Functional insights into ABC-F proteins that mediate antibiotic resistance in Gram-positive bacteria

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Finally, I would like to dedicate this thesis to my family. You have always believed in me and I couldn't have done it without you.

Abstract

Members of the ABC-F subfamily of ATP-binding cassette (ABC) proteins mediate resistance to a broad array of clinically-important antibiotic classes that target the ribosome of Gram-positive pathogens. The mechanism by which the ABC-F proteins mediate antibiotic resistance is poorly defined, although two hypotheses have been proposed; drug efflux and ribosomal protection. Here, this mechanism of resistance was investigated using a combination of bacteriological and biochemical techniques. Results obtained from the bacteriological assays provided preliminary data in support of ribosomal protection. Subsequently, the heterologous expression and purification of two ABC-F proteins, Vga(A) and Lsa(A), allowed the function of these proteins to be assessed in staphylococcal transcription-translation (T/T) reactions. Addition of Vga(A) and Lsa(A) to T/T assays subject to antibiotic inhibition caused drug specific, dose-dependent, rescue of translation. Several previously described resistance phenotypes attributed to these proteins were successfully recapitulated in T/T assays, corroborating the idea that rescue of translation observed *in vitro* is representative of the action of these proteins in whole cells. Finally, ribosome binding assays showed Lsa(A) to be capable of displacing antibiotics from staphylococcal ribosomes. Collectively, the experiments described in this thesis provide the first direct evidence to support a mechanism of ARE ABC-F resistance based on ribosomal protection.

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Abbreviations

The following abbreviations have been used throughout this thesis;

ABC	ATP-binding cassette
AMP-PNP	Adenosine 5'-(β,γ-imido)triphosphate
ATP	Adenosine triphosphate
BHI	Brain-heart infusion
DNA	deoxyribose nucleic acid
Ef-Tu	Elongation factor Tu
C	Celcius
cv	column volumes
g	*gravity*
g	gram
HEPES	2-[4-(2-hydroxyethyl)piperzin-1-yl]ethanesulfonic acid
IMAC	immobilised metal ion affinity chromatography
kDa	kilodaltons
LBA	Luria-Bertrani agar
LBB	Luria-Bertrani broth
M	molar
MDR	multidrug-resistant
mg	milligram
mg	milligram
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	Minimum inhibitory concentration
ml	milliltre
mM	millimolar
MWCO	Molecular weight cut off
NaCl	sodium chloride
NaH_2PO_4	soduim dihydrogen phosphate
NBD	Nucleotide binding domain
NEB	New England Biolabs
Ni-NTA	nickel-nitrilotriacetic
OD	optical density
nM	nanomolar
PCR	polymerase chain reaction
pH	potential hydrogen
pI	isoelectric point
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl suplhate polyacrylamide gel electrophoresis
TAE	Tris base, acetic acid, EDTA.
TBE	Tris, Borate, EDTA
Tm	melting temperature
TMD	Trans-membrane domain
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
v / v	volume per volume
w / v	weight per volume
µg	microgram
µl	microlitres
µM	micromolar
µm	micrometer

1. Introduction

1.1 The importance of antibacterial agents and the rise of antibiotic resistance

The advent of modern antimicrobial chemotherapy represented a seminal moment in the ongoing fight against infectious disease. Prior to the widespread integration of antibiotics into the toolkit of modern medicine, clinicians lacked effective options for the treatment of bacterial infections and bacterial pathogens were a major cause of morbidity and mortality. In 1930, around 1 in 5 deaths in the USA was attributable to conditions associated with bacterial infection such as pneumonia, tuberculosis, gastrointestinal disease, and childbirth-associated septicaemia (Murphy, 1931). However, by the end of the 20th century, lower respiratory tract infections were the only bacterially associated disease to be ranked among the top 10 causes of death within the USA (Wenzel and Edmond, 2000). Similarly, in 1941 staphylococcal bacteraemia was associated with an 80% mortality rate, but by 2004 only 20% of systemic staphylococcal infections resulted in death (van Hal *et al*., 2012, Skinner and Keefer, 1941). These dramatic decreases in mortality associated with bacterial infections coincide with entry into the "antibiotic era", which began with the introduction of Salvarsan for treatment of syphilis in the 1910s (Aminov, 2010), and the subsequent introduction of the sulphonamides and penicillin into widespread clinical use during the 1930s and 40s respectively (Domagk, 1986, Chain *et al*.). The following three decades (1940 – 1970) represented a "Golden Era" of antibiotic discovery, in which the majority of antibiotic classes in clinical use today were identified (Aminov, 2010), heralding an end to the "age of pestilence" and enabling modern medical procedures such as anti-cancer chemotherapy, organ transplantation and routine surgery (Omran, 2005).

The enormous progress made in the field of antimicrobial chemotherapy during the 20th century is now in danger of being compromised. The increasing prevalence of bacterial strains resistant to multiple classes of antibiotics threatens to bring about a post-antibiotic

era, in which clinicians once more lack the necessary tools to treat bacterial infection and deaths resulting from bacterial disease begin to increase. As of 2014, antimicrobial resistance is estimated to result in 700 000 deaths annually world-wide, with this figure predicted to rise as high as 10 million if current trends in development of antibiotic resistance are left unchecked, these figures include infections caused by drug resistant malaria and HIV in addition to bacteria (HM Government review on antimicrobial resistance, 2014). In the developing world, community acquired infections such as tuberculosis caused by drug resistant *Mycobacterium tuberculosis* pose a significant challenge; the estimated 480,000 cases of drug resistant TB in 2013 threaten to erode the current treatment success rate of 86% (WHO, 2014). Whereas, within industrialised nations infections caused by multi-drug resistant bacteria within are predominantly hospital-associated, with the so called ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) posing a particular threat (Boucher *et al*., 2009).

The clinical impact of antibiotic resistance is exacerbated by a lack of new antibiotic classes coming to market. Between 1940 and 1970 more than 20 new classes of antibiotics were developed, but since then only three novel classes have entered the clinic (Coates *et al*., 2011). Additionally, the development of analogues of existing classes is not proceeding quickly enough to match the accumulation of resistance (Boucher *et al*., 2009, Coates *et al*., 2011). The combination of the increasing frequency of infections caused by drug-resistant bacteria and the lack of new antibiotics has led to numerous calls to action (Commission, 2011, Wise, 2011, Spellberg *et al*., 2008). These reports all emphasise the urgent need to develop new classes and analogues of antibiotics, particularly those active against Gram-negative bacteria. A complementary strategy to develop inhibitors of resistance mechanisms to be used in combination with existing antibiotics has also been proposed (Pages *et al*., 2005, Drawz and Bonomo, 2010). Efforts to develop antibiotics and inhibitors

to circumvent currently existing resistance can be informed and accelerated by a thorough understanding of how antibiotic-resistance mechanisms work.

1.1.1 Antibiotic targets and mechanisms of antibiotic resistance

Antibiotics act by interfering with essential cellular biochemistry and physiology, thereby causing cell death or cessation of growth. In order to exert these effects, the majority of antibiotics inhibit or compromise one of five essential cellular targets; the cell membrane, nucleic acid synthesis, folic acid biosynthesis, peptidoglycan biosynthesis, or protein synthesis (Figure 1.1) (Wright, 2010). As these bacterial targets are either absent or different in eukaryotic cells, antibiotics are able to selectively inhibit bacteria whilst causing minimal toxicity to a mammalian host.

It was already understood in the early years of the antibiotic era that bacteria could in some cases resist the growth-inhibitory and lethal effects of antibiotics, a situation that can compromise treatment of infection in the patient (Abraham and Chain, 1940). The considerable threat that this poses to effective antibacterial chemotherapy has prompted intensive research efforts towards understanding the phenomenon of antibiotic resistance. An important facet of these efforts has been to dissect the mechanisms underlying antibiotic resistance; in other words, to establish exactly how bacteria evade the inhibitory action of antibiotics. Aside from yielding information that is of fundamental biological interest and providing novel insights into evolution, these studies also serve an important practical purpose; they offer essential intelligence for those engaged in antibacterial drug discovery to enable the development of new approaches to circumvent or overcome antibiotic resistance.

The ability of a bacterium to resist inhibition or killing by an antibiotic may result from inherent properties of the cell (‘intrinsic’ or ‘natural’ resistance), or may evolve in a bacterial population that was previously antibiotic-susceptible (‘acquired’ resistance). In both cases,

the mechanism by which resistance is ultimately achieved are the same; by preventing, or mitigating the effects of, an antibiotic binding to its cellular target. However, this effect can be achieved in a number of ways; the mechanisms by which bacteria resist antibiotics can be grouped into four major categories, and are summarised in Figure 1.1.

The work described in this study is concerned with a group of antibiotic resistance proteins that mediate resistance to ribosomally-targeted protein synthesis inhibitors. Accordingly, antibiotics that target the ribosome, and resistance mechanisms to these antibiotics, are discussed in more detail below (Sections 1.2 and 1.3).

