

4.2. Introduction

The present arsenal of antibacterial agents used clinically were identified based on whole-cell screening assays. However, since the 'Golden Age' of antibacterial discovery, this approach has been ineffective as the same classes of antibiotics have been repeatedly identified (Miesel *et al.,* 2003). An alternative strategy for finding hits is, structure based drug design (SBDD) and the initial use of cell-free biochemical assays followed by biological characterisation of the selected hit compound (Miesel *et al.,* 2003, Simmons *et al.,* 2010). This approach provides the opportunity to screen thousands of compounds with diverse chemical structures against a particular target. The success of SBDD has been proven by the discovery of new therapeutics for HIV/AIDS (Simmons *et al.,* 2010). The availability of structural data for HIV protease facilitated the discovery of five protease inhibitors which are now used clinically (Dorsey *et al.,* 1994, Roberts *et al.,* 1990). In SBDD, three main methods aid in the identification and design of novel compounds. One approach involves the chemical modification of known inhibitors of a particular target to enhance binding affinity. The second approach is based on the docking of small molecules in the active site of a suitable target and the third strategy exploits the *de novo* design of inhibitor scaffolds (Simmons *et al.,* 2010). These fragments are then docked with the target protein and subsequently used to generate whole molecules. However, the most important point to consider for any structure-based design work, is the selection of an appropriate target (Simmons *et al.,* 2010).

The aim of NABARSI was to exploit the conserved nature of these enzymes to develop novel aaRS inhibitors exhibiting multi-target activity and focussed its effort in designing inhibitors that target the catalytic site of IleRS and LeuRS. In order to identify and design candidate antibacterial agents targeting aaRS, an analysis of sequence conservation in LeuRS and IleRS across various bacterial species was carried out by collaborators at InhiBox (London, UK). The analysis was performed to identify highly conserved regions in the active site of the enzymes

as these are likely to be essential for enzyme activity and thus minimising the chances of spontaneous mutation. The InhiBox database of commercial compounds included 3,346,844 compounds, which all had drug-like structures. Compounds were then chosen based on similar pharmacophoric features to known aaRS inhibitors. This was done by calculating the distances between defined essential pharmacophoric points (Inhibox). The top 100,000 compounds were then assessed using the GOLD docking software (Jones *et al.,* 1997) against IleRS and LeuRS (PDB IDs: 1ffy and 3zgz). Following the assignment of docking scores, target binding properties of these compounds were assessed for their ability to inhibit purified IleRS and LeuRS enzymes in *in vitro* assays respectively, by collaborators at Omnia (Barcelona, Spain). This resulted in a total of 65 candidate aaRS inhibitors, whose biological activity were further evaluated in this chapter.

A crucial part of the biological evaluation of novel antibacterial drug candidates involves confirming that they demonstrate on-target activity (*i.e.* the antibacterial effect results specifically from activity of the agent upon the intended target), and ensuring that there is no evidence for off-target activity; the most common form of off-target activity involves membrane perturbation (Hurdle *et al.,* 2005, O'Neill and Chopra, 2004). Antibacterial activity of a candidate agent as a result of off-target activity is indicative of toxicity in eukaryotes, making them unsuitable candidate antibacterial agents and hence not worth pursuing for lead development. Thus, it is key to validate target specificity of candidate antibacterial agents (O'Neill and Chopra, 2004). The focus of this chapter was to delineate novel chemical entities (NCE) exhibiting specific activity from those demonstrating non-specific activity and to identify a hit compound by -

- determination of antibacterial activity against a panel of bacterial strains and clinical isolates of *Candida* spp. to demonstrate prokaryotic specificity

- assessing on-target activity and bacterial specificity using MMS, controlled target expression, the *Bac*Light™ assay and effect on equine red blood cells (RBCs)
- determination of the potential for the development of resistance to the hit NCE

4.3. Results and discussion

4.3.1. Assessing the antibacterial activity of candidate aaRS inhibitors

Based on initial docking studies, 10 compounds were chosen for further evaluation and were acquired from Molport, a chemical marketplace. Initial *in vitro* activity of the compounds against *S. aureus* and *E. coli* IleRS, LeuRS and ValRS was tested by collaborators at Omnia (Barcelona, Spain). The compounds in all cases exhibited inhibition of either staphylococcal IleRS or LeuRS but not both (Omnia, Barcelona, Spain). Based on these initial results, the compounds were sent to Leeds for determination of their antibacterial activity and target specificity. As these compounds only exhibited inhibition of staphylococcal IleRS or LeuRS *in vitro*, MICs were determined against *S. aureus* SH1000 and USA300. Table 1.1 shows the antibacterial activity of the 10 MolPort compounds against SH1000 and USA300. Compound 000-746-235 (‘235) and 002-369-439 (‘439) exhibited some degree of activity against both SH1000 and USA300 (MIC range = 16 mg/L - 32 mg/L). The other eight compounds did not exhibit significant antibacterial activity against SH1000 (MIC = ≥64 mg/L).

Table 4.1: Antibacterial activity of compounds purchased from MolPort, evaluated against two strains of *S. aureus;* SH1000 and USA300

	MIC (mg/L)	
COMPOUND NO	**SH1000**	**USA300**
003-125-137	64	128
000-746-235	16	32
007-710-050	64	128
005-778-538	128	>128
002-369-439	32	32
002-645-367	64	>128
002-918-928	32	64
002-918-933	64	64
003-032-514	64	>128
002-301-482	>256	>256

Screening paradigms would ideally have a cut-off for antibacterial activity to avoid wasting resources, both personnel and financial. However, at this stage any compound possessing a degree of antibacterial activity is promising, as it can act as a starting point for further chemical modification to produce derivatives with improved activity. Thus, efforts were made to establish the target specificity of all of the compounds.

The majority of antibacterial agents in clinical use exert their antibacterial activity by inhibiting bacterial macromolecular synthesis pathways (O'Neill & Chopra, 2004) and the first assay employed to determine specificity was the MMS assay. This assay follows the incorporation of radiolabelled precursors into major cellular macromolecules, and provides an indication whether the candidate compound exhibits antibacterial activity by inhibition of a particular biosynthesis pathway. The aim of this chapter was to identify compounds specifically inhibiting protein synthesis. In order to determine target pathway specificity of candidate inhibitors, their effect of DNA, RNA and protein biosynthesis was established. Eight

out of the nine compounds showed non-specific activity as determined in the MMS assay [Figure 4.1 (i)], *i.e.* the observed antibacterial activity of the compounds was a result of the inhibition of two or more biosynthesis pathways. Compound 002-918-928 did not show significant inhibition of either DNA, RNA or protein synthesis.

To establish whether the off-target activity of these compounds could be associated with their deleterious effect on the staphylococcal membrane, the compounds were tested in the *Bac*Light™ assay. This assay provides a quantitative measure of cell membrane integrity after a 10-min exposure to test compounds at 4 X MIC (see 2.4.1). Results presented in Figure 4.1 (ii) suggests that the observed off-target activity in the MMS assay could be linked to membrane damage upon exposure to the compounds. Although '235 did not perturb the staphylococcal membrane, it suppressed both protein and RNA synthesis [Figure 4.1 (i)], and compound 003-032-514 showed a similar profile. Although a profile like '235 suggests that it could induce the stringent response (where a reduction of RNA synthesis is observed), the goal was to indentify a compound with a similar inhibitory profile of MMS as mupirocin. As mupirocin is the only clinically used aaRS inhibitor and was used as a positive control in this experiment (Figure 4.1), these two compounds were not pursued any further as they were considered to lack specificity. Partners at LIOS however did synthesise some derivatives of '235, however the antibacterial activities could not be associated with LeuRS, as they did not show binding affinity for the enzyme *in vitro.* Hence, this particular class of compounds was not further pursued in the search of a novel aaRS inhibitor.

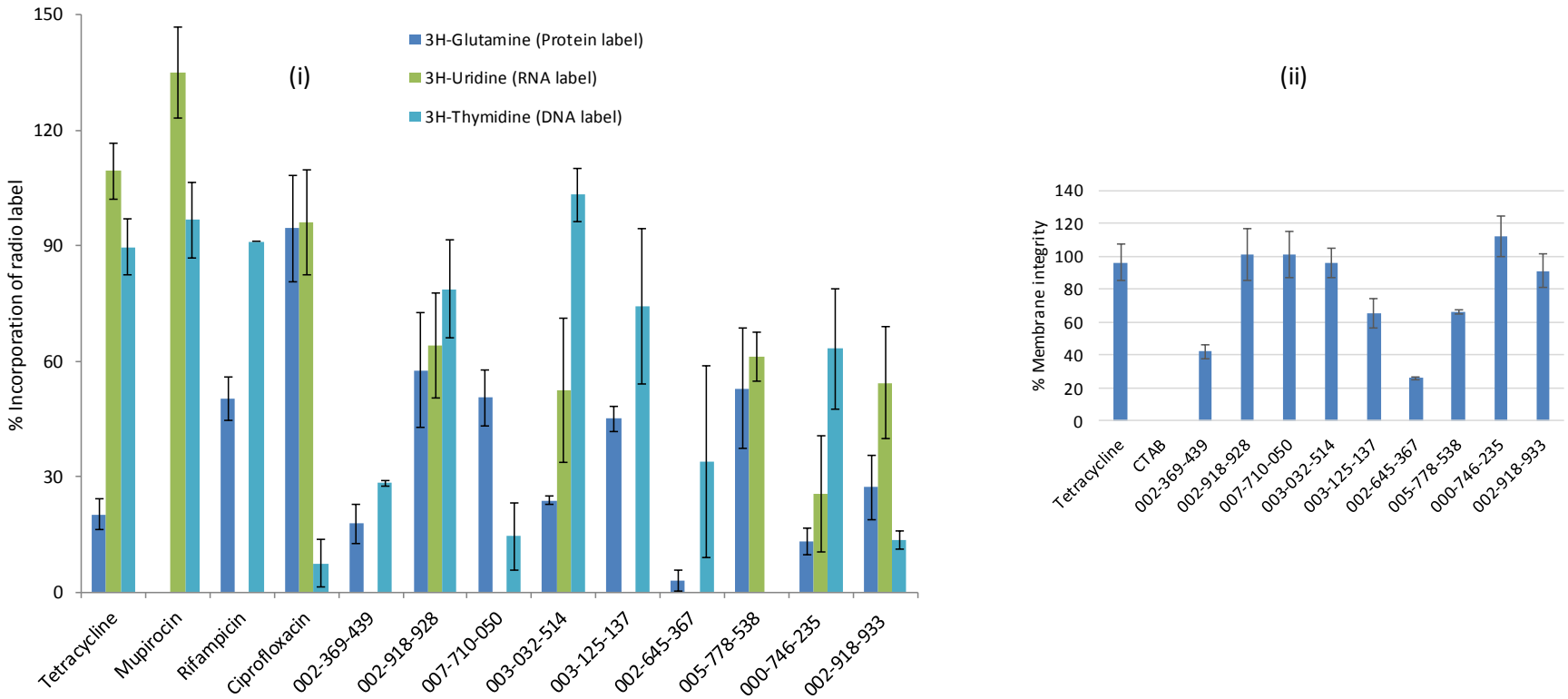

Figure 4.1: Effect of MolPort compounds and comparator agents on major macromolecular synthesis pathways and membrane integrity in SH1000. (i) The percent incorporation of ^{3}H-Glutamine, ^{3}H-Uridine and ^{3}H-Thymidine in protein, RNA and DNA synthesis have been assessed (mean of at least three independent replicates; error bars represent standard deviation); (ii) the percent integrity of staphylococcal membrane after 10 minute exposure to 4 X MIC of compounds (mean of three independent replicates; error bars represent standard deviation)

4.3.2. Investigating the activity of novel aminoacyl-adenylate analogues

As discussed in Chapter 1, analogues of aminoacyl adenylates have been shown to exhibit antibacterial activity. Traditionally, these analogues have been generated by the introduction of a stronger sulphamoyl linkage, but collaborators in NABARSI used different sub-groups as shown in Figures 4.2 and 4.3. The aminoacyl-adenylate intermediate can be divided into three subgroups comprising of an adenosine part, phosphonate group and the natural amino acid, respectively. Following the limited success of initial screening of compounds (4.3.2), rational design of aminoacyl-adenylate analogues using pharmacophore fingerprint virtual screening, where key binding residues of existing inhibitors with cognate target are used to generate a reference scaffold. This reference is then used for identifying and designing compound libraries. This approach led to the development of the IK580 series of compounds (Figure 4.2). Three of these analogues (Figure 4.3) exhibited good inhibiting activity against the purified *E. coli* LeuRS enzyme *in vitro*, but lacked antibacterial activity against *E.* coli BW25113.

It is often the case in screening studies that, compounds are discarded solely based on the agents' inability to penetrate the outer membrane of Gram-negative organism or because they are substrates of broad spectrum efflux systems like AcrAB-TolC. To rule out the possibility that the lack of antibacterial activity is a result of the presence of pumps, strains deficient in pump components were used to determine the activity of the candidate antibacterial agents. These strains represent ideal bacterial systems to determine activity of candidate antibacterial agents against *E. coli*, as the compromised efflux activity increases the penetrability and accessibility of a candidate antibacterial agent. When the antibacterial activity of the three compounds was assessed against BW25113 Δ*tolC,* BW25113 Δ*acrA* and BW25113 Δ*acrB,* only IK698 exhibited activity against the *tolC* deficient strain of BW25113 (MIC = 16 mg/L).

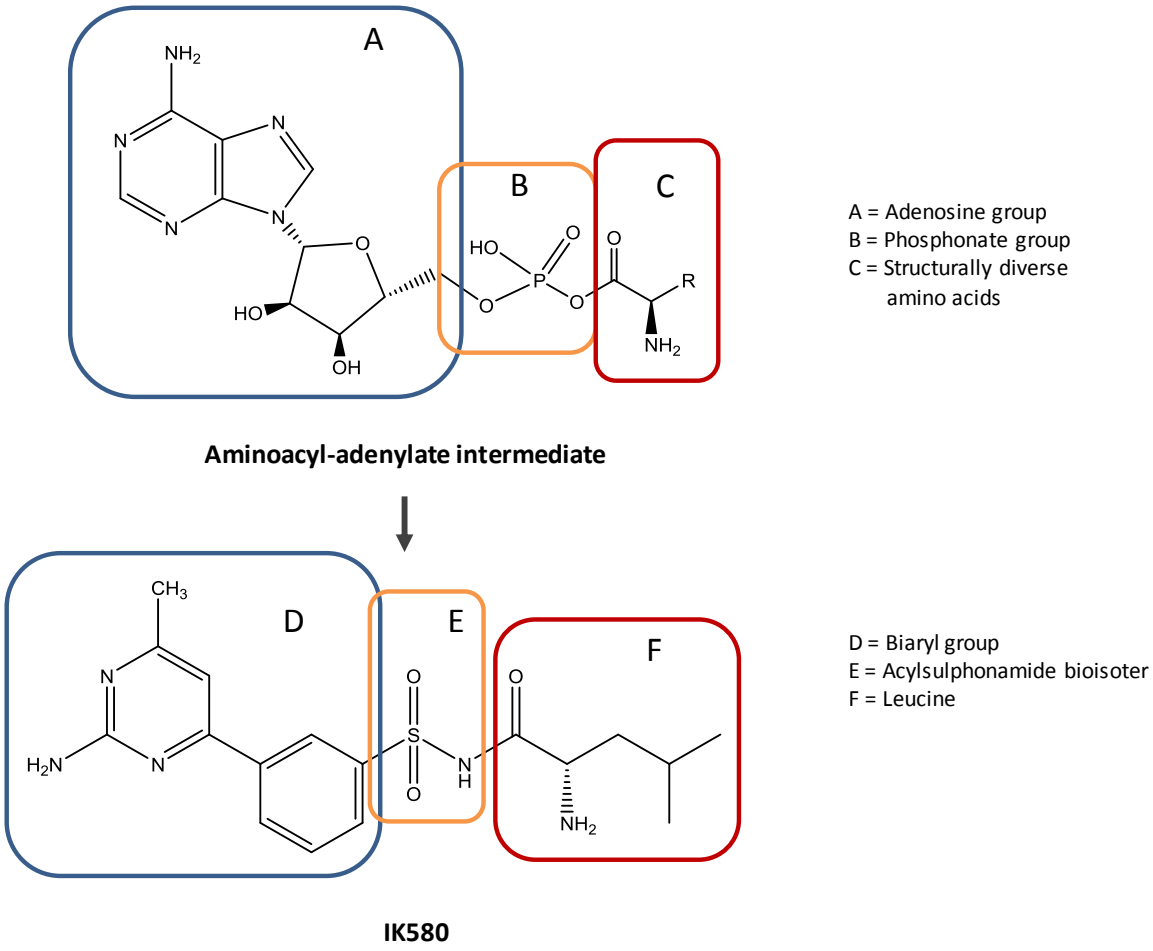

Figure 4.2: Rational design of aminoacyl-adenylate intermediate analogues by replacing the structural sub-groups A, B or C to D, E or F

This indicates that although IK698 exhibits antibacterial activity against *E. coli*, it represents a substrate for efflux involving TolC, but via a pump other than AcrAB. The other two compounds, IK580 and IK681, lacked activity against BW25113, BW25113 Δ*acrA* and BW25113 Δ*acrB* (Table 4.2).