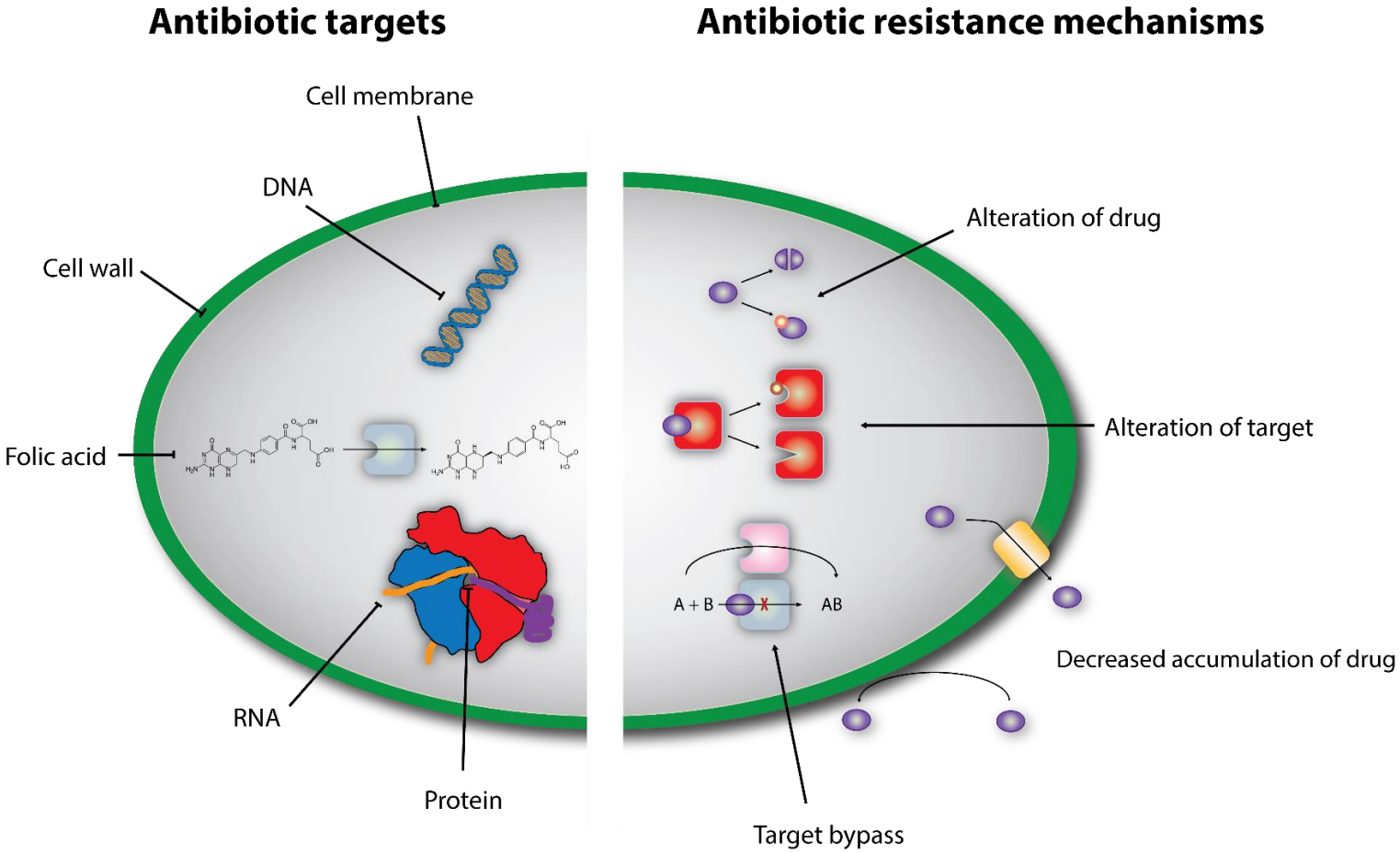

Figure 1.1. The major sites of antibiotic action, and mechanisms by which bacteria resist antibiotics. The majority of antibiotics elicit their inhibitory effects through action at one of the following cellular targets; the cell membrane or cell wall, DNA or RNA synthesis, folic acid biosynthesis, or protein synthesis (left of figure). Bacteria resist these inhibitory effects through alteration of the antibiotic, alteration of the antibiotic target, utilisation of an alternative molecule to bypass the inhibitory effect to the drug, and/or, decreasing cellular permeability and/or increasing efflux to decrease drug accumulation within the cell (right of figure). Figure adapted from (Wright, 2010).

1.2 The ribosome as an antibiotic target

Protein synthesis is essential for bacterial growth. Growing cells use ~50% of the energy they consume to make proteins, and 20–40% of protein synthesis in *Escherichia coli* (*E. coli*) is devoted the production of ribosomes and other translation factors (Russell and Cook, 1995). The ribosome is an RNA-based macromolecular machine around which the multi-step process of protein synthesis is centred. It provides a platform on which amino acids are polymerised into polypeptides according to the genetic information contained within messenger RNA (mRNA). Given the vital nature of protein synthesis, and the key role of the ribosome within this process, it is perhaps unsurprising that the ribosome is one of the main antibiotic targets in the bacterial cell.

The bacterial 70S ribosome is composed of two subunits; a smaller 30S subunit, made up of the 16S ribosomal RNA (rRNA) and 21 proteins, and a larger 50S subunit containing two rRNAs (the 23S and 5S rRNAs) and 33 different proteins (Schluenzen *et al*., 2000, Wilson and Nierhaus, 2005). The 30S subunit contains the decoding centre, where anti-codons within mRNA are paired with cognate transfer RNA (tRNA). By contrast, the 50S subunit provides the peptidyl-transferase centre (PTC), which catalyses formation of the peptide bond, and the peptide exit tunnel, where nascent polypeptides leave the ribosome. At the interface between the two subunits lies three pockets which bind tRNA; the acceptor site (A-site), the peptidyl site (P-site) and the exit site (E-site) (Ban *et al*., 2000, Wilson and Nierhaus, 2005).

Protein synthesis can be divided into four phases; initiation, elongation, termination, and recycling. During initiation, the 30S and 50S subunits assemble, forming the 70S ribosome and establishing the reading frame of mRNA through positioning of the initiator tRNA (fMet-tRNA) on the start codon in the P- site. This process is promoted by three initiation factors; IF1, IF2 and IF3. The elongation step involves the cyclic movement of tRNA from the A- to P- to E- site. At the A-site incoming amino-acyl tRNA, which is brought to the ribosome by

elongation factor Tu (EF-Tu), is screened and selected in accordance with the corresponding codon being displayed by mRNA. The P-site contains the peptidyl-tRNA, however, during catalysis of peptide bond formation in the PTC, the growing peptide chain is transferred from the peptidyl-tRNA in the P-site to the amino-acyl tRNA in the A-site. A second elongation factor, EF-G, drives translocation of tRNAs from the P- to E- site and A-to P site, leaving the A-site free to accommodate the next incoming tRNA. Eventually, the mRNA at the A-site will display a stop codon, resulting in progression to the termination and recycling phases. During termination, release factors 1 and 2 (RF1 and RF2) act to hydrolyse the peptidyl-tRNA bond, releasing the nascent polypeptide to leave through the polypeptide exit tunnel in the 50S subunit. Finally, a third release factor (RF3) causes dissociation of RF1 and RF2 and the 70S ribosome is split into its constituent subunits by EF-G in tandem with the ribosome recycling factor. The ribosomal subunits and ribosome associated factors, are then free to begin another round of protein synthesis (Schmeing and Ramakrishnan, 2009, Wilson, 2014a, Starosta *et al*., 2014) (see Figure 1.2 for an overview of translation).

Despite the complexity of protein synthesis, the majority of antibiotics that target this process act to inhibit elongation. This is true of all clinically important classes of protein synthesis inhibitors, namely the aminoglycosides, amphenicols, fusidic acid, lincosamides, macrolides, oxazolidinones, type A and B streptogramins, and tetracyclines (Figure 1.2) (Wilson, 2009, Wilson, 2014). Although some compounds do target the initiation phase (e.g. avilamycin, edeine, evernimicin, kasugamycin, pactamycin, thermorubin, and thiostrepton), due to problems of poor solubility and toxicity they are not currently in clinical use (Wilson, 2014a). Inhibitors of termination and recycling frequently have a more pronounced effect during elongation, with the exception of fusidic acid and blasticidin S, which inhibit recycling and termination, respectively, at lower concentrations than are required to inhibit elongation (Suematsu *et al*., 2010, Svidritskiy *et al*., 2013).