Table 4.2: Antibacterial activity of IK compounds against BW25113 and Keio strains

	MIC (mg/L)			
Compound No.	**BW25113**	**BW25113 Δ*tolC***	**BW25113 Δ*acrA***	**BW25113 Δ*acrB***
IK 580	>128	>128	>128	>128
IK 681	>128	>128	128	128
IK698	>128	16	>128	>128

Determination of target specificity of a candidate antibacterial agent can be achieved by overexpressing its cognate bacterial target, as described in Chapter 3. To determine if IK698 exhibits antibacterial activity by inhibiting LeuRS, MICs were determined against BL21 Δ*tolC* (pCA24N:*leuS*), the strain overexpressing *E. coli leuS*. IPTG induced overexpression of *leuS* on pCA24N in BL21 Δ*tolC* led to an 8-fold reduction in susceptibility to IK698 (MIC = 128 mg/L), indicating that LeuRS is indeed the target of IK698.

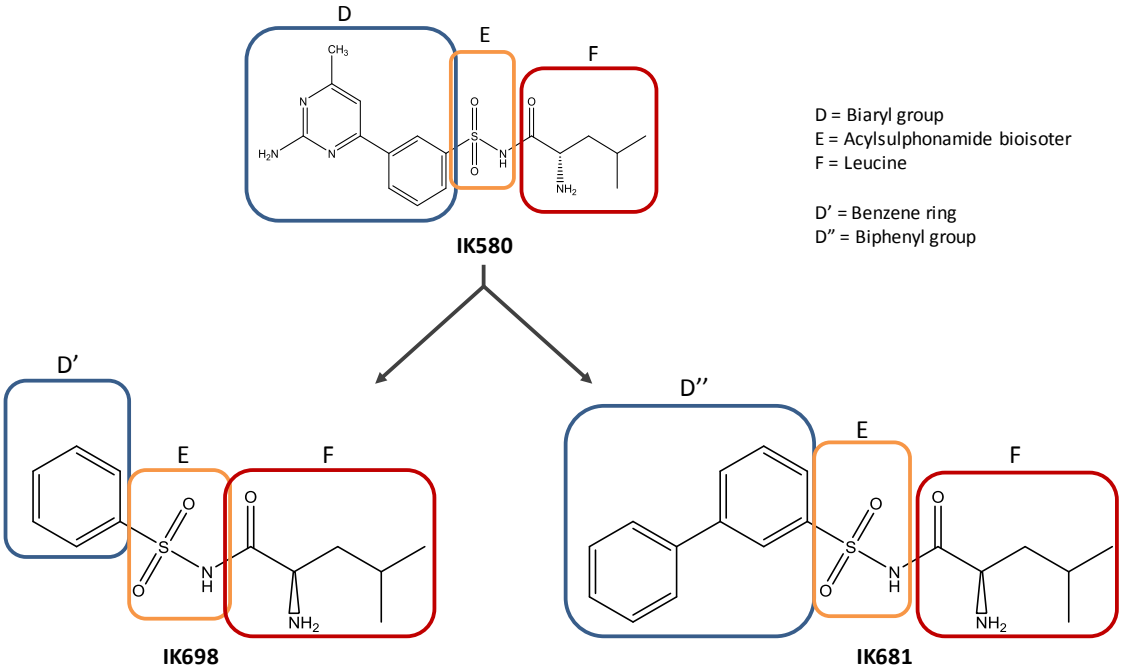

Figure 4.3: Structural derivatives of IK580, where only sub-group D has been substituted to D' and D" in case of IK698 and IK681 respectively.

Assessment of spontaneous resistance to candidate antibacterial agents is an important part of their pre-clinical evaluation, as it provides information regarding the potential for reduction in therapeutic effectiveness in the clinic. To investigate whether a mutation(s) in *leuS* leads to insusceptibility to IK698 in BW25113 Δ*tolC*, spontaneous mutants against the compound were selected at 4X MIC of IK698. The mutants exhibited a >32-fold reduction in

susceptibility to IK698 (MIC = >512 mg/L). The mutants were stable on passage *i.e.* the strains did not lose the mutation overtime.

The *leuS* gene from seven mutants was PCR amplified and subjected to DNA sequence determination. No nucleotide changes were identified upstream or within the coding sequence of the gene when compared with the sequence from wild-type BW25113. Although this was an unexpected observation, it has been found that resistance to candidate antibacterial agents can occur via mechanisms other than alteration in the target gene. For example, while investigating the basis of resistance to the experimental LeuRS inhibitor, GSK'052, O'Dwyer *et al.,* isolated *E. coli* strains where insusceptibility to the compound was not associated with mutations in *leuS*; instead the insusceptibility to GSK'052 in these strains was due to mutations which led to overexpression of pump proteins, thus facilitating efflux of the antibacterial agent (O'Dwyer *et al.,* 2015).

To establish the underlying mechanism of resistance to IK698, the entire genome from these mutants was subjected to DNA sequence determination using Illumina HiSeq (Leeds Institute of Molecular Medicine, Leeds, UK). Analysis of whole genome sequence data from these mutants showed a single mutation upstream of the *yhhY* locus ($G_{-38}T$), which encodes a putative acetyltransferase that shares 20.3% protein identity with the ribosome protein serine-acetyltransferase, RimL, which acetylates the N-terminal serine of ribosomal protein L7/L12. The presence of this mutation was confirmed by PCR and DNA sequence determination using Sanger sequencing (Sanger, 1977). It has previously been reported that RimL is associated with reduced susceptibility to the translation inhibitor, microcin C (McC) (Kazakov *et al.,* 2014). RimL mediates resistance to McC in *E. coli* by acetylating the primary amine group of processed McC-aspartate intermediate; it also confers resistance to aminoacyl sulfamoyl adenylates via the same mechanism (Kazakov *et al.,* 2014). The addition of an acetyl group at this position is likely to affect hydrogen bond formation, leading to

steric clashes with the enzyme backbone as seen in the case of MccE mediated resistance to microcin C (Agarwal *et al.,* 2011). The authors also showed that YhhY acetylates isoleucyl and leucyl sulfamoyl adenylates, suggesting an alternative mechanism of rescue from non-hydrolysable adenylates. Given that IK698 is a rationally designed derivative of the leucyl adenylate intermediate, it seems plausible that YhhY recognises the compound as a substrate for acetylation and hence leads to insusceptibility. To confirm that this mutation is indeed responsible for the observed insensitivity to IK698, the mutation ($G_{-38}T$) was engineered upstream of the *yhhY* locus of BW25113 Δ*tolC* using ssDNA mediated recombination (see 2.5.15). Introduction of this mutation resulted in a strain exhibiting the same degree of susceptibility to IK698 (MIC = >512 mg/L) as the spontaneous mutants generated previously.

Given the likely function of the protein encoded by *yhhY,* it can be speculated that the $G_{-38}T$ mutation leads to overexpression of *yhhY*, leading to a resistance phenotype. To determine if overexpression of *yhhY* by increasing gene dosage results in reduced susceptibility to IK698, *yhhY* from wild-type BW25113, including its ribosome binding site and promoter, was PCR-amplified and ligated into the promoter-less multi-copy plasmid, pIMAY, and introduced into BW25113 Δ*tolC.* The resultant strain, BW25113 Δ*tolC* (pIMAY:*yhhY*), exhibited the same degree of reduced susceptibility as the mutants and BW25113 Δ*tolC* ($yhhY_{G-38T}$). Together these results show that overexpression of *yhhY*, a putative acetyltransferase could be an alternative resistance mechanism to novel aaRS inhibitors. Thus, from these findings it can be concluded that IK698 is a specific inhibitor of *E. coli* LeuRS, resistance to which occurs via possible acetylation and not due to mutations in the target, *leuS*, which encodes LeuRS.

4.3.3. Assessing the antibacterial activity of IK580 and IK698 derivatives

As the first set of compounds exhibited non-specific activity or lacked penetrability or were substrates for efflux, concerted efforts were made to design further chemical derivatives of IK580 and IK698 series of compounds. Attempts to improve the activity of these two series led to the development of 44 candidate aaRS inhibitors. Due to dissolution of one of our collaborators, Omnia (Barcelona, Spain) towards the end of 2015, the primary screens for assessing the antibacterial activity of these inhibitors was completed in Leeds.

The antibacterial activity of the remaining 44 candidate inhibitors was tested against *E. coli* W0153 (TolC deficient) and *E. coli* W0159 (RfaC deficient) in the first instance. Strains W0153 and W0159 contain *asmB1*, an allele of *lpxC*, which leads to reduced lipopolysaccharide (LPS) synthesis. Deficiency of either TolC or RfaC in conjunction with the presence of *amB1,* results in compromised outer membrane biogenesis and efflux activity; this increases the likelihood of accessibility and penetrability of candidate antibacterial agents in *E. coli.* Forty of 44 analogues lacked antibacterial activity against these hyper-permeable *E. coli* strains (MIC = ≥128 mg/L). However, four compounds (LL20, LL49, DG539 and MZ411) exhibited some activity against W0153 and W0159 (MIC = 16 – 32 mg/L, Table 4.2). In addition, all four hits exhibited good binding affinity against purified *E. coli* LeuRS as determined by collaborators at LIOS (Riga, Latvia), suggesting that they should be specific for protein synthesis and LeuRS at a cellular level.

Table 4.3: Antibacterial activity of IK580 and IK698 analogues

	MIC (mg/L)			
Compound No	**W0153**	**W0159**	**BW25113**	**SH1000**
DG539	32	32	32	>128
LL20	16	16	16	>128
LL49	16	16	16	>128
MZ411	32	64	32	>128

While LL49 is a derivative of IK580, where the acylsulfonamide group of IK580 is substituted for a N-acylsulfinylamide; LL20, DG539 and MZ411 are meta-substituted derivatives of IK698 (Figure 4.4). The four compounds did not show a loss of antibacterial activity when MICs were determined against wild-type BW25113 (Table 4.3). The lack of change in activity against BW25113 suggests that these derivatives are not substrates for efflux pumps nor is their entry into the cell hindered by the presence of the Gram-negative outer membrane. However, the compounds lacked activity against *S. aureus* SH1000 (MIC = >128 mg/L, Table 4.3), suggesting that the four hits are specific for *E. coli*. Unfortunately, none of the compounds exhibited multi-target activity as determined by binding affinity against purified IleRS and LeuRS, which was one of the major goals for NABARSI. To assess the target specificity and selectivity of these four hits, further MoA studies were carried out, which are discussed in the subsequent sections in this chapter.

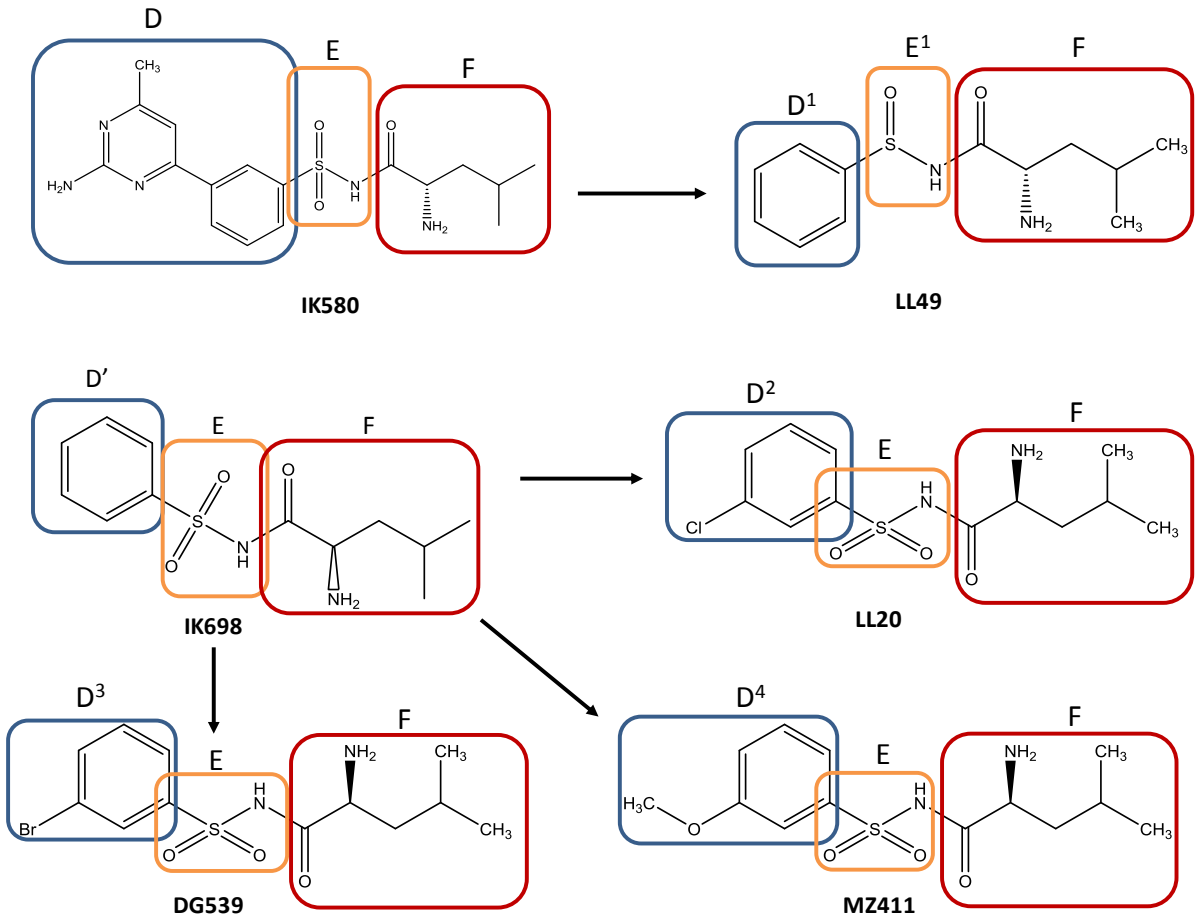

Figure 4.4: Structural analogues of IK580 and IK698. In LL49, the adenosine group (D) is substituted for a benzene ring and the acylsulphonamide group (E) is substituted with an N-acylsuphinyl amide (E^1). LL20, DG539 and MZ411 are meta-substituted benzene derivatives of IK698; where a Chlorine (Cl), Bromine (Br) and aldehyde has been added respectively.

4.3.4. Assessing target specificity of the IK580 and IK698 derivatives

To establish whether the four hits specifically inhibit protein biosynthesis in *E. coli*, incorporation of radiolabelled precursors into major cellular macromolecules was observed. A simplified MMS assay was used to determine target specificity of the four compounds. This was done due to time constraints and hence the inhibitory profile of the compounds was determined solely against protein and fatty acid synthesis pathways. At 4X MIC, LL20, DG539

and MZ411 specifically inhibited protein synthesis as did the known protein synthesis inhibitor, tetracycline, with no substantial or modest effect on fatty acid synthesis (Figure 4.5). The results from the MMS assay thus suggest that these compounds exert their antibacterial activity by inhibition of protein synthesis.

Establishing that these compounds inhibit protein synthesis, it was important to determine that the observed antibacterial activity was a direct result of inhibition of LeuRS. To investigate whether the antibacterial activity of the four identified hits was a result of inhibition of LeuRS, MICs were determined against the *E. coli* strain overexpressing LeuRS. As anticipated, overexpression of LeuRS resulted in ≥4-fold reduction in susceptibility to LL20, LL49, DG539 and MZ411 (MIC range = 64 mg/L – 128 mg/L) compared to BW25113 and BL21 (pCA24N), confirming that the hits exert their antibacterial activity specifically through the inhibition of *E. coli* LeuRS (Table 4.4).

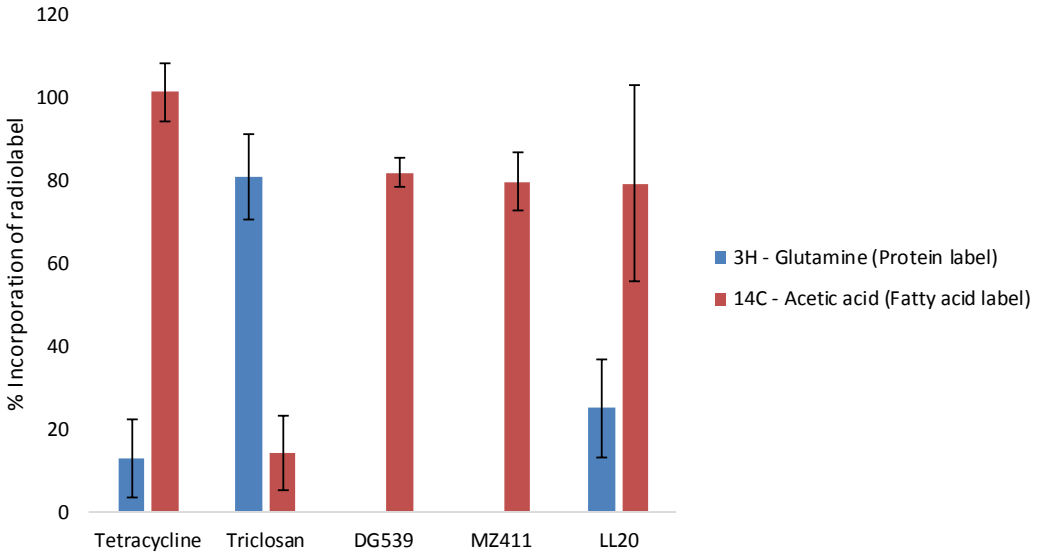

Figure 4.5: Effect of hits and comparator agents on macromolecular synthesis pathways in *E. coli*. Percentage incorporation of ^{3}H and ^{14}C into *E. coli* protein and fatty acid are shown (mean of at least three independent replicates; error bars represent standard deviation).

Table 4.4: Assessing the susceptibilities of strains overexpressing *leuS* to NCE's

	MIC (mg/L)	
Compound	**BL21 (pCA24N)**	**BL21 (pCA24N:*leuS*)**
LL20	16	64
LL49	16	128
DG539	16	128
MZ411	8	128

4.3.5. Assessment of membrane damage and bacterial specificity of LL20, LL49, DG539 and MZ411

Perturbation of the bacterial membrane is the most common reason for off-target activity. To rule out off-target activity as a result of membrane perturbation, the ability of the hits to cause membrane damage in *E. coli* was determined using the *Bac*Light™ assay. Exposure of BW25113 to 4X MIC of tetracycline, an established protein synthesis inhibitor, did not show any perturbation of the membrane as opposed to 4X MIC of CTAB, which is used as a positive control for membrane perturbation [Figure 4.6 (i)].

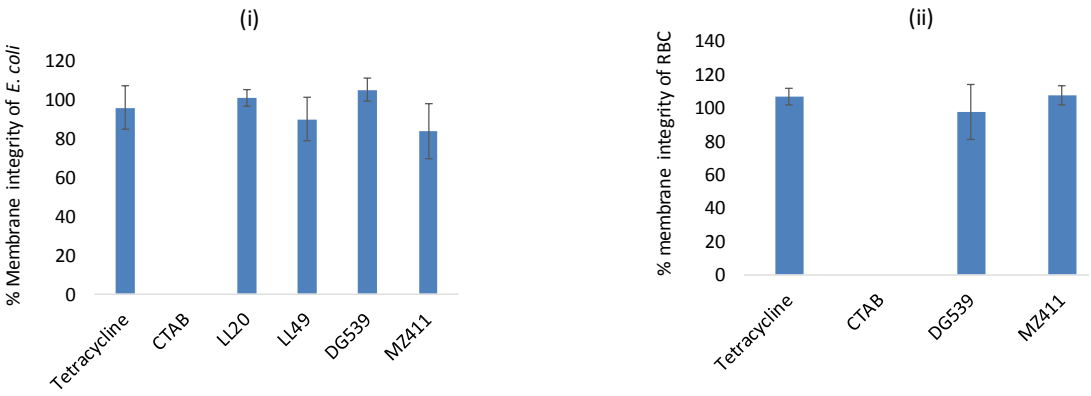

Figure 4.6: Assessing the effect of hits on prokaryotic and eukaryotic membranes at 4X MIC. (i) shows the effect of the hits on the *E. coli* membrane after 10 min; (ii) shows the effect of the hits on equine RBCs after 60 min (mean of at least three independent replicates; error bars represent standard deviation). **LL20 and LL49 could not be used in the haemolysis assay due to insufficient amounts.*

Compared to the controls, neither LL20, LL49, DG539 nor MZ411 showed significant perturbation of bacterial membrane integrity, implying that these compounds do not affect the integrity of bacterial membranes [Figure 4.6 (i)]. The lack of an adverse effect on the bacterial membrane is a crucial and desirable feature for novel antibacterial drug candidates, as identification of compounds perturbing the membrane is a common occurrence in antibacterial drug discovery programmes (Payne *et al.,* 2007, Silver 2011). The structural and functional similarities of prokaryotic and eukaryotic membranes imply that candidate antibacterial drugs exhibiting membrane damage are not suitable for development as antibiotics (Maher and McClean, 2006) and hence have no clinical significance.

MICs against *Candida* spp. were also determined to evaluate prokaryotic specificity. In addition to maintaining close to 100% membrane integrity, the hits had no activity against *Candida* spp. at the highest concentration tested (MIC = >128 mg/L), indicative of selective activity against bacteria. However, to further assess the selectivity of these compounds, the

haemolysis assay was employed to determine the affect they had on equine red blood cells (RBCs). Tetracycline, which does not damage the membrane, caused no haemolysis, whilst treatment of RBCs with CTAB resulted in haemolysis [Figure 4.6 (ii)]. An hour exposure of RBCs to 4X MIC of hits did not show any membrane damage compared to the negative control CTAB [Figure 4.6 (ii)]. Unfortunately, the effect of LL20 and LL49 on equine RBCs could not be tested due to unavailability of sufficient compound. The negligible effect on equine RBC integrity reinforces the idea that these compounds are bacteria-specific inhibitors.

4.3.6. Establishing the resistance liability and mechanism of resistance to LL20, LL49, DG539 and MZ411 in *E. coli*

To determine the ease with which resistant mutants can arise to these potential lead compounds, spontaneous mutants of *E. coli* resistant to the four hits were selected. Mutants arose at a frequency of (1-4) X 10^{-8}. This frequency is also observed in the case of reference aaRS inhibitors (mupirocin and GSK'052), where mutations in *ileS* and *leuS* confer resistance in staphylococci and *E. coli* respectively (Hurdle *et al.,* 2004, Hernandez *et al.,* 2013). A mutation frequency of 10^{-8} is also indicative of a single cellular target (Silver, 2011). The spontaneous mutants generated displayed a 4 to 8-fold decrease in susceptibility to the corresponding NCE compared with the susceptibility of BW25113 to the compounds (Table 4.5). The high frequency resistance to the 4 hits implies that they would be unsuitable for clinical use as mono-therapeutic agents. However, it has been suggested that high rates of mutational resistance to aaRS inhibitors could be overcome by the combination of two aaRS inhibitors (Randall *et al.,* 2016) or by using them at high concentrations to overcome mutation liability, as is the case for the topical agent, mupirocin (Cookson, 1998).

Table 4.5: Antibacterial susceptibilities of spontaneous mutants to the hits

	MIC (mg/L)				
Strains	**LL20**	**LL49**	**DG539**	**MZ411**	**GSK'052**
BW25113	16	4	32	32	.25
LL20 mutants	64	4	≥128	ND	≥2
LL49-1 mutants	≥64	16-32	≥128	ND	≥2
DG539 mutants	≥64	16	≥128	ND	≥2
MZ411 mutants	32	4	64	≥128	1-2

Although these compounds have a single target in LeuRS, combination of two aaRS inhibitors is likely to mitigate the high rate of spontaneous resistance. However, given all four compounds are derived from the same parental compound, there is the likelihood for the development of cross resistance between the compounds. To investigate if the four hits could be potential candidates for combination therapy, cross resistance studies were carried out. The mutants exhibited a certain degree of cross resistance between the four compounds (Table 4.5), indicating that they are not suitable for combination therapy. The mutants also showed reduced susceptibility to GSK'052 (MIC = 2 mg/L, Table 4.4) compared to BW25113 (MIC = 0.25 mg/L, Table 4.5), implying that there is potential for the development of cross resistance to other LeuRS inhibitors.

In an attempt to understand the molecular basis of resistance to the four hits, *leuS* from the generated mutants was subject to PCR amplification and subsequent sequence determination. Analysis of the data generated from DNA sequence determination of *leuS* from the respective mutants did not identify any polymorphisms which could be associated with the observed reduced susceptibility to the compounds. Although this is an unexpected result, it is not unusual as shown previously in section 4.3.3, where resistance to IK698 was mediated by up-regulation of a putative acetyltransferase, *yhhY.* Based on these previous findings and the origins of the hits, the sequence of the *yhhY* locus from the respective

mutants was determined. However, no mutations could be identified at this locus either, implying that YhhY is not associated with the reduced susceptibility of these mutants. To determine if RimL mediates resistance to the four hits, *rimL* from the mutants generated against LL20, LL49, DG539 and MZ411 was PCR amplified and subject to sequence determination. No mutation could be identified within the coding sequence or upstream of *rimL,* implying that the acetyltransferase, RimL, is also not responsible for resistance in the spontaneous mutants.

To elucidate the underlying reason for the insusceptibility of these mutants, genomic DNA was isolated and was subjected to sequence determination by Illumina HiSeq (Leeds Institute of Molecular Medicine, Leeds, UK) to identify any mutations; no mutations were identified upon analysis of WGS data. This could possibly be due to incomplete coverage or simply that the analysis of the reads is inappropriate. Although unexpected not to find a mutation in the genome, it could be possible that resistance in these mutants is lost over time when bacteria are grown without drug selection. A possible explanation for the inability to identify a mutation is, the change in the first instance results in a certain degree of fitness cost and hence is lost overtime, without selection. Whether this was true in the case of these mutants could have been checked by passaging the strains in non-selective media overtime and then determining its susceptibility to the compounds. However, owing to time and financial constraints this was not be investigated any further.

4.4. Conclusions

As a consortium, NABARSI set out to identify candidate aaRS inhibitors with multi-target activity against bacterial class I aaRS enzymes (IleRS, LeuRS and ValRS). The goal was to identify and synthesise a single compound with the ability to inhibit two of the mentioned aaRS enzymes. Although we were unable to achieve this, NABARSI did identify four hits with antibacterial activity. The four compounds, LL20, LL49, DG539 and MZ411, were specific for *E. coli* LeuRS and did not cause membrane perturbation of bacterial or equine red blood cells. In addition, these compounds inhibited protein synthesis and susceptibility to the compounds decreased upon over-expression of LeuRS, suggesting that these are target specific aaRS inhibitors. However, like other antibacterial agents with a single target, resistance to the compounds arose rapidly and observations in this chapter also suggest the possibility of cross-resistance between the hits. The liability of these compounds to resistance and cross-resistance will need to be considered when designing derivatives of the same to improve their antibacterial activity. Unfortunately, the basis of resistance to the compounds could not be linked to *leuS* or *yhhY;* neither could it be connected to mutations in any other loci on the *E. coli* chromosome, suggesting that these mutations could be transient and undergo reversion. To summarise, the four hits identified in this chapter, LL20, LL49, DG539 and MZ411 represent themselves as candidate compounds that can be used as scaffolds for modification and subsequent development of lead compounds with enhanced antibacterial activity. However, before proceeding to designing more potent derivatives, the basis of resistance to these four hits should be further explored to elucidate the underlying reason(s) for insusceptibility observed in *E. coli* mutants resistant to the compounds.

Chapter 5

Investigating the molecular basis of resistance to the aaRS inhibitors, mupirocin and GSK2251052 (GSK'052), in *Staphylococcus aureus*

Work presented in this chapter has been published in part in Gupta *et al.* (2016)

Gupta A, Monteferrante C, Rasina D, Leitis G, Randall CP, Tomlinoson JH, Jirgensens A, Goessens WHF, Hays JP and O'Neill AJ. 2016. A ploymorphsim in *leuS* confers reduced susceptibility to GSK2251052 in a clinical isolate of *Staphlyococcus aureus*. **Antimicrobial Agents and Chemotherpay, 60**:3219-3221

Chapter 5: Investigating the molecular basis of resistance to the aaRS inhibitors, mupirocin and GSK2251052 (GSK'052), in *Staphylococcus aureus*

5.1. Abstract

Resistance to the aaRS inhibitors, mupirocin and GSK2251052 (GSK'052) can result from mutations in the native *ileS* and *leuS* genes, respectively. Additionally, mupirocin resistance can result from horizontal acquisition of the *mupA* or *mupB* genes. Work described in this chapter investigates, a) the basis of mupirocin resistance in MupA and MupB and, b) resistance to GSK'052 in *S. aureus*. Expression of either *mupA* or *mupB* was able to sustain growth of an *ileS* conditional mutant in the absence of the inducer, IPTG, suggesting that they can support growth when expression of *ileS* is switched off. Allelic replacement of staphylococcal *ileS,* with either *mupA* or *mupB* resistance determinants, yielded the strains, SH1000 Δ*ileS*::*mupA* and SH1000 Δ*ileS*::*mupB,* which were not less fit than SH1000 (*W*=0.92 ± 0.03 and 0.98 ± 0.05, respectively). These results confirm that MupA and MupB are fully functional, mupirocin-insensitive aminoacyl tRNA-synthetases. Although the mupirocin insensitivity of MupA/B could not be linked to a single amino acid residue, the predicted structure of MupA/B suggested the presence of an extra domain not present in native IleRS. Truncation studies at the C-terminal end of the proteins suggest that the extra domain could be responsible for mupirocin insensitivity.

GSK'052 selects for resistant mutants in *S. aureus* at a frequency of 5.4×10^{-8}, and resistance was found to pre-exist in the clinic. The identified mutations mediate high-level resistance to GSK'052 and decrease the susceptibility to the compound by >32-fold. The observed insusceptibility of the clinical isolate, *S. aureus* 1372, was shown to be associated with an existing single amino acid polymorphism (P_{329}) in LeuRS, which does not directly interact with

the compound but is likely to cause conformational changes in LeuRS resulting in reduced susceptibility.

5.2. Introduction

Antibiotic resistance is a perennial concern as it reduces the efficacy of the clinical use of antibiotics. This chapter focusses on the two known areas of resistance to aaRS inhibitors, (a) mutations in the target gene and, (b) horizontal acquisition of resistance determinants such as *mupA* and *mupB*. Understanding the molecular basis of antibiotic resistance proteins is likely to provide useful information regarding key amino acid residues that are directly involved with resistance to cognate antibiotics. This in turn can serve as a platform to provide information and guidance in the development of novel candidate antibacterial agents or more effective derivatives of antibiotics currently used.

5.2.1. Resistance to mupirocin

Resistance to the anti-staphylococcal agent mupirocin can arise due to non-synonymous mutations in native staphylococcal *ileS* ($G_{1762}T$ or $G_{1891}T$), or by horizontal acquisition of the *mupA* or *mupB* determinants, leading to low-level (MIC: 8–256 mg/L) (Hurdle *et al.,* 2005, Vondenhoff *et al.,* 2011) and high-level resistance (MIC: >256 mg/L), respectively (Hodgson *et al.,* 1993, Gilbart *et al.,* 1993, Eltringham 1997). MupA and MupB share 30% and 25.4% identity, respectively, with native staphylococcal IleRS (Gilbart *et al.,* 1993, Seah *et al.,* 2012) and it has therefore been suspected that these are alternate mupirocin-insensitive IleRS enzymes.