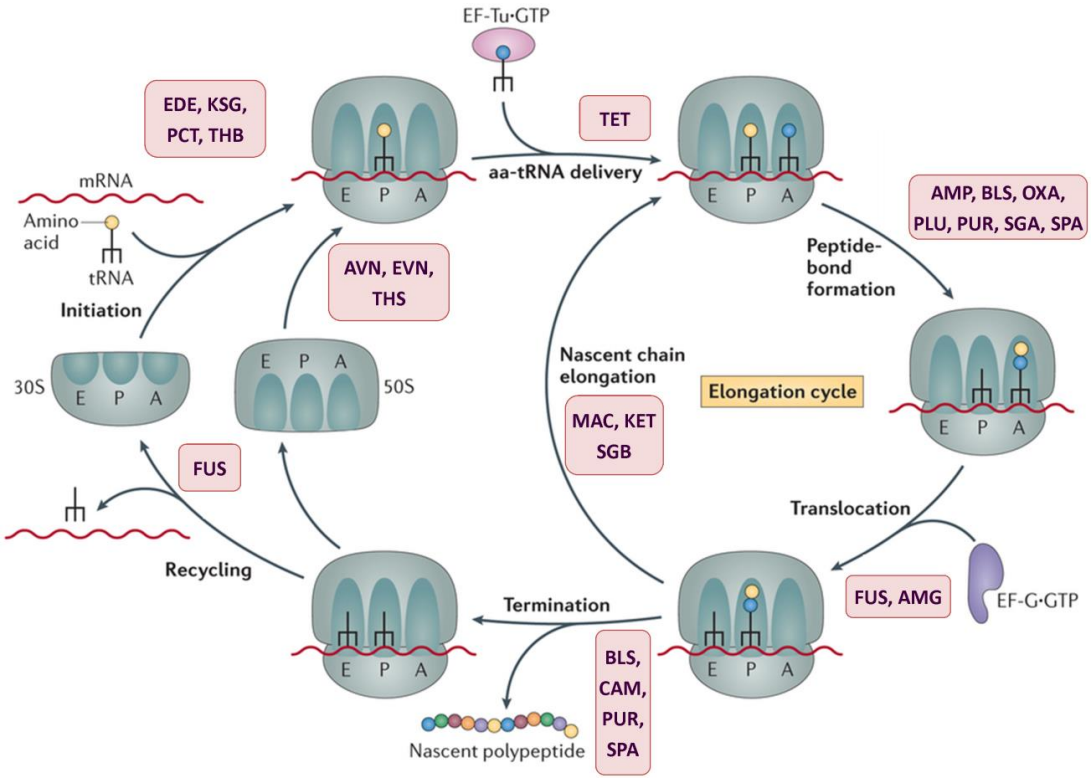

Figure 1.2. Sites of antibiotic inhibition during protein synthesis. During initiation of translation, the 30S and 50S ribosomal subunits assemble to from the 70S ribosome and the fMet-tRNA is positioned on the start codon in the P- site. At the 30S subunit, this phase of translation is inhibited by edeine (EDE), kasugamycin (KSG), pactamycin (PCT), and thermorubin (THB), while avilamycin (AVN), evernimicin (EVN), and thiostrepton (THS) act at the 50S subunit. The elongation cycle requires delivery of the cognate tRNA into the A-site, a process that is inhibited by antibiotics of the tetracycline class (TET). Peptide bond formation between the peptidyl-tRNA at the P-site and amino-acyl tRNA at the P-site occurs at the PTC and is inhibited by amphenicols (AMP), blasticidin S (BLS), oxazolidinones (OXA), pleuromutilins (PLU), puromycin (PUR), type A streptogramins (SGA), and sparsomycin (SPA). Translocation of tRNAs is catalysed by EF-G and is inhibited by antibiotics of the aminoglycoside (AMG) class and fusidic acid (FUS), although this antibiotic primarily acts to prevent recycling. Elongation of the nascent chain is the target of antibiotics in the macrolide (MAC), ketolide (KET), and type B streptogramin (SGB) class. Although, it should be noted that selected macrolides also inhibit peptide bond formation. Termination of protein synthesis is inhibited by peptidyl-transferase targeted antibiotics such as BLS, CAM, PUR and SPA, whereas, FUS acts to trap EF-G in complex with the ribosome and thereby prevent recycling. Figure adapted from (Wilson, 2014).

1.2.1 Antibiotics that target the ribosome

Although the ribosome is a large and complex structure, the sites at which antibiotics bind to mediate their inhibitory effects are relatively few. In the 30S subunit, antibiotics primarily bind close to the positions at which mRNA and tRNA are accommodated into the ribosome. In this manner, they mediate inhibition of translation by either blocking the association of tRNAs with the A-site, as is the case for tetracyclines, or by preventing translocation, as exemplified by aminoglycosides (Brodersen *et al*., 2000). Within the 50S subunit, the majority of antibiotic classes exert their inhibitory effects through binding sites in the vicinity of the PTC. Exceptions include the orthomycins and thiopeptides, which target sites away from the PTC and interfere with the association of initiation and elongation factors (Belova *et al*., 2001, Mikolajka *et al*., 2011). The binding sites of antibiotics at the PTC overlap the binding sties of A-site tRNA (amphenicols, lincosamides, oxazolidinones, puromycin, and sparsomycin), P-site tRNA (blasticidin S), both A- and P-site tRNA (pleuromutilins and type A streptogramins), or are found adjacent to the PTC at a constriction in the peptide exit tunnel (macrolides, type B streptogramins) (Figure 1.3). At the PTC, antibiotic binding acts to interfere with the correct positioning of the amino-acyl moieties at the ends of tRNAs, thereby inhibiting peptide bond formation (Polacek and Mankin, 2005, Wilson, 2009, Wilson, 2014). By contrast, type B streptogramins and 14-membered ring macrolides do not interfere with the PTC reaction, but instead act to prevent exit of the growing peptide chain from the ribosome, prompting premature termination of protein synthesis and the release of peptidyl-tRNA from the ribosome (Di Giambattista *et al*., 1989, Hansen *et al*., 2002, Mao and Robishaw, 1971).

The structures of a representative member of each class of ribosomal antibiotic that has been used in clinical or veterinary medicine are shown in Figure 1.4, Page 11.

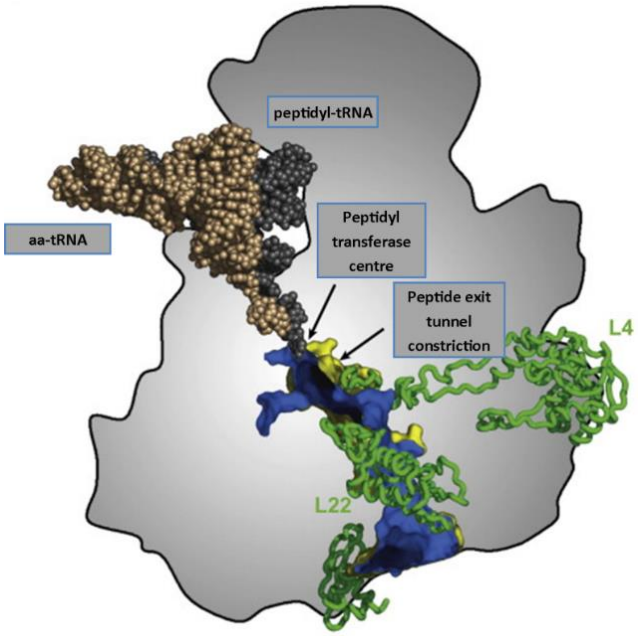

Figure 1.3. The points at which the majority of antibiotics bind the 50S subunit of the ribosome. The PTC (indicated) is the target of amphenicols, blasticidin S, lincosamides, oxazolidinones, pleuromutilins, puromycin, streptogramin As, and sparsomycin. Whereas, macrolides and streptogramin Bs bind in proximity to a constriction in the peptide exit tunnel (indicated). A schematic of the 50S subunit is shown in grey. The amino acyl- and peptidyl- tRNAs are represented as copper or dark grey spheres respectively. A surface representation of the peptide exit tunnel is shown, yellow surface represents the outside of the tunnel and blue surface represents the tunnel interior. Ribosomal proteins L4 and L22, which contact the wall of the tunnel, forming a constriction, are shown as green ribbons. Figure adapted from (Jenni and Ban, 2003).