Indeed there are examples where resistance proteins possessing a degree of sequence conservation compared with their native homologue are unable to replace the native protein. This phenomenon is seen in the cases of MecA (PBP2a), TetM and TetO. MecA and TetM or TetO confer resistance to β-lactams and tetracycline respectively, but are not functionally active forms of penicillin-binding protein 2 (PBP2) or elongation factor-G (EF-G),

i.e. they cannot replace its native counterpart in its entirety or without having an adverse effect on growth. PBP2 possesses both transpeptidase and transglycosylase activity, whereas MecA can only carry out transpeptidase activity. Both these enzymatic activities are essential functions for PBP2 and hence MecA is unable to replace PBP2 in its entirety and is not a functional alternative for PBP2. Similarly, there is evidence to show that TetM/O cannot replace native EF-G, which aids in translocation of tRNA and mRNA during protein systhesis. Despite sharing high-level similarity with the GTP-binding domain found in EF-G (Burdett 1991, Manavathu *et al.,* 1990), and possessing GTPase activity, the introduction of a plasmid expressing TetM is unable to rescue *Bacillus subtilis* and *E. coli* strains consisting of a temperature sensitive and mutated EF-G, respectively (Burdett 1991). In addition, chimeric studies of TetO and EF-G, show that the proteins are functionally divergent and each possess structural determinants specific for its function (Thakor *et al.,* 2008). These respective studies conclusively show that TetM/O are unable to replace native EF-G as they are unable to carry out translocation of tRNA and mRNA during protein synthesis.

However, in the case of MupA/B there is no direct demonstration *in vivo* to support the idea that these proteins can replace staphylococcal IleRS in its entirety. To date only fast protein liquid chromatography (FPLC) analysis of total cell extracts from mupirocin-susceptible (Mup^S) and mupirocin-resistant (Mup^R) strains, has provided evidence to support this assumption (Gilbart *et al.,* 1993). Chromatograms of cell extracts from the Mup^s strain showed a single IleRS activity peak, whereas two distinct activity peaks were observed in the case of extracts obtained from the Mup^R strain (Gilbart *et al.,* 1993). The IleRS activity of one peak was similar to what was seen in the Mup^S strain; the other fraction exhibited half the IleRS activity compared to the susceptible strain, suggestive of the presence of an additional IleRS (Gilbart *et al.,* 1993). However, there is still a lack of conclusive evidence confirming that MupA/B can completely replace IleRS function in *S. aureus*. This chapter investigates whether MupA/B are alternate, functional IleRS enzymes and can replace staphylococcal

IleRS in its entirety without having any deleterious effect on bacterial growth. Establishment of such a system will also provide a platform to better understand the underlying reason(s) for mupirocin-insensitivity in these proteins.

5.2.2. Resistance to GSK'052

The experimental broad spectrum inhibitor, GSK'052, exhibits antibacterial activity by disrupting the integrity of the editing function in the bacterial leucyl tRNA-synthetase enzyme (LeuRS). It was considered as a therapeutic agent for treatment of Gram-negative infections (Hernandez *et al.,* 2013). Despite possessing many of the pre-requisites of an effective antibacterial agent, such as target specificity, selectivity and lack of membrane damage, resistance in *E. coli* rapidly arises to this compound, as seen in Phase II clinical trials involving adults suffering from complicated urinary tract infections (O'Dwyer *et al.,* 2015), making it an unsuitable candidate for monotherapy of Gram-negative infections. This is perhaps unsurprising, as it has been shown that GSK'052 selects for resistant mutants in *E. coli* at a rate of 10^{-8} *in vitro* (Hernandez *et al.* 2013). GSK'052 also exhibits antibacterial activity against Gram-positive organisms (MIC range = 2–4 mg/L) (Hernandez *et al.,* 2013), but resistance to the compound has not been characterised in these organisms. Assuming that the mode of action would be the same in Gram-positive organisms, we would expect similar frequencies for generating spontaneous mutants against the compound. To investigate if GSK'052 has potential clinical application as an anti-staphylococcal agent, experiments were performed to assess the resistance liability of GSK'052 in *S. aureus in vitro* and determine if resistance to the compound is pre-existing in clinical isolates of *S. aureus*.

5.2.3. Aims of the work presented

The primary aims of the research presented in this chapter were to, (a) determine if MupA and MupB are functional aaRS enzymes that can entirely substitute for the native staphylococcal IleRS; (b) identify which residues/parts of these proteins are responsible for the functionality and insensitivity to mupirocin and, (c) assess the resistance liability of GSK'052 *in vitro* and in clinical strains of *S. aureus*.

5.3. Results and discussion

5.3.1. Effect of MupA and MupB on mupirocin susceptibility in SH1000

High-level resistance to mupirocin in *S. aureus* is associated with the expression of either *mupA* or *mupB* on a plasmid (Seah *et al.,* 2012). Initial studies thus sought to confirm that ectopic expression of either *mupA* or *mupB* in SH1000 conferred mupirocin resistance. Both *mupA* and *mupB* were ligated into the staphylococcal expression vector pSK5487 for constitutive expression from P_{qacR} and introduced into SH1000. The resultant strains, SH1000 (pSK5487:*mupA*) and SH1000 (pSK5487:*mupB*), exhibited reduced susceptibility to mupirocin (MIC = >64 mg/L) when compared with SH1000 (pSK5487) (MIC = 0.125 mg/L). These results corroborate literature reports that MupA and MupB confer resistance to mupirocin.

5.3.2. Assessing whether MupA and MupB are functional IleRS enzymes

To establish whether MupA and MupB can take over the function of native staphylococcal IleRS, the constructs pSK5487:*mupA* and pSK5487:*mupB* were introduced into SH1000 (↓*ileS*) (the *ileS* conditional mutant) to assess if MupA and MupB could rescue growth in the absence of IleRS. The *ileS* conditional mutant is dependent on IPTG for growth (as shown in Chapter 3). Therefore, in the absence of the inducer, only expression *in trans* of a functional version of the synthetase would be able to restore growth. The expression of either *mupA* or *mupB* in this strain restored growth in the absence of IPTG compared with SH1000 (↓*ileS*) carrying the empty vector, pSK5487 (Figure 5.1).

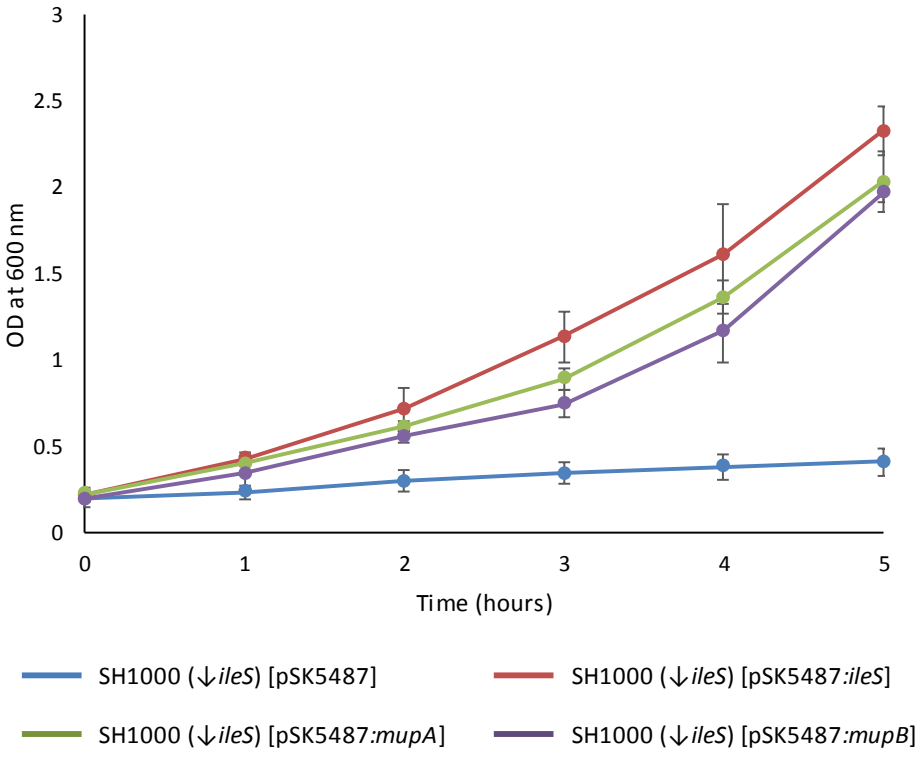

Figure 5.1: Ability of MupA and MupB to restore growth of *ileS* conditional mutant in the absence of inducer, IPTG. Growth was monitored over a course of 5 h, with OD_{600} readings being recorded every hour during the time period. Graph is a result of triplicate experiments where error bars represent SD

The rescue of growth by MupA and MupB in SH1000 (↓*ileS*) was comparable to that seen for SH1000 (↓*ileS*) harbouring pSK5487:*ileS*. The ability of MupA and MupB to restore growth in the absence of staphylococcal IleRS in SH1000 (↓*ileS*) implies that the two mupirocin-insensitive proteins are functional and alternate IleRS enzymes.

To exclude the possibility of rescue because of residual expression of *ileS* and confirm that *mupA* and *mupB* can indeed support growth of *S. aureus* in the absence of native *ileS,* the allelic exchange vector, pIMAY (Monk *et al.,* 2012), was used to replace the staphylococcal *ileS* in SH1000 in its entirety with either *mupA* or *mupB.* Replacement of native staphylococcal *ileS* resulted in two viable strains, SH1000 Δ*ileS*::*mupA* and SH1000 Δ*ileS*::*mupB.* The effect of these exchanges was assessed by determining mupirocin MIC and pair-wise competition against SH1000 to establish relative fitness cost (*W*). Figure 5.1 shows that SH1000 (↓*ileS*) [pSK5487:*mupA*] and SH1000 (↓*ileS*) [pSK5487:*mupB*] grow marginally slower compared with SH1000 (↓*ileS*) [pSK5487:*ileS*]. Thus, relative fitness cost was determined to assess whether swapping native *ileS* with either *mupA* or *mupB* had any deleterious effect on growth/fitness compared with wild-type SH1000. The resultant strains*,* SH1000 Δ*ileS*::*mupA* and SH1000 Δ*ileS*::*mupB,* showed reduced susceptibility to mupirocin (MIC = >64 mg/L) compared with SH1000 (MIC = 0.125 mg/L). As such, *t*he complete replacement of *ileS* with either *mupA* or *mupB* had a modest or no effect on the fitness of the strains compared with wild type SH1000 *(W*=0.92 ± 0.03 and 0.98 ± 0.05, respectively). These findings confirm that MupA and MupB are functional, mupirocin-insensitive IleRS enzymes that can completely substitute for the essential staphylococcal IleRS.

5.3.3. Investigating the basis of mupirocin resistance in MupA and MupB

5.3.3.1. Exploring the potential role of the amino acid residue at position 631 in mupirocin-susceptibility in MupA and MupB

Given that both MupA and MupB are functional, mupirocin-insensitive IleRS enzymes, the next objective was to determine the basis of mupirocin insusceptibility observed in MupA and MupB. It has been shown previously that spontaneous mupirocin-resistant mutants of *S. aureus* harbour point mutations in *ileS* encoding the amino acid substitutions, $V_{588}F$ and $V_{631}F$ (Hurdle *et al.,* 2004). It can therefore be speculated that if natural allelic variants exist at the same amino acid residues in MupA/B, relative to native IleRS, replacement of the same should result in a mupirocin susceptible enzyme. To determine if amino acid residues at these positions were different in MupA and MupB compared with IleRS, the sequences of all three proteins were aligned using Clustal Omega (Figure 5.2).

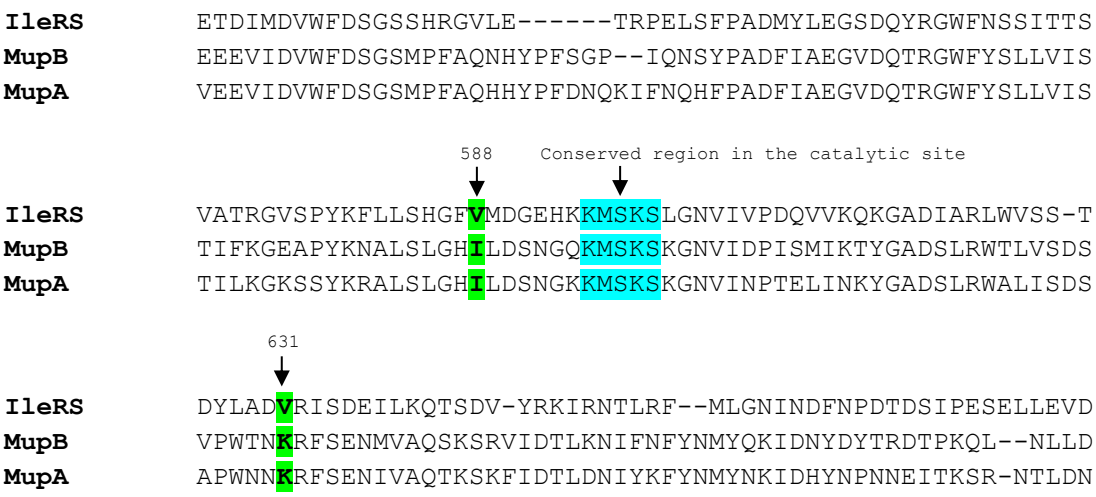

Figure 5.2: Part of protein sequence alignment of staphylococcal IleRS, MupA and MupB, showing residues at position 588, 631 and the conserved KMSKS region in the catalytic site. Amino acid residues at positions 588 and 631 are highlighted in green; the KMSKS region is highlighted in blue. Alignment was generated using Clustal Omega.

Sequence alignment of MupA/B with IleRS showed that residues at positions 588 and 631 in MupA and MupB are occupied by an isoleucine and lysine, respectively. Residue I_{588} was not considered to be responsible for mupirocin-insensitivity in MupA and MupB as isoleucine and valine are similar, in both structure and overall charge. Hence, it was assumed that substituting an isoleucine for a valine residue in either MupA or MupB would not have a significant impact on the mupirocin sensitivity of the enzymes. However, given that amino acid substitutions at position 631 in staphylococcal IleRS also confer resistance to mupirocin in clinical isolates of *S. aureus*, residue K_{631} was chosen as the most likely residue associated with mupirocin-insensitivity in MupA and MupB. Although not like phenylalanine but considerably different to valine, it was hypothesized that lysine, a positively charged amino acid, different from valine in structure and charge, could be responsible for resistance to mupirocin in MupA and MupB.

To test if K_{631} in MupA and MupB is responsible for reduced mupirocin susceptibility, the substitutions $AAA_{1864\text{-}1866}GTA$ ($K_{631}V$) and $AAA_{1870\text{-}1872}GTA$ ($K_{631}V$) were engineered into pSK5487:*mupA* and pSK5487:*mupB,* respectively, using site-directed mutagenesis. If K_{631} is indeed associated with mupirocin-insensitivity in MupA and MupB, substitution of the lysine residue with valine should result in a mupirocin-sensitive enzyme. To test if this engineered substitution leads to a loss of mupirocin resistance in MupA/B, the constructs were introduced into SH1000 and mupirocin susceptibilities was determined. The resultant strains SH1000 (pSK5487:$mupA_{AAA1864\text{-}66GTA}$) and SH1000 (pSK5487:$mupB_{AAA1870\text{-}72GTA}$), exhibited the same degree of mupirocin-susceptibility compared with SH1000 (pSK5487:*ileS*) (MIC = 0.125 mg/L). The mupirocin susceptibility of these strains suggest that the introduction of $K_{631}V$ in either MupA or MupB leads to a loss of mupirocin insensitivity. However, an alternate explanation for the loss of mupirocin insensitivity could be the result of a non-functional protein due to the amino acid substitution at position 631 in MupA/B. To confirm that $MupA_{K631V}$ and $MupB_{K631V}$ retain aaRS functionality, the pSK5487 constructs were introduced

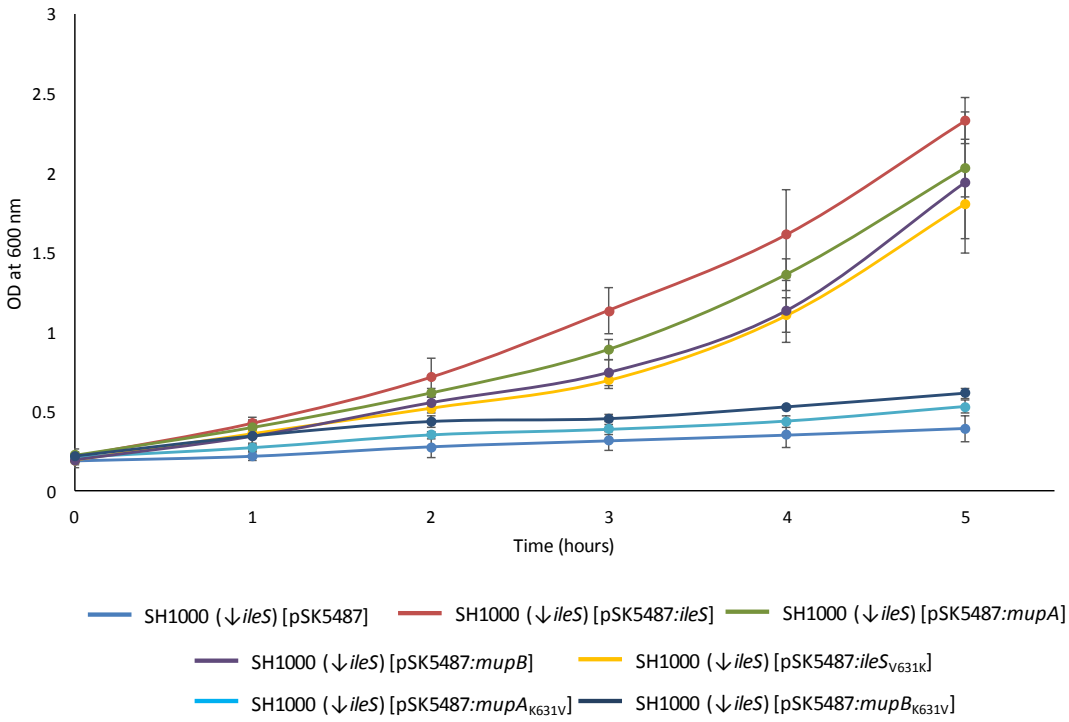

Figure 5.3: Restoration of growth of SH1000 (↓*ileS*) in the absence of IPTG by native IleRS, MupA, MupB and their mutagenized counterparts over a five-hour period. Growth was monitored every hour at 600 nm. Data is based on triplicates, where error bars represent SD.

into SH1000 (↓*ileS*). Both, MupA$_{K631V}$ and MupB$_{K631V}$ were unable to restore growth of SH1000 (↓*ileS*) when compared with the same strain harbouring native MupA and MupB, implying that replacement of lysine at position 631 affects the synthetase activity of MupA and MupB. The loss of functionality could be a result of mis-folding due to replacement of lysine, leading to disruption of aminoacylation activity. To determine whether the reciprocal change in *ileS* had a similar effect, the substitution GTT$_{1891\text{-}1893}$AAG (V$_{631}$K) was engineered into pSK5487:*ileS* using site-directed mutagenesis, and introduced into SH1000 and SH1000 (↓*ileS*) respectively. Introduction of the substitution, V$_{631}$K, leads to a functional IleRS as it is able to restore growth of SH1000 (↓*ileS*) in the absence of IPTG, at a level comparable to wild-type staphylococcal IleRS (Figure 5.3). Although IleRS$_{V631K}$ retains activity, there was no change in mupirocin susceptibility in SH1000 (pSK5487:*ileS*$_{GTT1891\text{-}93AAG}$) (MIC = 0.125 mg/L), implying that K$_{631}$ is not linked to mupirocin resistance.

5.3.3.2. Investigating the role of the C-terminal domain in mupirocin-insensitivity in MupA and MupB

The relative lengths of *mupA* (3075 bp) and *mupB* (3102 bp) compared to native staphylococcal *ileS* (2754 bp) suggest that the resultant proteins will be larger than IleRS (918 amino acid residues). Alignment of the three proteins, IleRS, MupA and MupB, show that both MupA and MupB possess extra amino acids and are larger than IleRS by 107 and 146 amino acid residues, respectively. Based on the protein sizes it can be speculated that these additional residues form part of an existing domain or a distinct domain. For ease of explanation, the additional amino acid residues will be referred to as an extra domain in the following sections of the chapter. To determine if these additional residues form an extra domain in MupA/B, a predicted structure of MupA/B was generated by I-TASSER (Zhang Lab, University of Michigan, USA). Alignment of the predicted structure of MupA/B with staphylococcal IleRS using PyMOL suggest that these extra residues form an extra domain (Figure 5.4). The presence of this speculative additional domain in MupA/B may participate in mupirocin resistance.

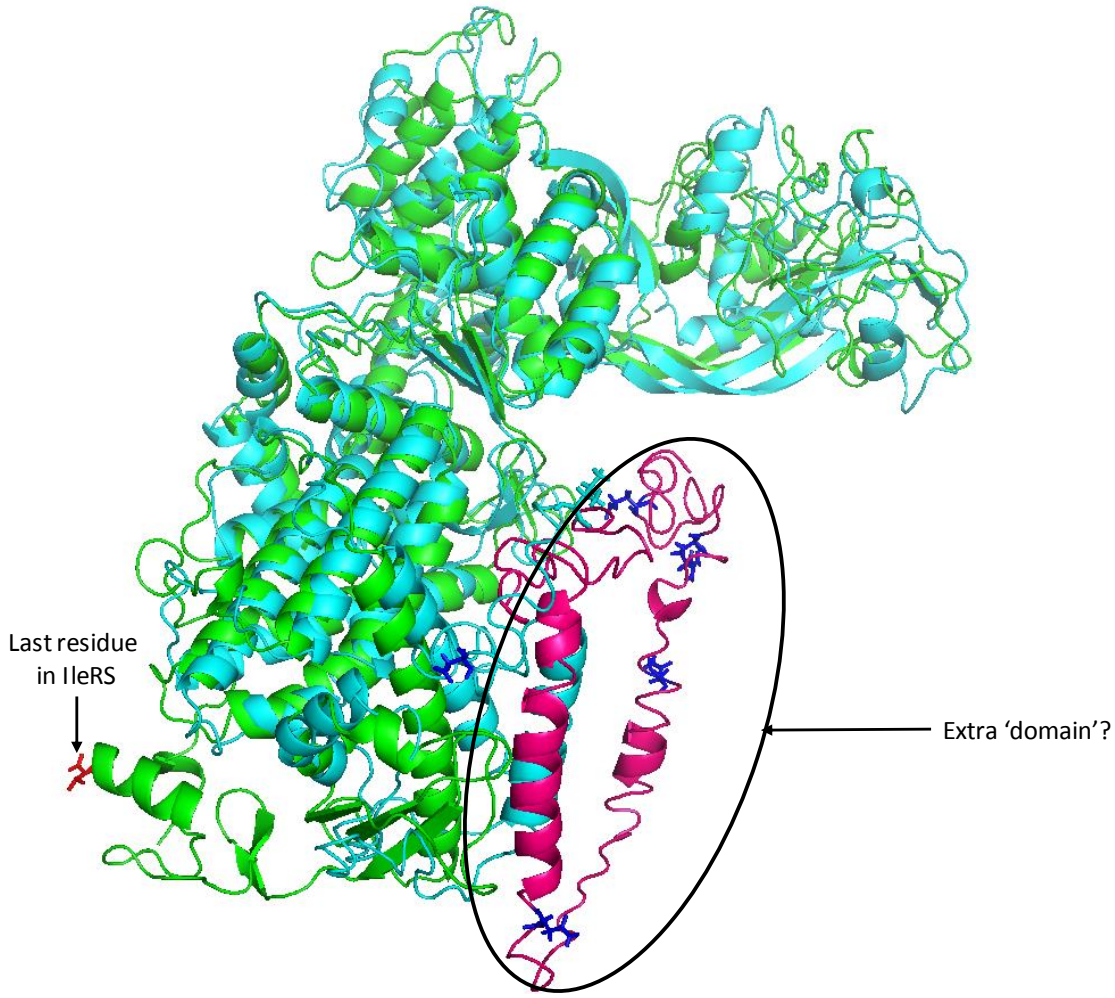

Figure 5.4: Comparison of the structure of IleRS (green, PDB ID: 1FFY) and the predicted structure of MupB (cyan and pink). The pink region of the predicted structure represents the putative extra 'domain' in MupB.

To determine whether this extra domain is involved in mupirocin insensitivity in MupA and MupB, the additional sequence in these two proteins was fused to the C-terminus of staphylococcal IleRS, yielding the chimeric proteins, IleRS_A1 and IleRS_B1 (Figure 5.5).

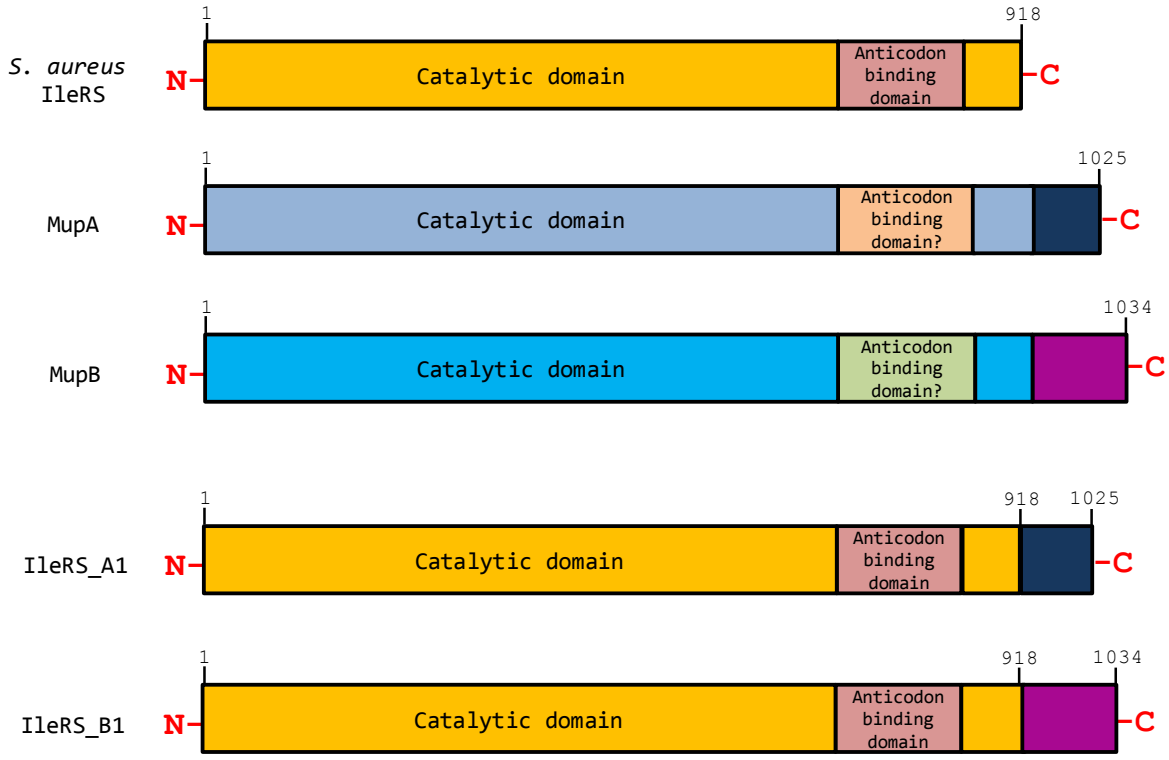

Figure 5.5: IleRS_MupA/B chimeric proteins. Additional residues at the C-terminal are highlighted in dark blue and pink for MupA and MupB respectively.

The chimeras were generated by subjecting pSK5487:*ileS* to inverse PCR at the stop codon of *ileS,* followed by ligation of the additional C-terminal coding region of either MupA or MupB. The modified synthetase genes encoding chimeric proteins IleRS_A1 and IleRS_B1, were individually expressed in SH1000 from the P_{qacR} promoter on pSK5487. Cells expressing either chimera were sensitive to mupirocin (MIC of 0.125 mg/L). This was in contrast to cells expressing intact MupA or MupB (MIC = >64 mg/L) but similar to those expressing IleRS (MIC = 0.125 mg/L). A potential problem in generating these chimeras is that fusion of IleRS with MupA/B could possibly lead to the disruption of domains within the protein. The lack of a shift in mupirocin MIC for SH1000 harbouring the chimeras suggest that fusing IleRS and MupA/B results in a non-functional synthetase or a functional synthetase lacking the mupirocin resistance phenotype or the expression of these chimeric proteins are toxic in SH1000. To determine whether the chimeric proteins retain functionality of the wild type

synthetase, they were introduced in SH1000 (↓*ileS*) and the ability to restore growth in the absence of IPTG was monitored (Figure 5.6). The chimeric proteins were unable to restore growth of SH1000 (↓*ileS*), implying that the chimeras generated lacked catalytic activity.

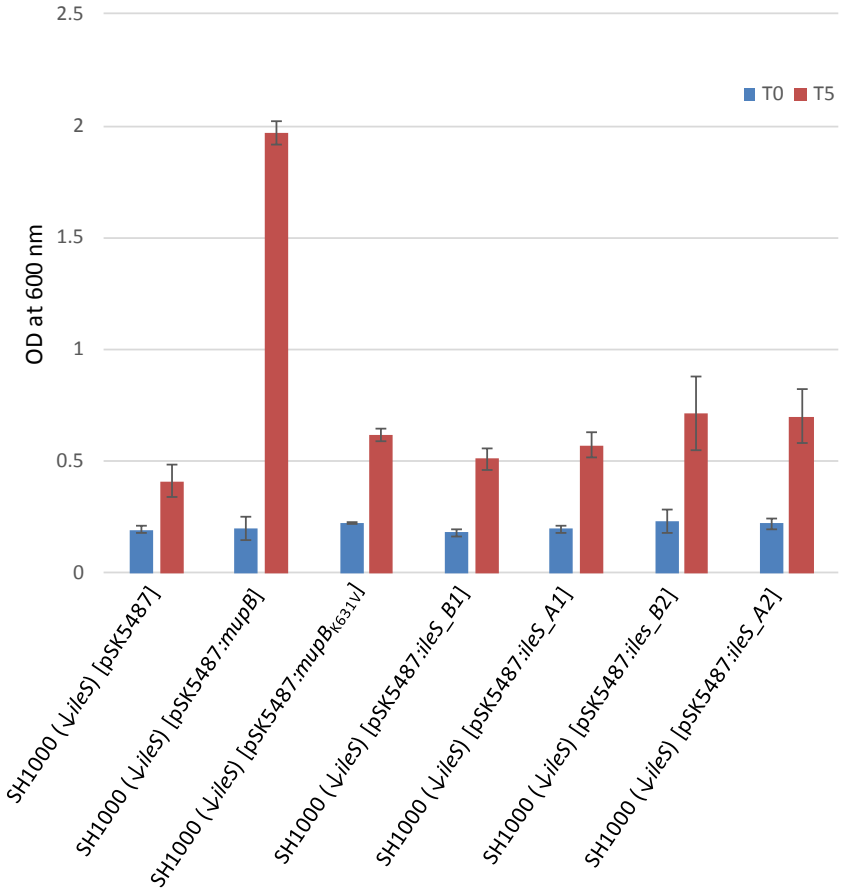

Figure 5.6: Ability of the IleRS_MupA and IleRS_MupB chimeras to restore growth in the SH1000 (↓*ileS*) background, in the absence of IPTG, compared to native MupB, MupA, mutagenized MupB, mutagenized MupA and the empty vector. Cultures were grown for five hours and OD_{600} was recorded at time 0 (T0) and the 5th hour (T5). Results are represented as the mean of triplicate experiments where error bars correspond to SD.

It is likely that the resultant chimeric proteins lead to disruption of the structure and function of native IleRS. To try and avoid this, I decided to generate IleRS_A/B chimeras around the KMSKS motif, which is conserved in all three proteins as shown in Figure 5.2. This region shares the highest degree of identity, suggesting that fusion of MupA/B with IleRS might

provide better recognition of amino acid residues, in turn promoting normal protein folding. The chimeric proteins were generated using the front end of IleRS (up to the KMSKS region) and MupA/B (starting from the KMSKS region up to the stop codon), as shown in Figure 5.7.