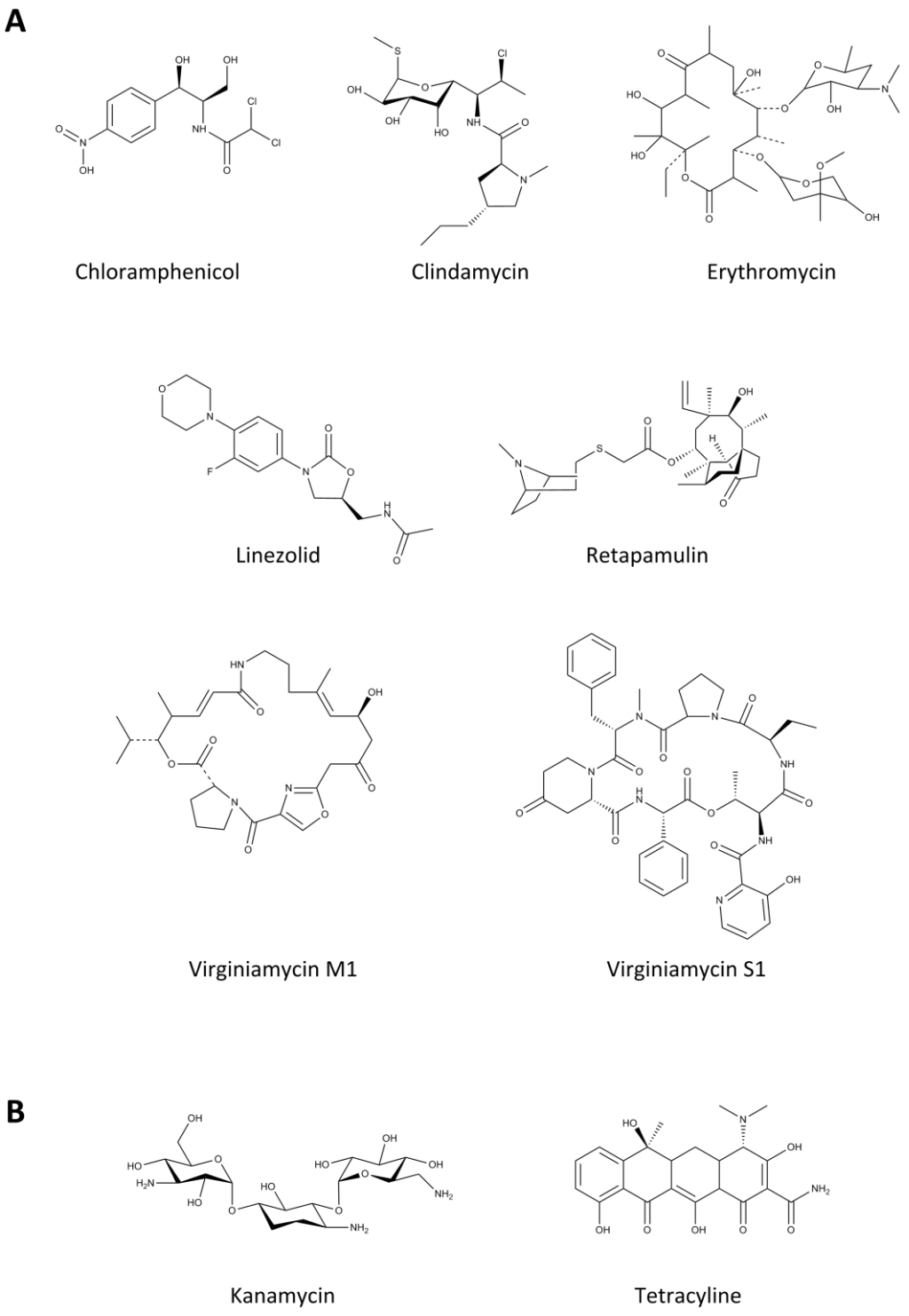

Figure 1.4. Chemical structures of representative members of clinically used antibiotics that target the ribosome. (A) Classes targeting the 50S subunit; phenicols (chloramphenicol), lincosamides (clindamycin), macrolides (erythromycin), oxazolidinones (linezolid), pleuromutilins (retapamulin), type A streptogramins (virginiamycin M1), and type B streptogramins (virginiamycin S1). **(B)** Classes targeting the 30S subunit; aminoglycosides (kanamycin) and tetracyclines (tetracycline).

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1.3 Mechanisms of resistance to ribosomal antibiotics

Ribosomally active antibiotics play important roles in clinical and veterinary medicine. Of the 30S targeted antibiotic classes, aminoglycosides and tetracyclines are important agents for the treatment of both Gram-positive and Gram-negative infections. Of the antibiotic classes that bind the 50S subunit in the vicinity of the PTC, seven are used in clinical and veterinary medicine for the treatment of primarily Gram-positive infections, namely the amphenicols, lincosamides, macrolides, oxazolidinones, pleuromutilins, and type A and B streptogramins. Although all of these antibiotics have potent antibacterial effects and form a large proportion of the antibiotic armamentarium, the development of resistance poses a threat to their continued efficacy.

Most Gram-negative bacteria are intrinsically resistant to 50S targeted antibiotics due to the inability of these predominantly hydrophobic drugs to penetrate the Gram-negative outer membrane (Wilson, 2014). By contrast, the mechanisms by which Gram-positive and sensitive Gram-negative bacteria resist the action of ribosomal antibiotics can be separated into three groups; alteration of the target, through modification and dissociation; alteration of the drug, through modification or destruction; and decreased accumulation, through efflux. Examples of each mechanism are described below.

1.3.1 Resistance through antibiotic modification and degradation

A number of plasmid-borne enzymes have been identified that mediate drug inactivation through destruction or degradation of aminoglycosides, amphenicols, lincosamides, macrolides, both type A and B streptogramins, and tetracyclines. However, to date, no resistance genes encoding enzymes that modify or destroy antibiotics belonging to either the pleuromutilin or oxazolidinone class have been identified. Drug modification generally acts through the addition of functional groups which prevent the antibiotic from binding to

its target site, whereas drug degradation relies on cleavage of chemical bonds that are integral to the compounds activity.

1.3.1.1 Inactivation of aminoglycosides

Resistance to the aminoglycoside antibiotics in both Gram-positive and Gram-negative pathogens is predominantly mediated by modification of the drug through *N*-acetylation, *O*-adenylation or *O*-phosphorylation. Addition of these bulky chemical groups to any one of a number of OH or NH_2 groups on the aminoglycoside molecule disrupts the interaction of the drug with its target, the ribosomal RNA. Over 100 aminoglycoside modifying enzymes (AMEs) catalysing such reactions have been identified, and they are grouped into three classes according to the type of chemical modification catalysed: aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphotransferases (APHs) (Ramirez and Tolmasky, 2010).

Some AMEs are bifunctional enzymes, possessing the ability either to perform more than one type of chemical modification, or to modify more than one type of substrate. The enzyme AAC (6')-APH(2") is one such bifunctional AME, and is one of the most common mediators of aminoglycoside resistance in *S. aureus*. The N-terminal portion of this enzyme possesses AAC activity, whilst the C-terminal domain has APH activity. The dual catalytic activity of this enzyme appears to have arisen as a consequence of fusion of two ancestral genes to yield a single, bifunctional enzyme possessing a broad substrate profile that encompasses nearly all the members of the aminoglycoside class (with the exception of streptomycin and spectinomycin) (Zhang *et al*., 2009).

1.3.1.2 Inactivation of amphenicols

The most frequently encountered inactivating modification of chloramphenicol is acetylation of the drug via chloramphenicol acetyltransferases (CATs). CATs can be classified into two groups based on differences in their structure: class A and class B (also known as xenobiotic

CATs). Both groups of enzyme act by catalysing O-acetylation of the 3-hydroxyl group of chloramphenicol. The synthetic chloramphenicol analogue florfenicol is refractory to the action of CATs due to the replacement of the hydroxyl group at C-3 by a fluorine moiety. (Schwarz *et al*., 2004).

1.3.1.3 Inactivation of macrolides

Erythromycin is the target of esterases that render the compound inactive through hydrolysis of its macrolactone ring. The genes encoding these enzymes were originally identified in *E. coli* and have been designated *ere(A)* and *ere(B)* (Ounissi and Courvalin, 1985, Arthur *et al*., 1986). Although *ere* mediated degradation is not a common resistance mechanism, *ere* genes confer a very high-level of resistance (≥1600 µg / ml in *E. coli*) (Nakamura *et al*., 2000) and have been found on mobile genetic elements [that provide a means of dissemination (Biskri and Mazel, 2003, Plante *et al*., 2003). It is of note that an erythromycin esterase has also been identified in a clinical strain of *S. aureus* (Wondrack *et al*., 1996).

A second enzymatic mechanism for inactivation of macrolides is phosphorylation. Three genes encoding macrolide kinases have been identified; two in *E.coli,* [*mph(A) and mph(B)*]*,* and one in *S. aureus*; [*mph(C)*]. Genes encoding macrolide kinases are not currently widespread, but confer high-level (>2 mg/ml) resistance to 14- and 16- member macrolides and ketolides (Matsuoka and Sasaki, 2004, Wright, 2005).

1.3.1.4 Inactivation of lincosamides

Lincomycin nucleotidyltransferases catalyse inactivation of lincomycin and clindamycin through adenylation. To date, seven distinct lincomycin nucleotidyltransferases have been identified and designated *lnu(A)* to *(F)* (http://faculty.washington.edu/marilynr/ermwebA.pdf, last accessed 28th September 2015). Although the majority of these genes are confined to Gram-positive bacteria, *lnu(F)* was identified in *E. coli* (Zhao *et al*., 2014).