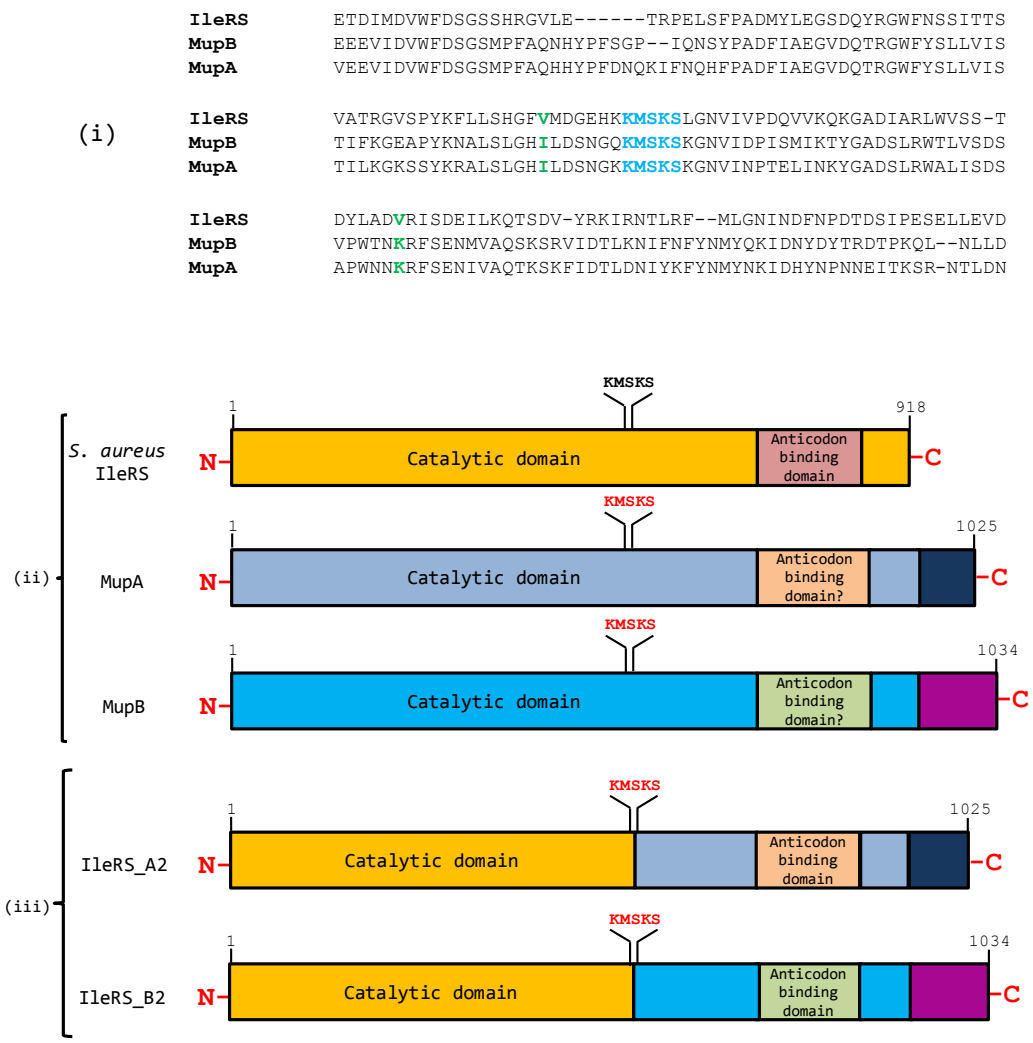

Figure 5.7: IleRS_MupA/B chimeric proteins by fusion around the KMSKS motif. (i) shows the alignment of the three proteins around the KMSKS motif; (ii) shows the schematic of staphylococcal IleRS, MupA and MupB. The KMSKS regions in MupA and MupB are highlighted in red; (iii) shows the IleRS_MupA/B chimeric proteins.

The resultant chimeras were unable to provide resistance to mupirocin and restore growth in the absence of the inducer, IPTG, in SH1000 and SH1000 (↓*ileS*), respectively. The inability of the chimeras to restore growth of the *ileS* conditional mutant (Figure 5.6) suggest that fusion of MupA/B (from the KMSKS motif up to the stop codon) to IleRS possibly results in disruption of the catalytic site or mis-folding, therefore yielding an inactive protein. However, the loss of synthetase activity and lack of mupirocin-resistance, resulting from the possible mis-folding of the chimera's remains speculative, as no structural data is available for either MupA or MupB. To better understand the role of the extra 'domain' in MupA and MupB, truncations were made within the region to generate smaller versions of the protein. The effect of these truncations on functionality and mupirocin-insensitivity of MupA and MupB have been discussed in the next section.

5.3.3.3. Investigating the effect of truncations at the C-terminal end of MupA and MupB

To determine whether the extra amino acids present in MupA and MupB play a role in their function and mupirocin-insensitivity, truncations of the putative resistance domain were generated and assessed. Inverse PCR was used to generate five *mupB* fragments, truncated from the C-terminal end in the construct pSK5487:*mupB* (Figure 5.8). The constructs were introduced in SH1000 by electroporation to establish mupirocin susceptibility. All five truncated versions resulted in a loss of mupirocin resistance, showing mupirocin susceptibility comparable to wild-type SH1000 (MIC = 0.125 mg/L). This suggests that the additional amino acid residues do play a role in mupirocin resistance in MupB. However, the other possibility is that the truncations result in a non-functional protein and hence the reduced susceptibility to mupirocin. To establish whether all five truncations retain functionality, the constructs were introduced in SH1000 (↓*ileS*) by electroporation and the ability to restore growth of the strain in the absence of the inducer (IPTG) was determined.

Only one truncation of MupB was able to partially restore growth. Figure 5.9 (ii) shows that MupB with a size of 968 amino acids was the only truncation which resulted in restoration of growth in the absence of IPTG in the SH1000 (↓*ileS*) background. Although, restoration was not equivalent to that observed upon expression of native MupB, this truncation still achieved considerable restoration of growth compared with SH1000 (↓*ileS*) expressing

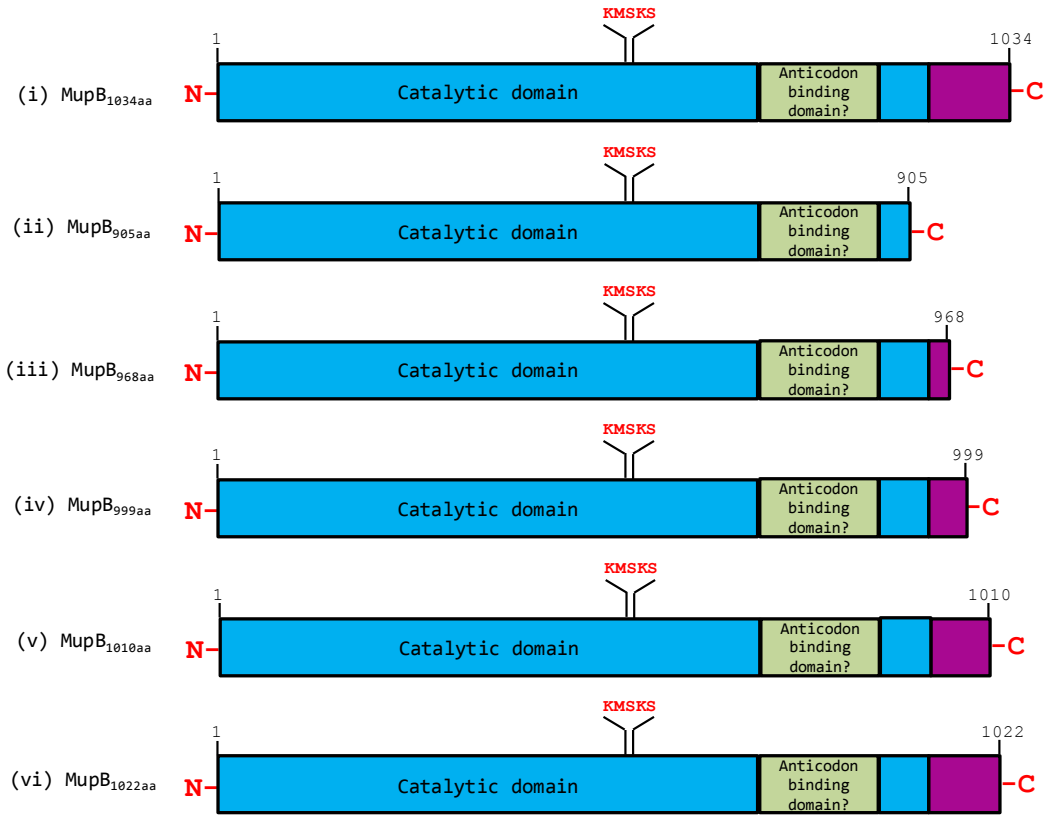

Figure 5.8: Schematic of MupB fragments, truncated from the C-terminal end using inverse PCR. (i) represents native MupB; (ii) – (vi) represent MupB fragments of different lengths

$MupB_{K631V}$ or the other four truncations or the empty vector. However, when a similar truncation was made at the same residue resulting in MupA with 963 amino acids, it did not lead to restoration of growth (Figure 5.10). This suggests that although MupA and MupB are similar, there are structural differences between them.

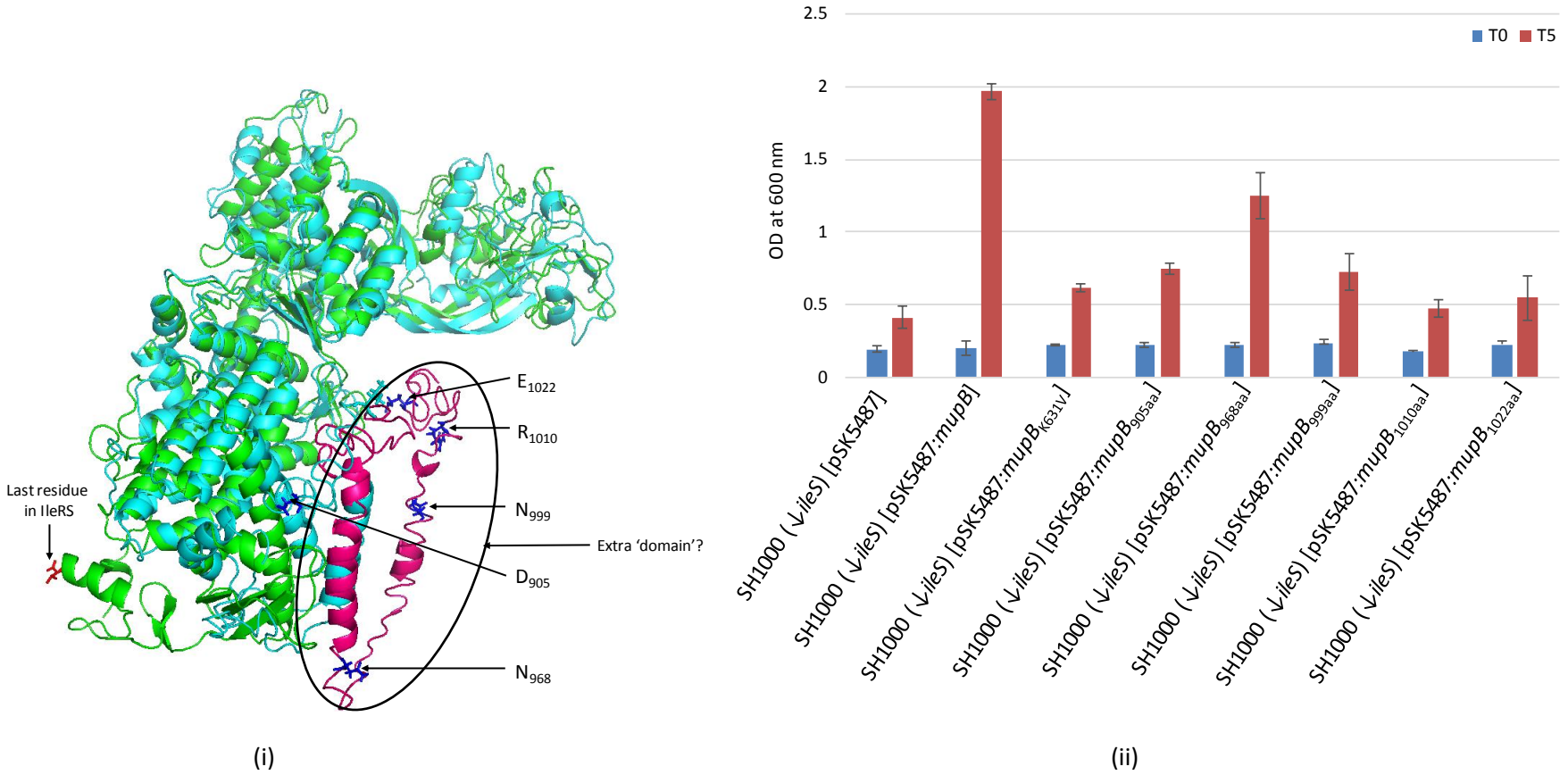

Figure 5.9: Ability of truncated versions of MupB to restore growth in the SH1000 (↓*ileS*) background, in the absence of IPTG, compared with native MupB, mutagenized MupB and the empty vector. (i) shows the residues at which truncations were made; (ii) shows restoration of growth of SH1000 (↓*ileS*) expressing the different pSK5487 constructs. Cultures were grown for five hours and OD_{600} was recorded at time 0 (T0) and 5 h (T5). Error bars represent SD based on triplicate experiments.

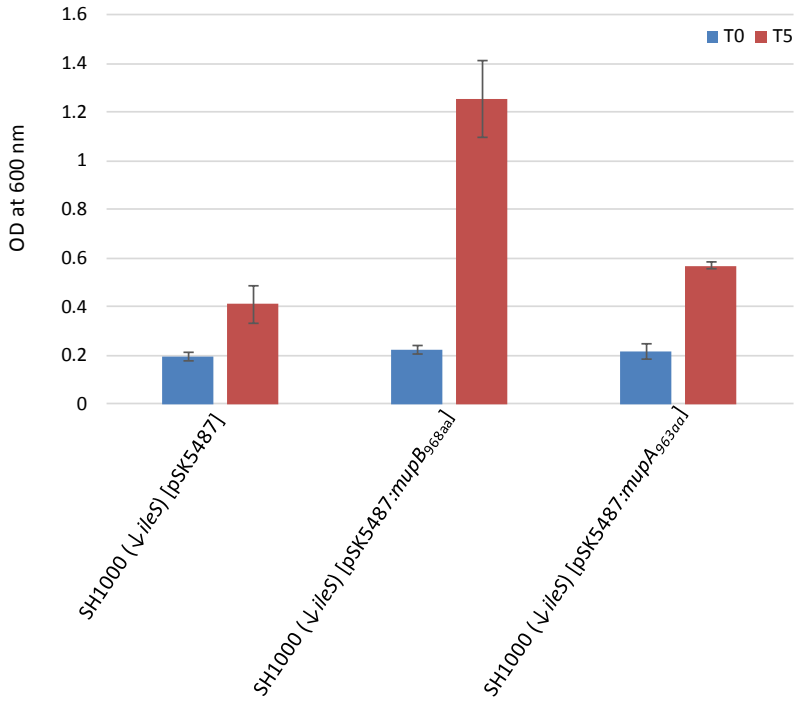

Figure 5.10: Ability of MupA comprised of 963 amino acids to restore growth in the SH1000 (↓*ileS*) background, in the absence of IPTG, compared to native MupB comprised of 968 amino acids, and the empty vector. Cultures were grown for five hours and OD_{600} was recorded at time 0 (T0) and the 5th hour (T5). Error bars represent SD based on triplicate data.

The truncation of MupB to generate a partially functional protein when comprised of 968 amino acids instead of 1034 amino acids suggests that the extra domain could possibly be responsible for the functionality and mupirocin-insensitivity observed in these proteins. However, this approach requires further investigation and attempts are being made to solve the structure of these two mupirocin-insensitive proteins, which is likely to provide better insight on the role of the extra domain in MupA and MupB. If the molecular basis and structural mechanics of mupirocin resistance in MupA and MupB can be understood, it could pave the way for the development of more effective mupirocin derivatives, which are able to overcome the structural differences between MupA/B and staphylococcal IleRS.

5.3.4. Investigating the propensity for GSK'052 resistance to arise in staphylococci

5.3.4.1. Generating and characterising spontaneous mutants of S. aureus resistant to GSK'052

GSK'052 was developed for treatment of Gram-negative infections; unfortunately the compound rapidly selects for resistance in *E. coli*, thus making it unsuitable for monotherapy (Hernandez *et al.,* 2013). The liability of GSK'052 to rapidly select for resistance is a common feature of single target inhibitors and similar frequencies of resistance development is observed in the case of the anti-staphylococcal agent, mupirocin (Hurdle *et al.,* 2004a). However, in addition to being active against Gram-negative organisms, GSK'052 also exhibits activity against staphylococci (Hernandez *et al.,* 2013), implying that it could be considered as a potential anti-staphylococcal agent. Given that GSK'052 has the same target (LeuRS) in staphylococci, it is likely that resistance can develop at the same frequency as observed in *E. coli.* Hence, before it can be considered for treatment of staphylococcal infections, it is vital to determine the frequency of resistance development in staphylococci and the impact this might have on the antibacterial activity of GSK'052.

To assess the resistance liability of GSK'052 in staphylococci, mutation frequency of SH1000 to the compound *in vitro* was determined. GSK'052 exhibited an MIC of 4 mg/L against SH1000, and spontaneous mutants resistant to GSK'052 were selected at a frequency of 5.4 x 10^{-8}, a rate comparable to that previously observed in *E. coli* (MF = 7.5 x 10^{-8}) (Hernandez *et al.,* 2013). All 25 spontaneous mutants tested exhibited high-level resistance to GSK'052 (MIC = >128 mg/L). A similar fold increase (>32-fold) in MIC is seen in *E. coli* resistant mutants (O'Dwyer *et al.,* 2015). Sequence determination of *leuS* revealed that all the amino acid substitutions identified reside within a region of LeuRS that has also been associated with resistance in the case of *E. coli* mutants (Table 5.1 and Figure 5.11) (O'Dwyer *et al.,* 2015). These results indicate that GSK'052 would not be a suitable mono-therapeutic agent for the

treatment of staphylococcal infections. However, if approved as an antibacterial agent in future, GSK'052 could be used as part of combinatorial therapy to mitigate resistance liability (Randall *et al.,* 2016).

Table 5.1: Summary of amino acid substitutions identified in laboratory isolated *S. aureus* mutants and its effects on GSK'052 susceptibility

Nucleotide substitutions in *leuS*	Amino acid substitutions in LeuRS	No. of identified strains (n=25)	Fold increase in MIC
$G_{671}A$	$G_{224}E$	1	>32
$C_{677}T$	$S_{226}F$	2	>32
$G_{683}A/T$	$G_{228}E/V$	2	>32
$C_{737}A/T$	$T_{246}K/M$	2	>32
$C_{752}A/G$	$T_{251}K/R$	2	>32
$T_{980}A$	$V_{327}G$	1	>32
$T_{991}C$	$Y_{331}F/H$	2	>32
$T_{991}C$, $A_{992}T$	$A_{335}E/P$	3	>32
$C_{1013}T$	$A_{338}V$	5	>32
$A_{1038}G$	$D_{346}G$	1	>32
$A_{1236}T$	$K_{412}N$	4	>32

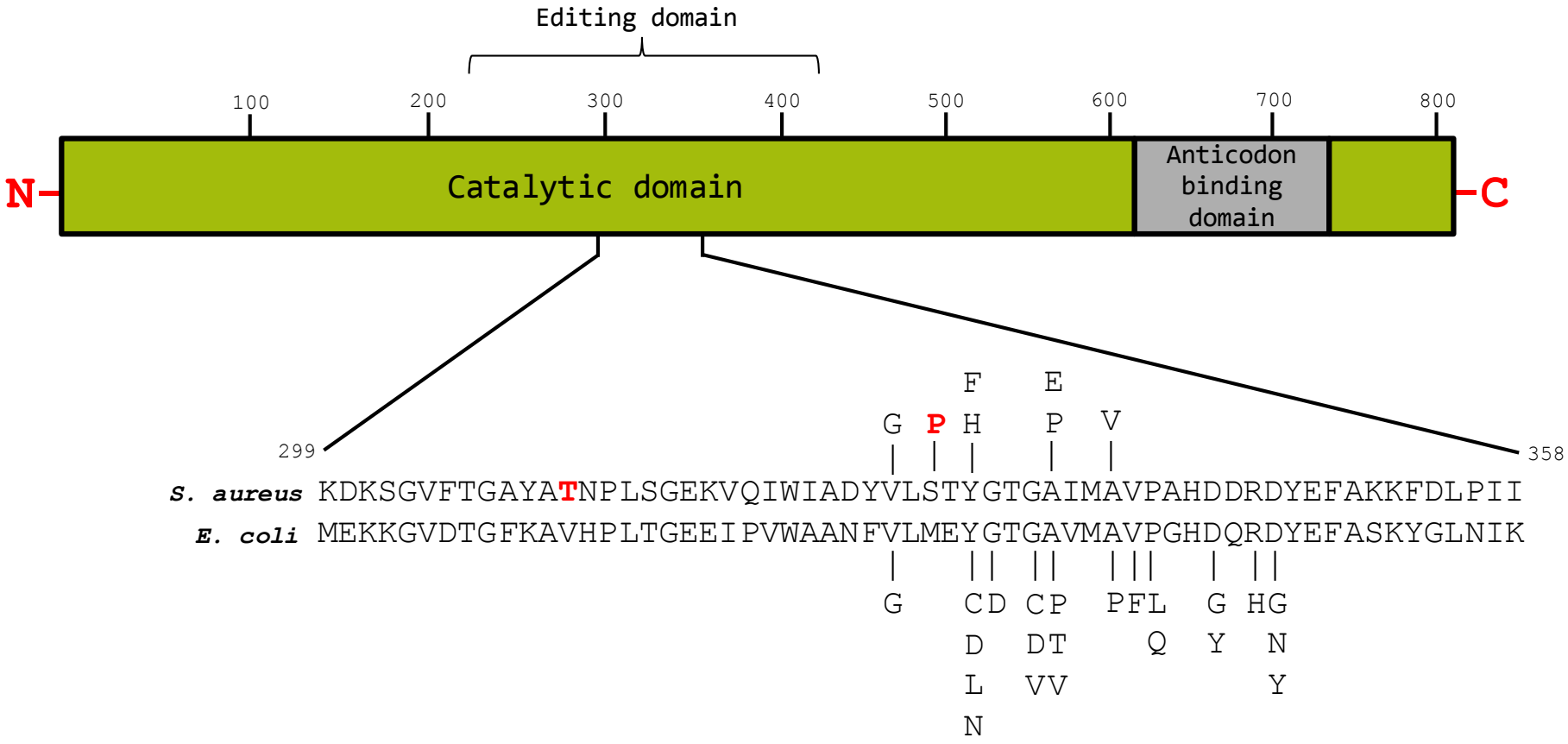

Figure 5.11: Schematic of the LeuRS protein, with a close-up on the editing domain, showing the amino acid substitutions that mediate reduced susceptibility to GSK'052 in *S. aureus* and *E. coli* (O'Dwyer *et al.*, 2015). The residue numbering corresponds to the *S. aureus* protein sequence, and amino acid substitutions observed in spontneous resistant mutants of *S. aureus* and *E. coli* to GSK'052 are denoted above and below the sequence, respectively

5.3.4.2. Assessing the susceptibility of S. aureus clinical strains to GSK'052

For GSK'052 to be considered as an anti-staphylococcal agent, it is also important to identify if resistance to the compound pre-exists in the clinic. The presence of resistance in the clinic would make GSK'052 a less viable option for development as anti-staphylococcal agent. To assess the antibacterial activity of GSK'052 against staphylococcal strains, a panel of *S. aureus* clinical strains (*n*=52) was tested using broth micro dilution determined by Dr Carmine Monteferrante at Erasmus MC University, Rotterdam, The Netherlands. The isolates were recovered from patients at the Erasmus MC University Medical Centre, Rotterdam, The Netherlands, between November 2009 and May 2010. Clinical trials for GSK'052 were carried out in the USA, Canada, Czech Republic, France, Italy, Spain and Russia between 2011 and 2014. Therefore, the 52 clinical strains originate from a country in which GSK'052 has never been trialled before and also predates the clinical evaluation of GSK'052. Consequently, it can be assumed that these strains have never been exposed to the compound.

In addition to these 52 strains, GSK'052 was also tested against well characterised clinical isolates of staphylococci such as MRSA252, Mu50, UAMS-1 and USA300. GSK'052 exhibited a MIC of 2 to 4 mg/L against all isolates except for strain 1372 (MIC 16 mg/L). This degree of reduced susceptibility (4-8 fold) is equivalent to that observed in some GSK'052 resistant *E. coli* strains selected *in vivo* in phase II clinical trials. The reduced susceptibility of strain 1372 to GSK'052 suggests that resistance to this compound pre-exists in the clinic implying that it would not be an ideal candidate for treatment of staphylococcal infections.

5.3.4.3. Analysis of leuS from S. aureus 1372 and characterisation of a novel polymorphism in the encoded LeuRS

To determine the mechanism responsible for the reduced susceptibility of 1372 to GSK'052, *leuS* was amplified from the strain and its sequence determined. Analysis of $leuS_{1372}$ in comparison with $leuS_{SH1000}$ showed four single nucleotide polymorphisms that resulted in the amino acid polymorphisms $T_{311}I$, $S_{329}P$, $A_{553}G$ and $Y_{735}F$. To determine if any of these polymorphisms in *leuS* was responsible for the reduced susceptibility to GSK'052, *leuS* from both SH1000 and 1372 were amplified using appropriate oligonucleotide primers. The amplified genes were introduced into staphylococcal expression vector, pLOW, for over-expression in SH1000, resulting in SH1000 (pLOW:$leuS_{SH1000}$) and SH1000 (pLOW:$leuS_{1372}$). Expression of the latter caused a substantial reduction in GSK'052 susceptibility (MIC 64 mg/L) compared to SH1000 (pLOW:$leuS_{SH1000}$) (MIC 16 mg/L). This shows that the reduced susceptibility to GSK'052 is likely to be a result of a polymorphism in LeuRS in 1372.

Amongst the four polymorphisms identified, the $A_{553}G$ and $Y_{735}F$ polymorphisms are also found in MRSA252 and Mu50, two clinical strains of *S. aureus* with annotated genome sequences (Genbank accession no. BX571856 and BA000017, respectively). When these two strains were tested with GSK'052, neither was more resistant than SH1000 (GSK'052 MIC = 4 mg/L), implying that these two polymorphisms are not responsible for the reduced susceptibility observed in case of *S. aureus* 1372. Based on the sequence analysis and MIC determinations of GSK'052 against strains overexpressing $leuS_{SH1000}$ and $leuS_{1372}$ respectively, I sought to determine which of the other two residues was responsible for the observed reduced susceptibility. Residue P_{329} was the likely candidate as it resides within the editing domain of LeuRS, a region where majority of the amino acid substitutions have been identified in LeuRS of clinical isolates of *E. coli* resistant to GSK'052 during clinical trials (Figure 5.11). In addition, it also resides in the region where most of the amino acid substitutions occur in spontaneous resistant mutants of SH1000 to GSK'052 (Figure 5.11).

Thus, the P_{329} polymorphism was engineered in pLOW:*leuS*$_{SH1000}$ by site directed mutagenesis and introduced in SH1000, which exhibited a similar reduction in susceptibility to GSK'052 (MIC 64 mg/L) as SH1000 (pLOW:*leuS*$_{1372}$). The decrease in susceptibility to GSK'052 due to the introduction of P_{329} in pLOW:*leuS*$_{SH1000}$ implies that it is the residue responsible for the insusceptibility of strain 1372.

The insensitive nature of LeuRS in strain 1372 to GSK'052 is probably due to the position of P_{329} within the editing domain of the enzyme. To understand how this residue affects the antibacterial activity of GSK'052, the crystal structure of *Thermus thermophilus* LeuRS bound to the parent compound (AN2690) of GSK'052 (PDB ID: 2V0C) was examined. AN2690 forms an adduct with $tRNA^{leu}$, thus trapping it in the editing site of the protein (Rock *et al.,* 2007). Residues close to the novel P_{329} polymorphism are involved in binding to the tRNA portion of this adduct. In particular, the preceding residue, L_{329} (in *T. thermophilus* LeuRS), forms two hydrogen bonds with A76 of $tRNA^{leu}$ (Figure 5.12) (Rock *et al.,* 2007).

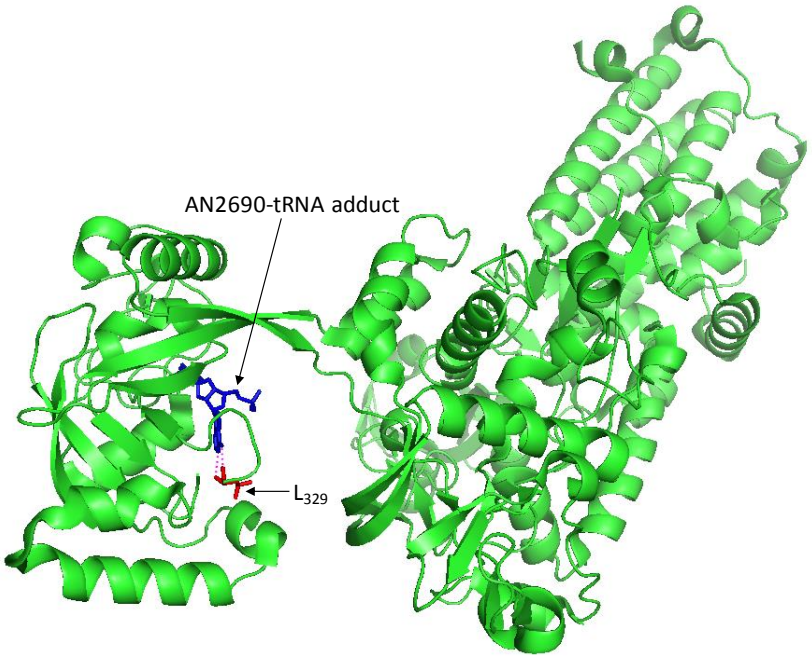

Figure 5.12: Structure of *T. thermophilus* LeuRS with the AN2690-$tRNA^{leu}$ adduct in the editing site. Dashed magenta lines represent hydrogen bonds between A76 and L_{329}, one of the key bonds which leads to the trapping of the adduct in the editing site

That being the case, the presence of a more rigid, proline residue at this position could constrain the backbone in such a way that the conformation of L_{329} is changed, making it incapable of forming these hydrogen bonds. Loss of these hydrogen bonds in turn would result in reduced affinity for A76; and as affinity for AN2690 is dependent on tRNA, this will also result in reduced affinity of the drug for its target, in turn leading to reduced susceptibility to the compound. Based on the above evidence, it can be speculated that this novel polymorphism in LeuRS in strain 1372 is unlikely to bind directly to the drug.

The P_{329} polymorphism in LeuRS observed in *S. aureus* 1372 is not found in any other *S. aureus* strain with an annotated genome in the public databases. This feature of reduced susceptibility to an experimental drug due to polymorphisms within the drug target prior to exposure to the drug is rare and not usually observed (Watters *et al.,* 2006).

5.4. Conclusions

Resistance to any antibiotic is inevitable due to the evolutionary nature of bacteria. However, attempts can be made to better understand these resistance mechanisms to aid in the development of new antibiotics before a new line of defence mechanism is identified. Results presented in this chapter show for the first time that the mupirocin-resistant proteins – MupA and MupB can completely replace the native staphylococcal IleRS enzyme without having an adverse effect on bacterial growth. This proves that these two proteins are indeed catalytically active, alternate, mupirocin-insensitive IleRS enzymes. The results presented in this chapter suggest that the mupirocin-insensitivity nature of MupA and MupB is not associated with the K_{631} residue; a residue which is prone to substitution in spontaneous resistant mutants of *S. aureus*. The inability to generate functional chimeric proteins does not undermine the hypothesis that the extra residues play a role in the enzymatic properties

of MupA and MupB. Truncation studies of MupB, based on structural predictions by I-TASSER, showed that truncating the protein at residue 968 leads to loss of mupirocin insusceptibility but maintains partial functionality compared to full length MupB. These results are promising, and could potentially inform a platform to generate analogues of mupirocin that can overcome the presence of the extra domain in MupA/B and still maintain their potent antibacterial activity.

The ability to rapidly generate high-level *S. aureus* resistant mutants to GSK'052 *in vitro* and the pre-existence of resistance to GSK'052 in a clinical isolate of *S. aureus*, suggests that it is not a suitable candidate for monotherapy of staphylococcal infections. The latter is a result of a single amino acid polymorphism (P_{329}) in the LeuRS enzyme, which likely results in reduced binding affinity of GSK'052. These observations emphasise the need for thorough pre-clinical evaluation of any candidate antibacterial agent; and also emphasizes the need for assessing the activity of candidate antibacterial compounds against clinical isolates to identify any pre-existing mechanisms mediating reduced susceptibility.