1.3.1.5 Inactivation of streptogramins

The *vat* genes encode enzymes that inactivate type A streptogramins through acetylation (Wright, 2005). Whereas the *vgb* genes encode lyases that degrade type B streptogramins (Allignet *et al*., 1988). Type A and B streptogramins are usually co-administered, as binding of a streptogramin A antibiotic to the ribosome increases the affinity of the streptogramin B binding site, resulting in synergy between the two compounds (Harms *et al*., 2004). Inactivation of one of the two compounds results in a small decrease in susceptibility to the synergistic mixture. However, plasmids encoding both *vgb* and *vat* genes have been shown to confer full resistance to synergistic streptogramin combinations (Allignet *et al*., 1998, Allignet and El Solh, 1999).

1.3.2 Resistance through target site modification

Modification of the binding sites of antibiotics that target the PTC and peptide exit tunnel decreases the affinity of the drugs for their target. These alterations can occur through post-transcriptional modification of 23S rRNA, via mutations in genes encoding rRNA and proteins, or through a direct interaction of a protein or peptide with the ribosome.

1.3.3.1 Mutations in rRNA and ribosomal proteins

The majority of ribosome targeting antibiotics interact exclusively with rRNA (Wilson, 2009, Wilson, 2014). Thus, mutations to rRNA genes can result in modification of the conformation of antibiotic binding sites and thereby lead to decreased affinity of the drug. Most bacterial pathogens possess multiple alleles of rRNA genes organised into operons, with the notable exceptions of *Helicobacter pylori* (Versalovic *et al*., 1996)*, Mycobacterium* spp*.* (Menendez *et al*., 2002) and select propionibacteria, which have only one or two rRNA operons [(Ross *et al*., 1997)]. Thus the effects of resistance conferring mutations in a single rRNA gene are frequently masked by the multiple copies of wild-type drug susceptible rRNA being simultaneously expressed. In Gram-positive pathogens such as *Staphylococcus* and

Streptococcus spp., which possess six and four rRNA operons respectively, mutation of the majority of rRNA alleles is generally required for resistance. For example, the MICs of macrolide and oxazolidinone antibiotics have been shown to increase in parallel with the number of mutated rRNA operons (Prunier *et al*., 2003, Besier *et al*., 2008, Marshall *et al*., 2002).

However, mutations do not always have to arise independently; the processes of mutation and recombination may also act together to evolve an antibiotic-susceptible 23S rRNA to a more resistant form. This is exemplified by mutational resistance to linezolid that has emerged in bacterial genera harbouring four to six copies of the 23S rRNA gene (Eliopoulos *et al*., 2004). Such linezolid-resistant isolates carry resistance mutations in domain V of the 23S23S rRNA, and the mutations are usually found in two to five of the alleles encoding the 23S rRNA. These multiple resistance alleles are not the result of independent mutational events, but are instead the consequence of 'gene conversion'; following emergence of a resistance mutation in a single 23S rRNA allele, this mutation is then introduced to one or more of the 'susceptible' alleles by homologous recombination within the cell, thereby converting them to the resistant form (Lobritz *et al*., 2003).

Although ribosomal proteins do not normally interact directly with ribosome targeted antibiotics, mutations to genes encoding ribosome proteins, which are normally present as a single copy, can in some instances result in resistance. This appears to arise indirectly, through changes in protein structure inducing conformational changes in rRNA. For example, mutations in in *rplD* and *rplV*, which encode ribosomal proteins L4 and L22, have been shown to confer macrolide resistance in clinical strains of *Streptococcus pneumoniae, Haemophilus influenzae,* and *Staphylococcus aureus* (Peric *et al*., 2003, Prunier *et al*., 2005, Franceschi *et al*., 2004). The L4 and L22 proteins converge to form a narrowing in the peptide exit tunnel adjacent to the macrolide-binding site (Figure 1.3, Page 10) and mutations in these proteins

result in distortions to the erythromycin-binding pocket and decreased affinity of the ribosome for the drug (Lovmar *et al*., 2009a).

1.3.3.2 Modification of rRNA

Modification of single nucleotides within the 23S rRNA in the vicinity of the PTC by mono or dimethylation can prevent a range of antibiotics from binding their target sites. Methyltransferase enzymes of the erythromycin rRNA methylase (Erm) family mediate resistance to macrolide, lincosamide and streptogramin B antibiotics (MLS_B phenotype) via methylation of a single adenine of the 23S rRNA (nucleotide position 2058 in *E. coli*). The Erm family represent the most widespread mechanism of resistance to macrolides and lincosamides in pathogenic bacteria, and to date over 30 different members of this family have been identified (Roberts, 2008). Another methylase, termed Cfr (for chloramphenicol/florfenicol resistance), methylates nucleotide A2053 (*E. coli* numbering) in the 23S rRNA. The *cfr* gene was originally identified as a plasmid-borne amphenicol resistance determinant in *Staphylococcus sciuri*, however, further analysis of the *cfr* phenotype showed that the methylase also mediates resistance to lincosamides, oxazolidinones, pleuromutilins and streptogramin As ($PhLOPS_A$ phenotype), providing the first example of horizontally-acquired resistance to the oxazolidinone linezolid (Long *et al*., 2006). Recently, a Cfr homologue, Cfr(B), was identified in two clinical isolates of *Enterococcus faecium* (75% amino acid identity to Cfr). As with c*fr*, c*fr(B),* was found on a mobile genetic element and when expressed in *S. aureus* also conferred the $PHLOPS_A$ phenotype (Deshpande *et al*., 2015). Although linezolid resistance is currently rare amongst clinical strains (Mendes *et al*., 2014) the potential for wide spread dissemination of *cfr* genes has necessitated the development of second generation oxazolidinones, such as tedizolid and radezolid, which are refractory to Cfr-mediated resistance (Shaw and Barbachyn, 2011, Mendes *et al*., 2014).

The detection of *cfr* organised into an operon also encoding *erm(B)* (termed the *mlr* operon) in the clinical methicillin-resistant *Staphylococcus aureus* strain CM05 is a particularly worrying development (Locke *et al*., 2012, Smith and Mankin, 2008). The *mlr* operon mediates resistance to members of all seven clinically used antibiotic classes that bind in the vicinity of the PTC and is present in a in a highly mobile region of the chromosome, providing potential for further dissemination (Locke *et al*., 2012).

Methylation of the 16S rRNA can also lead to antibiotic resistance. This mechanism is of particular importance for self-protection in aminoglycoside producing Streptomyces spp. and Micromonospora spp. (Cundliffe, 1989). These organisms possess chromosomally encoded 16S methyltransferases (16S-RMTases) that methylate rRNA at numerous different positions (Wachino and Arakawa, 2012). At present, this resistance mechanism is not frequently found in pathogenic bacteria, however, clinical isolates of *Acinetobacter baumannii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showing high-level aminoglycoside resistance, due to horizontally acquired 16S-RMTases, have been identified (Galimand *et al*., 2003, Yokoyama *et al*., 2003, Tada *et al*., 2014).

1.3.3.3 Dissociation of drugs from the ribosome

Protein mediated dissociation of antibiotics from their ribosomal binding site represents an important mechanism of resistance to the tetracyclines. A family of determinants termed the ribosomal protection proteins (RPPs) mediate resistance to tetracyclines in both Gram-positive and Gram-negative bacteria (Connell *et al*., 2003a). To date, more than ten RPPs have been identified (http://faculty.washington.edu/marilynr/tetweb1.pdf last accessed 28th September 2015), and detailed study of the two most prevalent RPPs [Tet(M) and Tet(O)] has provided much of our understanding of this family of resistance proteins (Dönhöfer *et al*., 2012, Spahn *et al*., 2001). RPPs exhibit homology to bacterial house-keeping G-proteins that participate in protein synthesis, particularly the enzyme elongation factor-G

(EF-G) that associates transiently with the ribosome to bring about translocation, and their site and mode of binding to the ribosome appear to closely mimic that of EF-G. However, whilst domain IV of the EF-G protein acts to reach into the decoding site of the ribosome where it prompts translocation by driving the movement of A-site tRNA to the P-site, the equivalent domain of the RPPs directly interacts with the binding site of tetracycline, thereby acting to displace the drug from the ribosome (Dönhöfer *et al*., 2012, Spahn *et al*., 2001).