Chapter 6

Final conclusions and future work

Chapter 6: Final conclusions and future work

6.1. Final conclusions

The steady rise in antibiotic resistance amongst both Gram-positive and Gram-negative pathogens has diminished the efficacy of clinically used antibiotics, including antibiotics of last resort such as colistin. In addition, identifying hit compounds in screening platforms amongst thousands of compounds is challenging to begin with and the issue is made worse by the difficulty in taking such hits to the end of clinical trials. The situation is also suffering from the lack of investment from big pharma in antibiotic development. Based on the current antibiotic discovery/development void, it has been predicted that by 2050, 10 million deaths a year will be a direct consequence of antibiotic resistance; a figure which surpasses the predicted number of deaths caused by cancer (8.2 million) (O'Neill, 2016). However, several approaches are being taken to identify, develop and feed novel compounds into the antibiotic pipeline in the hope that these novel antibacterial agents will halt the rapid development and spread of resistance. These include revisiting compounds which were previously not considered to be antibacterial agents (Ooi and O'Neill, 2016), awakening silent gene clusters in microorganisms (Rutledge and Challis, 2015, Reen *et al.,* 2015), and screening the human microflora (Zipperer *et al.,* 2016) and other under explored natural habitats with the intention of finding a novel compound with antibacterial activity (Ling *et al.,* 2015). In addition, one of the approaches suggested is multi-targeting of essential bacterial proteins (Silver, 2011). But is this a fool proof solution? Although not completely fool proof as it cannot negate the acquisition of resistance determinants on mobile genetic elements, a compound with multi-targeting activity will be less liable to the spontaneous development of resistance in pathogens compared to a compound with a single target. It is evident that there is a need for intensified research to ensure the development of novel

effective antibacterial agents and better understand the molecular/structural basis of resistance proteins to help guide design of new inhibitors.

It is hoped that this study has contributed to the research in the field. The results presented in this thesis provide further proof that the underexploited aaRS enzymes represent themselves as viable and validated targets for the development of candidate antibacterial agents. Although the initial goal of developing and characterising a multi-targeting aaRS inhibitor did not yield any success, our latter approach of looking for an inhibitor exhibiting whole cell antibacterial activity against either IleRS, LeuRS or ValRS yielded fruit in the form of four candidate antibacterial agents which specifically targeted *E. coli* LeuRS. The inability to develop a multi-targeting compound in spite of these enzymes being conserved, further highlights the challenges faced in antibiotic discovery. The NABARSI approach also highlights one of the major challenges faced in SBDD which is, one must always remember that crystal structures are frozen, whereas in solution a protein may exhibit flexibility which in turn might alter binding affinity. In addition, the lack of crystal structures of the desired target protein from the organism of choice may also pose an issue. In such circumstances, although the construction of homology models may circumvent the lack of structure, it might not be an accurate representation of the target protein. The lack of an *S aureus* LeuRS crystal structure meant that in some cases we went in blind when determining binding affinities *in vitro* and subsequent testing of antibacterial activity. It is abundantly clear that identifying and developing a new antibacterial agent is challenging. In an era of advanced structural, genomics and chemical synthesis knowledge, it might be prudent to utilise all the tools to enable a multi-facet approach to antibiotic discovery. Alternatively, one might consider taking a step in the direction of oncology – which has evolved to personalised medicine. This implies that researchers need a better understanding of a specific pathogen causing infection and in turn focus on a narrow spectrum agent. Perhaps this approach may also reduce the pressure to evolve resistance (Brown and Wright, 2016).

Furthermore, this study shows for the first time that overexpression of *yhhY,* a putative acetyltransferase can provide resistance to aaRS inhibitors, by acetylation of the inhibitor, thus reducing binding affinity for its target protein. The results presented in this thesis provide further evidence that the use of hypermorphic or hypomorphic bacterial strains are useful for drug discovery, as they provide an immediate indication of the target specificity and mechanistic basis of whole cell activity of a candidate antibacterial agent. The use of hypomorphs also helps establish the essentiality of target genes, making them more sensitive than simply overexpression of the gene encoding the target protein.

In addition, this study has provided useful information regarding the molecular basis of resistance to known inhibitors. The results provide direct and conclusive *in vivo* evidence that the mupirocin resistance proteins, MupA and MupB are indeed alternate, functional IleRS enzymes resistant to mupirocin. Examination of the predicted structures of MupA and MupB indicate that the extra amino acid residues in these proteins are likely to form an additional domain, in turn contributing to their mupirocin insensitivity. However, to conclusively say that this extra domain is responsible for mupirocin resistance, further experiments must be conducted which have been described in 6.2. Although the data obtained suggests that GSK2251052 is not a suitable candidate for the treatment of staphylococcal infections, it has highlighted the molecular basis of resistance in *S. aureus*. The results presented in this thesis and in the publication Gupta *et al.,* 2016, show that resistance to GSK'052 in *S. aureus* is also pre-existing in the clinic.

6.2. Future work

Over the past ten years, the aaRS enzymes have been exploited for the identification and development of candidate antibacterial, antifungal and anti-parasitic agents. The most noteworthy is Tavaborole, which also served as the starting scaffold for the experimental antibacterial agent, GSK'052. As such, the research described in this thesis provides further evidence that aaRS enzymes are viable targets for the development of novel antibacterial agents.

The sensitivity of the *S. aureus* hypomorph strains for *ileS, leuS, serS* and *thrS*, form a strong platform for the development and validation of similar conditional mutants in *E. coli.* Although these conditional mutants are important tools in antibacterial discovery, they can also be utilised to understand more fundamental processes such as the stringent response, which is triggered by inhibition of aaRS enzymes. The stringent response in *S. aureus* remains understudied and the hypomorphs generated in this thesis provide model systems to better understand the response without the dependence on antibiotics.

The identification and biological characterization of the four *E. coli* LeuRS inhibitors (LL20, LL49, DG539 and MZ411) provides the grounds for generating derivatives of the same with more potent antibacterial activity. The whole cell activity of these derivatives can be evaluated following the screening platform used for the work described in this thesis. It would also be prudent to analyse the sequence determinations obtained from the spontaneous mutants generated in this study (Chapter 4) differently, as this could help ascertain if any gene has been duplicated which in turn could be responsible for resistance to the candidate LeuRS inhibitors. In addition, passaging the mutant strains in non-selective media over a designated period might shed light on the stability of the mutations. With regard to '235, which inhibited both protein and RNA synthesis (which was not considered as a lead scaffold), it might be prudent to test its antibacterial activity against the *S. aureus*

hypomorphic strains to elucidate its target specificity. In addition, determining its effect on potassium leakage would also give an indication on whether it exhibits non-specific activity.

In addition, results presented in this thesis indicate that the speculative extra domain in MupA/B may play a pivotal role in mupirocin resistance. To assess this hypothesis the purification of MupA/B is necessary as this would help determine how the two proteins bind mupirocin compared to staphylococcal IleRS and identify how the extra amino acid residues affect this binding. In addition, understanding the underlying effect of $MupB_{968aa}$ on relative fitness and/or aminoacylation in SH1000 would also provide proof that the extra domain has a role in resistance and function. Based on the structural data, one could revisit the truncation studiesof MupA/B to identify the portion of the protein responsible for mupirocin-insensitivity. Finally, it might be fruitful to consider the use of GSK'052 as an antibacterial ointment, as the high concentration would negate the resistance liability of the compound. Alternatively, developing and characterizing combinatorial therapy regimes with different aaRS inhibitors could also provide an avenue for the development of a new antibacterial therapy.

In conclusion, the hunt for new antibacterial agents must go hand in hand with understanding antibiotic resistance mechanisms, policy making including proper antibiotic stewardship and funding.

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Appendix

Table A1: List of oligonucleotide primers used in this study. Underlined sequence indicate restriction sites and sequence highlighted in red indicate homology arms required for Gibson Assembly

ileS pEL up	ATCGTTATGAGCTCTAAATTTTTAAGGAGTGAAA	For cloning staphylococcal *ileS* into pEPSA5
ileS pEL low	TGCTTAGTGGATCCCAATTTCAATTATACAAGTG	
ileS pSK up	ACCCTAGCTTCGAAATGGATTACAAAGAAACGTTATTAATG	For cloning staphylococcal *ileS* into pSK5487
ileS pSK low	ACCCTAGCTTCGAACAATTTCAATTATACAAGTGATTTTACAA	
pSK MCS up	CGGATTTGAACGTTGCGA	To confirm the presence of desired insert in pSK5487
pSK MCS lw	TATCCCACTTTATCCAATTTTCGTT	
ileS upper	ATCGTTATGAGCTCTAAATTTTTAAGGAGTGAAA	For cloning staphylococcal *ileS* into pLOW
ileS lower	TGCTTAGTGGATCCCAATTTCAATTATACAAGTG	
leuS upper	ATCGTTATGAGCTCTTTTTTATTGAATAGGAGGA	For cloning staphylococcal *leuS* into pLOW
leuS lower	TGCTTAGTGGTACCATTTCAAAGTCCTCCTTAAA	
pLOW MCS upper	TATAGTTTTGGTCGTAGAGC	To confirm the presence of desired insert in pLOW
pLOW MCS lower	GTACTGAGAGTGCACCATAT	
pIMAY amp upper	GGGGGATCCACTAGTTCTAGAG	To generate linear pIMAY around the *SmaI* site
pIMAY amp lower	GGGCTGCAGGAATTCGATAT	
pIMAY MCS upper	CAAGAATAAACTGCCAAAGC	To confirm the presence of desired insert in pIMAY
pIMAY MCS lower	TGTGACGGAAGATCACTTCG	
Ile out fwd	TTCAGAAACAACTAAGAAGT	To confirm excision of staphylococcal *ileS* or *leuS* in *S. aureus* SH1000
Ile out rev	TGATACATTCAATTAACTCC	
Leu_outfwd	GAGAATAATACGCAACCTAA	
Leu_outrev	CTTTTTATTTCCAAACCTTC	
pLL39 amp upper	GGGGATCCTCTAGAGTCGAC	To generate linear pLL39 around the *Sma*I site for introducing P_{spac} ahead of the MCS
pLL39 amp lower	GGGTACCGAGCTCGAATTC	
pLL39 MCS upper	GCTTAGATCTAATCGAATTC	To confirm the presence of desired insert in pLL39 and pAR89
pLL39 MCS lower	GTTGTTCCAATAACTGATGA	
pAR89 upper amp	GGGGAATTCGGGGATCCTCT	To generate linear pAR89 around the *Sma*I site for

pAR89 lower amp	GGGCTCGAGAAGCTTACTAGTATCG	introducing staphylococcal *ileS* and *leuS* using Gibson assembly
Table A1 is continued on page		
Ile_par upper	GTGAGCGGATAACAATTAAGCTTATCGATACTAGTA AGCTTCTCGAGCCCTAAATTTTTAAGGAGTGAAA	For cloning staphylococcal *ileS* into pAR89
Ile_par lower	AAAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGG ATCCCCGAATTCCCCCAATTTCAATTATACAAGTG	
Leu_par upper	GTGAGCGGATAACAATTAAGCTTATCGATACTAGTA AGCTTCTCGAGCCCTTTTTTATTGAATAGGAGGA	For cloning staphylococcal *leuS* into pAR89
Leu_par lower	AAAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGG ATCCCCGAATTCCCCATTTCAAAGTCCTCCTTAAA	
Scv4	ACCCAGTTTGTAATTCCAGGAG	To confirm integration of pAR89 constructs in *S. aureus*
Scv10	TATACCTCGATGATGTGCATAC	
Scv8	GCACATAATTGCTCACAGCCA	
Scv9	GCTGATCTAACAATCCAATCCA	
serS upper	ATCGTTATGAATTCTTATTTGGGAAAGGATGAAG	For cloning staphylococcal *serS* into pMUTIN4
serS lower	TGCTTAGTGGATCCACATTGTATCTGCGTTACA	
thrS upper	ATCGTTATAAGCTTAAGCAAATAGGAGGGTTTAAC	For cloning staphylococcal *thrS* into pMUTIN4
thrS lower	TGCTTAGTGGATCCCAGTATGCACCTGCTGTAGA	
Erm prime	ATCTGTTGTTTGTCGGTGAA	To confirm the presence of desired insert in pMUTIN4
pMUT MCS	CCTCTTCGCTATTACGCC	
mupA upper	ACCCTAGCTTCGAATTGACAAAGAAATATTTAA ACACC	For cloning *mupA* into pSK5487
mupA lower	ACCCTAGCTTCGAAGTTTAATATAATAAGGA AATTT	
mupB upper	TACCATACTTCGAATTGGAAAACGAGAATATAATAG AAGAA	For cloning *mupB* into pSK5487
mupB lower	TACCATACTTCGAATTAATTTGTAAAGCTAGACATTA ACTGAAT	
mupA pI up	ATTAGGTAATGGTGAGAGCCTAGTAAAAGCATGTA TGTTATATCACTGGCAAGGAGTGAAAAAATTGACA AAGAAATATTTAAACACC	To clone *mupA* with homology arms to staphylococcal *ileS* including RBS into pIMAY for allelic replacement
mupA pI low	GAAGAGAGCTTTAATTTATATCATCTGTATGAGTAC TTTATACAATTTCATTTGTTTAATATAATAAGGAAAT TTTTA	
mupB pI up	ATTAGGTAATGGTGAGAGCCTAGTAAAAGCATGTA TGTTATATCACTGGCAAGGAGTGAAAAATTGGAAA ACGAGAATATAATAGA	To clone *mupB* with homology arms to staphylococcal *ileS* including RBS into pIMAY for allelic replacement
mupB pI low	GAAGAGAGCTTTAATTTATATCATCTGTATGAGTAC TTTATACAATTTCATTAATTTGTAAAGCTAGACATTA ACT	
MupAB out up	AATGAAAGTGCAACAGTTAG	To confirm replacement of staphylococcal *ileS* with either *mupA* or *mupB*
MupAB out low	TTCGTCCATTCAAAGTATAT	
ileS V631K up	TTTAGCTGAT*AAG*AGAATTTCTGATGAAATTTTAAA AC	To introduce $GTT_{1891\text{-}1893}AAG$ in staphylococcal *ileS*
ileS V631K low	TAGTCCGTACTACTTACCCAAAG	
mupA K631V up	ATGGAATAAC*GTT*AGATTCTCAGAAAATATAGTAG	To introduce $AAA_{1863\text{-}1865}GTT$ in *mupA*
mupA K631V low	GGAGCACTATCCGAAATTAAG	

mupB K631V up	TAACAAAAGA*GTTT*CAGAAAATATAGTAGC	To introduce $AAA_{1870\text{-}1872}GTT$ in *mupB*
mupB K631V low	TTCCATGGAGCACTATCC	
Table A1 is continued on page		
ileS chop1	ATCGTCTAACTTATCTACAACTTTAA	To generate linear pSK5487:*ileS* for use as background for chimeras
ileS chop2	CTTACCTTCACCGTCCATAA	
pSK chop	TTCGAAAGATGAAGGCAG	
A fuse1 up	ATCAATTATTTATCGTGTCACAAGTTAAAGTTGTAG ATAAGTTAGACGATTATATTACCTATGAGCTTAAAT TG	To generate chimeras between staphylococcal IleRS and MupA
A fuse1 low	TCGATACAAAACAAAGGCAGACTCATTTCAGTCTGC CTTCATCTTTCGAATTAATATAATAAGGAAATTTTTA TTTC	
A fuse2 up	CTTATAAATTCTTACTTTCTCATGGTTTTGTTATGGA CGGTGAAGGTAAGAAAATGTCTAAAAGTAAAGGAA	
B fuse1 up	ATCAATTATTTATCGTGTCACAAGTTAAAGTTGTAG ATAAGTTAGACGATTTTTTAGAATATGATGTAAAAC CGA	To generate chimeras between staphylococcal IleRS and MupB
B fuse1 low	TCGATACAAAACAAAGGCAGACTCATTTCAGTCTGC CTTCATCTTTCGAATTAATTTGTAAAGCTAGACATTA ACTG	
B fuse2 up	CTTATAAATTCTTACTTTCTCATGGTTTTGTTATGGA CGGTGAAGGTAAGAAAATGTCGAAAAGTAAAGGG	
Frag up A/B	TAATTCGAAAGATGAAGGC	For generating truncated versions of MupB and MupA
B Frag 1_{905aa}	GTCAAAAACTTTTTGAAGTTTA	
B Frag 2_{968aa}	GTTCTGTTGTTTTCTTAGTTGT	
B Frag 3_{999aa}	ATTTTCTTTCAATATATTGATATT	
B Frag 4_{1010aa}	TCTTTTCTCAAATTTTAGATTAT	
B Frag 5_{1022aa}	TTCTTTCTGATTAATTTTAAAAT	
A Frag 2_{968aa}	ACGTTGATTTATTGGTAAA	
yhhY up	TGCTTAGTGGTACCATTTACACCTTAGCGCAAAG	For cloning *yhhY* locus into pIMAY
yhhY low	ATCGTTATGAATTCCTCAGAAAAAGGGCACTATC	
yhhY MAGE	AAATGCATTTGACTCGCATTTGAAAGTCAATTATGT TGAAAGGGACATTTACCCCAAAGAGGACAAAGGA	To introduce the mutation $G_{-38}T$ in the *yhhY* locus