Resistance to antibiotics of the macrolide class can be mediated by the expression of short peptides of four to six amino acids (Tenson *et al*., 1996, Tenson *et al*., 1997, Tenson and Mankin, 2001, Verdier *et al*., 2002, Vimberg *et al*., 2004, Lovmar *et al*., 2006, Lovmar *et al*., 2009b). This resistance mechanism was discovered when a pentapeptide encoding open reading frame (ORF) within the gene encoding *E. coli* 23S rRNA was shown to confer low-level erythromycin resistance (Tenson *et al*., 1996). Subsequently, characterisation of random pentapeptide libraries has identified several alternative peptides capable of mediating resistance to different drugs within the macrolide class (Tenson *et al*., 1997). These peptides are thought to act *in cis,* directly upon the ribosomes from which they are translated, to mediate displacement of macrolide antibiotics from their binding sites (Tenson and Mankin, 2001, Verdier *et al*., 2002, Vimberg *et al*., 2004). The expression of macrolide resistance peptides could therefore increase the amount of drug-free ribosomes within the cell, permitting continued protein synthesis in the presence of macrolide antibiotics (Tenson and Mankin, 2001, Verdier *et al*., 2002, Vimberg *et al*., 2004). It is currently unclear whether this erythromycin resistance mechanism is physiologically important, as due to the association of 23S rRNA with ribosomal proteins and sequestration of the Shine-Dalgarno region of the ORF within the secondary structure of 23S rRNA, the pentapeptide encoding mini-gene is not expressed unless the 23S rRNA gene is subjected to mutation or fragmentation (Tenson and Mankin, 2001). No peptides that confer resistance to any other PTC-targeted drugs have been identified to date (Vimberg *et al*., 2004).

1.3.3 Resistance through active efflux

By restricting the accumulation of an antibiotic inside the cell, a bacterium may experience only low (sub-inhibitory) antibiotic concentrations at the drug target, even under conditions where extracellular concentrations of antibiotic are high. Bacteria have evolved an array of membrane-located proteins capable of actively transporting intracellular molecules across the membrane(s) and out of the cell. A subset of these efflux transporters includes within their substrate profile one or more antibiotic classes, and can therefore act to reduce the intracellular accumulation of antibacterial drugs. The transporter proteins involved in antibiotic resistance are distributed across five protein families; the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance-nodulation-cell division (RND) family, multidrug and toxin extrusion (MATE) family, and ABC family (Piddock, 2006, Li and Nikaido, 2009). To achieve active transport of antibiotics against a concentration gradient, these transporters require a source of energy; efflux proteins belonging to the SMR, MFS, MATE and RND families all utilise the proton-motive force generated by cellular metabolism to drive transport, whilst transporters of the ABC family derive the necessary energy from the hydrolysis of ATP.

In Gram-positive bacteria, resistance to targets that bind the ribosome at the PTC is mediated by MFS and ABC efflux systems, some of which are specific for one antibiotic class, whilst others are capable of recognising and exporting a wide range of structurally-diverse molecules.

1.3.2.1 Efflux of PTC-targeted antibiotics by MFS proteins

The MFS efflux proteins found in Gram-positive bacteria can be split into two groups, those that confer multidrug resistance (MDR) and those specific to a single antibiotic class. Examples of MDR MFS systems include the chromosomally encoded *S. aureus* proteins LmrS and MdeA. Expression of LmrS in an antibiotic-hypersensitive strain of *E. coli* has been shown to confer resistance to amphenicols, lincosamides, macrolides, and oxazolidinones, in

addition to a broad range of antibiotics and antibacterial compounds (Floyd *et al*., 2010). Similarly, overexpression of MdeA in *S. aureus* has been shown to result in resistance to streptogramins, as well as numerous non-ribosomal antibiotics (Huang *et al*., 2004). Although these MDR MFS proteins have shown to confer resistance *in vitro*, they have not yet been associated with antibiotic resistance in clinical strains of *S. aureus*.

In contrast, the tetracycline-specific Tet efflux proteins are the predominant mechanism of tetracycline resistance in Gram-negative bacteria and are also found in Gram-positive organisms. Tetracycline was the first antibiotic for which efflux was identified as a mechanism of resistance (McMurry *et al*., 1980). Since that time, over 20 different tetracycline efflux proteins have been described, and all are members of the MFS transporter family (http://faculty.washington.edu/marilynr/tetweb1.pdf, last accessed 28th September 2015). The widespread emergence of resistance to first and second generation tetracyclines, through efflux and ribosomal protection, led to a systematic search for a new generation of tetracycline analogues. The result was the glycylcyclines, of which the best known is tigecycline; these antibiotics are not susceptible to current efflux mechanisms. However, it has been demonstrated *in vitro* that mutations to pre-existing Tet efflux pumps can elicit resistance to glycylcyclines, suggesting a route by which resistance to these drugs may develop with sustained clinical use (Guay *et al*., 1994, Chopra and Roberts, 2001).

A second example group of drug specific MFS proteins with an important role in clinical antibiotic resistance can be found in the Mef proteins. These proteins mediate resistance to 14- and 15- membered macrolides, but not to 16- membered macrolides, type B streptogramins or lincosamides. This resistance profile is frequently observed in clinical strains of streptococci and is designated the M-phenotype (Sutcliffe *et al*., 1996). The resistance genes that underpin this resistance mechanism, *mef(A)* and *mef(E)* were originally identified in *Streptococcus pyogenes* (Clancy *et al*., 1996) and *Streptococcus pneumoniae* respectively (Tait-Kamradt *et al*., 1997).

1.3.2.2 Efflux of ribosomal antibiotics by ABC proteins

The first MDR ABC transporter to be characterised in bacteria was the LmrA protein of *Lactococcus lactis* (van Veen *et al*., 1998). Due the homology exhibited by LmrA to an ABC transporter frequently found to be upregulated in mammalian tumour cells (MDR1), LmrA has been the subject of multiple functional and structural studies (Poelarends *et al*., 2002, Federici *et al*., 2007). When expressed in an antibiotic-hypersensitive strain of *E. coli* LmrA confers resistance to a broad range of structurally dissimilar compounds, including multiple antibiotics, several of which target the PTC (Poelarends *et al*., 2002). However, the physiological function of this protein in its natural host is unclear (van den Berg van Saparoea *et al*., 2005), and, although a homologue has been identified in *S. aureus* (van Veen and Konings, 1998), LmrA mediated MDR is not currently common in pathogenic bacteria.

Members of a large subgroup of ABC proteins, termed the ABC-F or type II ABC subfamily, confer resistance to several structurally diverse classes of antibiotic that target the PTC and exit tunnel of the 50S ribosomal subunit (Kerr *et al*., 2005, Reynolds *et al*., 2003). Although frequently cited as mediators of efflux, a long standing controversy exists regarding the mechanism by which ABC-F proteins confer antibiotic resistance. Due to the lack of a definitive explanation for their mechanism of action, this subgroup of ABC proteins was chosen as the subject of this thesis and is discussed in more detail below.

1.4 The ABC-F sub-family of ABC proteins mediates resistance to ribosomally active antibiotics

Members of the ABC-F subgroup of ABC superfamily proteins specifically mediate resistance to PTC-targeted antibiotics through a poorly characterised mechanism and, for that reason, are the subject of this study. The classification of ABC-F proteins within the context of the ABC superfamily is discussed below (Section 1.4.2). Additionally the phylogeny, phenotype, and potential mechanism of ABC-F proteins involved in antibiotic resistance are outlined.

1.4.1 The ABC domain

Proteins of the ABC superfamily are universally distributed across all three domains of life (Dassa and Bouige, 2001). These proteins are named for their stereotypical ABC domains (also known as the nucleotide binding domain, NBD) that permit binding and hydrolysis of ATP, releasing energy to drive a wide variety of biological processes (Davidson *et al*., 2008, Dorrian, 2009). ABC domains, each around 200 amino acids in length, are characterized by five unequivocally conserved short sequence motifs, which should be present in the following order to qualify as an ABC ATPase: the Walker A motif, a highly conserved glutamine residue in the Q-loop, the signature motif which is characteristic of ABC ATPases, the Walker B motif and finally, a highly conserved histidine residue within the H-loop or Switch region (Davidson *et al*., 2008, Dorrian, 2009, Dassa, 2011) (Figure 1.5 A).

A single ABC domain is composed of two structurally distinct sub-domains: A RecA-like domain, which contains the Walker A and B motifs, and a helical domain that is unique to ABC ATPases and includes the signature motif (Figure 1.5 B) (Davidson *et al*., 2008, Dorrian, 2009, Dassa, 2011). The two domains are joined by two loops, one of which is the Q-loop (Davidson *et al*., 2008, Dorrian, 2009, Dassa, 2011) and is important for interactions of the ABC domain with other cellular proteins (Davidson *et al*., 2008, Chen *et al*., 2014). Crystal structures of ABCs bound to ATP have shown that ATP co-ordination requires formation of a "nucleotide-sandwich dimer" containing two ABC domains (Jones and George, 1999, Hopfner *et al*., 2000, Smith *et al*., 2002). The two monomers in the complex align in a "head to tail" orientation, resulting in the formation of two ATP binding pockets in which the Walker A and B motifs of one monomer act in concert with the signature motif of the other monomer to bind the nucleotide (Jones and George, 1999, Hopfner *et al*., 2000, Smith *et al*., 2002). Thus the binding and hydrolysis of ATP results in conformational changes throughout the complex that are used to elicit biological effects.

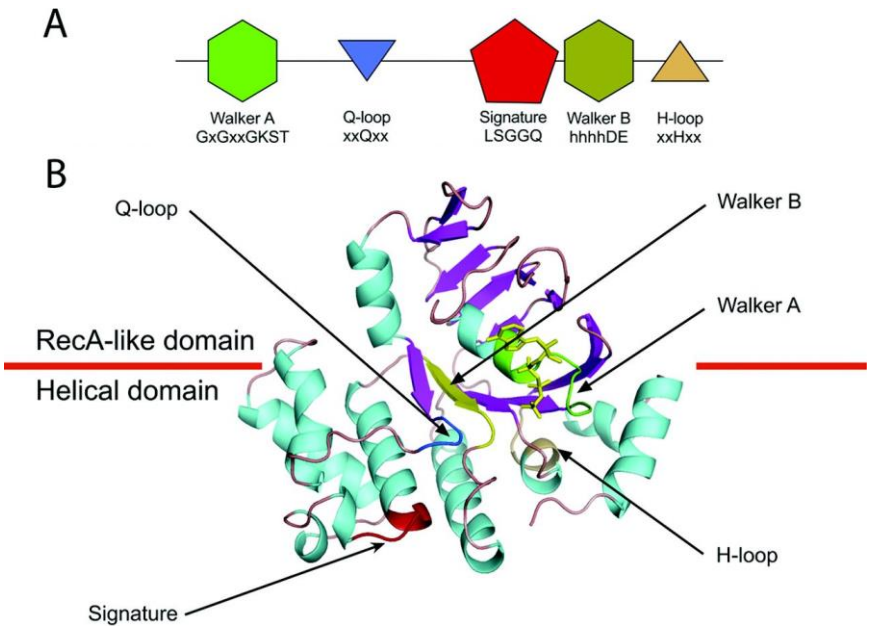


Figure 1.5. The motifs and domain architecture characteristic of ATP binding cassette proteins. (A) A linear representation of the five unequivocally conserved motifs characteristic of ABC ATPases. The Walker A motif and the Walker B motif, shown as large polygons, form the nucleotide-binding fold of the P-loop ATPase family. The signature motif is unique to ABC proteins and also interacts with ATP. The Q-loop and H-loop, represented as triangles, contain single covered residues. These residues make contacts with the γ-phosphate of ATP and the Q-loop is also involved in interactions with other cellular proteins. The consensus sequence of each motif is shown using the single-letter amino acid code, where x and h stand for any and hydrophobic amino acids respectively. **(B)** Localization of conserved motifs within the structure of an ABC monomer (HisP of *Salmonella typhimurium*, PDB: 1B0U (Hung *et al*., 1998)). The same colour code as for the linear representation is used. The RecA-like domain is shown in purple, the helical domain in cyan. Figure adapted from Dassa (2011).

1.4.2 Classification of ABC proteins

ABC transporters represent the architecture most frequently associated with ABCs. Here, two cytoplasmic, hydrophilic ABC domains associate with two hydrophobic trans-membrane domains (TMDs). The four domains may be expressed as separate polypeptides or as various configurations of domain fusions (Davidson *et al*., 2008, Dassa, 2011). The resulting functional complex uses the energy derived from ATP-hydrolysis to drive conformational changes that result in the cellular import or export of a wide variety of substrates (Jones and George, 1999). However, it has become increasingly evident that ABCs also participate in cellular processes other than transport (Dassa and Bouige, 2001).

Classification by Dean *et al*. (Dean *et al*., 2003) of ABC systems in accordance with sequence homology in eukaryotic ABC proteins has divided the ABC superfamily into seven sub-families, designated ABC-A to ABC-G (Dean *et al*., 2003, Kerr, 2004). The majority of these groups constitute ABC transport systems. However, two groups, ABC-E and ABC-F, are composed of proteins that possess tandemly repeated ABC domains and lack TMDs. ABC-E and ABC-F proteins have been shown to play non-transport roles in all three domains of life (Kerr, 2004).

More recently, an alternative system for classification of ABC proteins was proposed by Elie Dassa (Dassa, 2011) in which proteins are grouped based on sequence homology and function. This analysis includes sequences from all three domains of life, and separates ABC proteins into three major classes and multiple further sub-classes. The three classes appear to correlate to architecture and function of ABC proteins; class 1 is made up of the majority of ABC exporters, class 2 comprises soluble proteins with two tandemly repeated ABC modules and no recognised TMDs, and class 3 is predominantly composed of bacterial importers that require an extracellular or periplasmic substrate binding domain for their function (Figure 1.6).

The large subgroup of ABC proteins that specifically mediate resistance to PTC-targeted antibiotics are found within the ABC-F and class 2 groups of the Dean and Dassa classification schemes respectively. In accordance with the most current literature (Boel *et al*., 2014, Chen *et al*., 2014, Lenart *et al*., 2015), these proteins are referred to throughout this study as antibiotic resistance (ARE) ABC-F proteins.

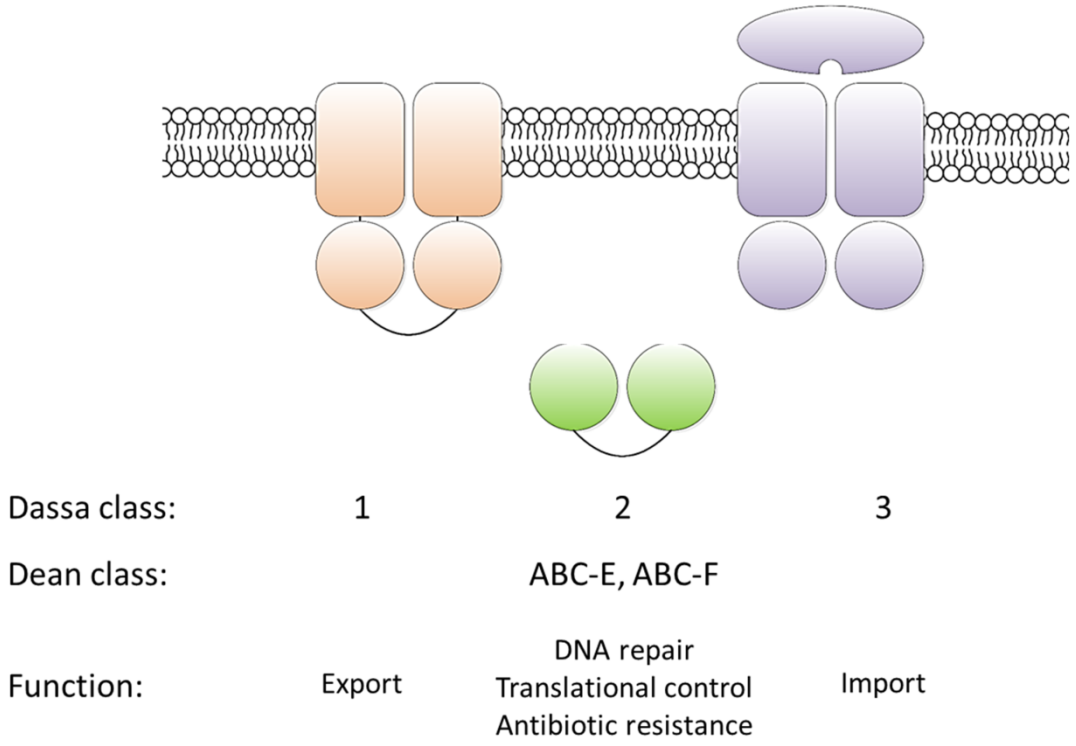


Figure 1.6. Classification of ABC proteins. The Dassa scheme of classification separates the ABC superfamily into three major classes. Class 1 and class 3 are canonical ABC transport proteins, comprising a functional complex of two hydrophobic TMDs and two hydrophilic intracellular ABC domains, class 3 proteins are frequently also associated with an extracellular or periplasmic SBD. In contrast, class 2 proteins, which comprise two tandemly repeated ABC domains, lack recognised TMDs. These Dassa classes broadly correlate with the function of their members, class 1 are primarily export proteins, whereas class 3 proteins are primarily involved with import within bacteria. Class 2 appear to play predominantly non-transport roles, although the mechanism of antibiotic resistance proteins within this class is unknown. Within the dean classification scheme, ABC proteins with tandemly repeated NBDs and no associated TMDs fall within the ABC-E and ABC-F sub-families, which correlate to class 2 in the Dassa system (Dean *et al*., 2003, Kerr, 2004, Dassa, 2011).

1.4.3 The ARE ABC-F proteins mediate resistance to ribosomally active antibiotics

In Gram-positive bacteria, a sub-group of ABC-F proteins mediates resistance to antibiotics that exert their action on the ribosome. These proteins are found in both antibiotic-producing bacteria (e.g. the streptomycetes), and in pathogenic bacteria that include the staphylococci, streptococci, enterococci, and, in one instance, the Gram-negative zoonotic pathogen *Pasteurella multocida*. (Figure 1.7). Collectively, the ARE ABC-F family of proteins mediates resistance to the majority of antibiotic classes that bind to either the PTC or peptide exit tunnel of the ribosome, including the ketolides (Reynolds and Cove, 2005), lincosamides (Novotna and Janata, 2006, Singh *et al*., 2002), macrolides (Ross *et al*., 1990), oxazolidinones (Wang *et al*., 2015), phenicols (Wang *et al*., 2015), pleuromutilins (Gentry *et al*., 2008), streptogramin As (Allignet *et al*., 1992, Singh *et al*., 2002), and streptogramin Bs (Ross *et al*., 1990) (Figure 1.7). However, no single ARE ABC-F determinant confers resistance to every listed class, and three phenotypic resistance profiles are distinguished in clinical isolates. Combined lincosamide, streptogramin A (and sometimes pleuromutilin) resistance, referred to as the LS_A (or LS_AP) phenotype is conferred by *vga*-, *lsa*- and *sal*- type genes (Tesse *et al*., 2013, Singh *et al*., 2002, Hot *et al*., 2014), concurrent resistance to macrolides, streptogramin Bs (and sometimes ketolides) (MS_B phenotype) by the *msr*-type determinants (Ross *et al*., 1990, Reynolds and Cove, 2005), and resistance to phenicols and oxazolidinones by the recently identified *optrA* gene (Wang *et al*., 2015) (Figure 1.7).

In antibiotic-producing bacteria, ARE ABC-F mediated resistance appears to be specific to the particular antibiotic produced by each species. However, as levels of resistance to other PTC-targeted antibiotics have predominantly not yet been determined, it is unclear whether these proteins mediate any further cross resistance. It is of note that the lincomycin-resistance determinant *lmr(C)* from *Streptomyces lincolnensis* has been shown to additionally confer low-level erythomycin resistance (Peschke *et al*., 1995), suggesting that ARE ABC-F proteins from antibiotic-producing bacteria may show similar resistance spectra to those found in bacterial pathogens.

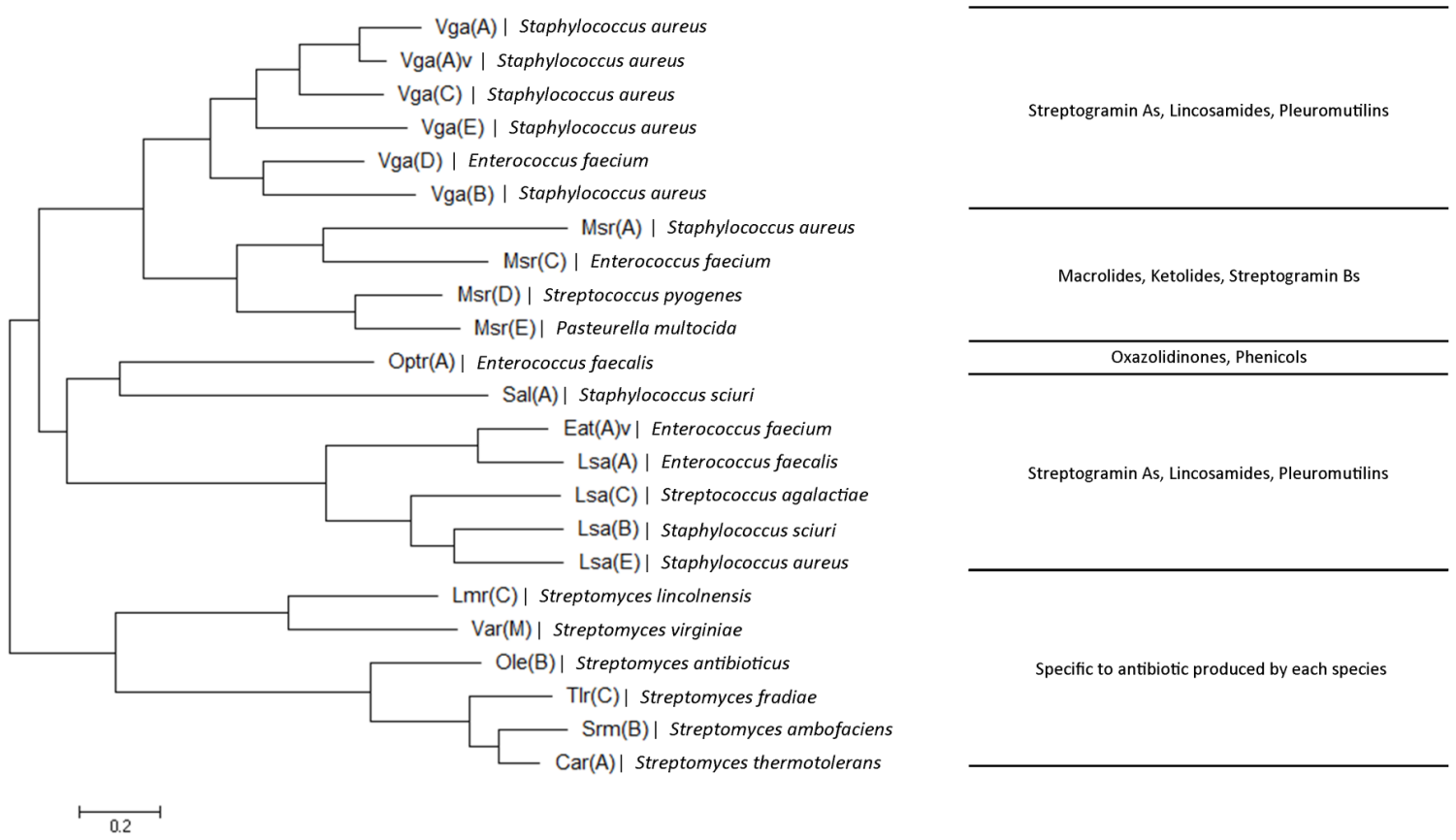


Figure 1.7. Phylogenetic tree and antibiotic resistance profiles of the ARE ABC-F proteins found in Gram-positive pathogens and producer organisms. An overview of the resistance phenotypes conferred by the different subgroups of determinant are shown at the right of the figure (although variations in individual resistance phenotypes within each subgroup are not). Alignments were performed using the MUSCLE alignment program (Edgar, 2004) and the phylogenetic tree was generated using the maximum likelihood method. Analysis was performed using the MEGA 6.0.6 software package (Tempe, AZ, USA).

1.4.4 Domain architecture of ARE ABC-F proteins

As with all members of the ABC-F sub-family, ARE ABC-F proteins comprise a single polypeptide containing two ABC domains separated by an inter-domain linker of ~80 amino acids and do not include any predicted membrane-spanning domains. Equally, genes encoding ARE ABC-F proteins are not linked in operons with any identified genes encoding TMDs. Within ABC-F proteins of Gram-positive pathogens, Pfam (Marchler-Bauer *et al*., 2015) identifies a conserved domain separate from the two ABC domains within the linker region (PF12848 or ABC_tran_2); however, this domain is not recognised in ARE ABC-F proteins of antibiotic-producing bacteria (Figure 1.8). The linker region of *msr*-type ARE ABC-F proteins is additionally identified as a Q-linker (Wootton and Drummond, 1989). Q-linkers comprise a class of interdomain sequences that are typically rich in glutamine and other hydrophilic amino acids and have a characteristic spacing of hydrophobic amino acids. Amongst ARE ABC-F proteins, the Q-linker appears to be confined to *msr*-type determinants as the inter-domain linkers of OptrA and Vga- or Lsa- type proteins do not meet these criteria. Studies of Vga(A) have shown the amino acid composition of sequence of the linker to influence the spectrum of resistance mediated by the protein. Initially, a Vga(A) variant, $Vga(A)_{LC}$, that conferred enhanced levels of resistance to lincosamide antibiotics in comparison to Vga(A), was identified in several clinical isolates of *Staphylococcus haemolyticus*. Sequence analysis showed the $Vga(A)_{LC}$ protein to differ from Vga(A) by seven amino acid substitutions, four of which were shown to influence the resistance phenotype and were clustered within an 18 amino acid region of the linker. Subsequently, a detailed mutational analysis of the Vga(A) interdomain linker found that the sequence of an eight amino acid stretch (amino acids 212 to 220) was responsible for determining different levels of resistance to streptogramin As, lincosamides and pleuromutilins. With one amino acid substitution (K219T) conferring high levels of resistance to antibiotics of all three classes.

Both OptrA and Vga-type proteins possess a C-terminal extension of ~40 and ~110 amino acids respectively. Deletion of the final 18 amino acids of this extension in Vga(A) resulted in a four-fold reduction in the level of resistance conferred to a streptogramin A antibiotic, suggesting that the C-terminal extension of both proteins may be of functional importance (Jacquet *et al*., 2008), although no corresponding modification of OptrA has been performed.

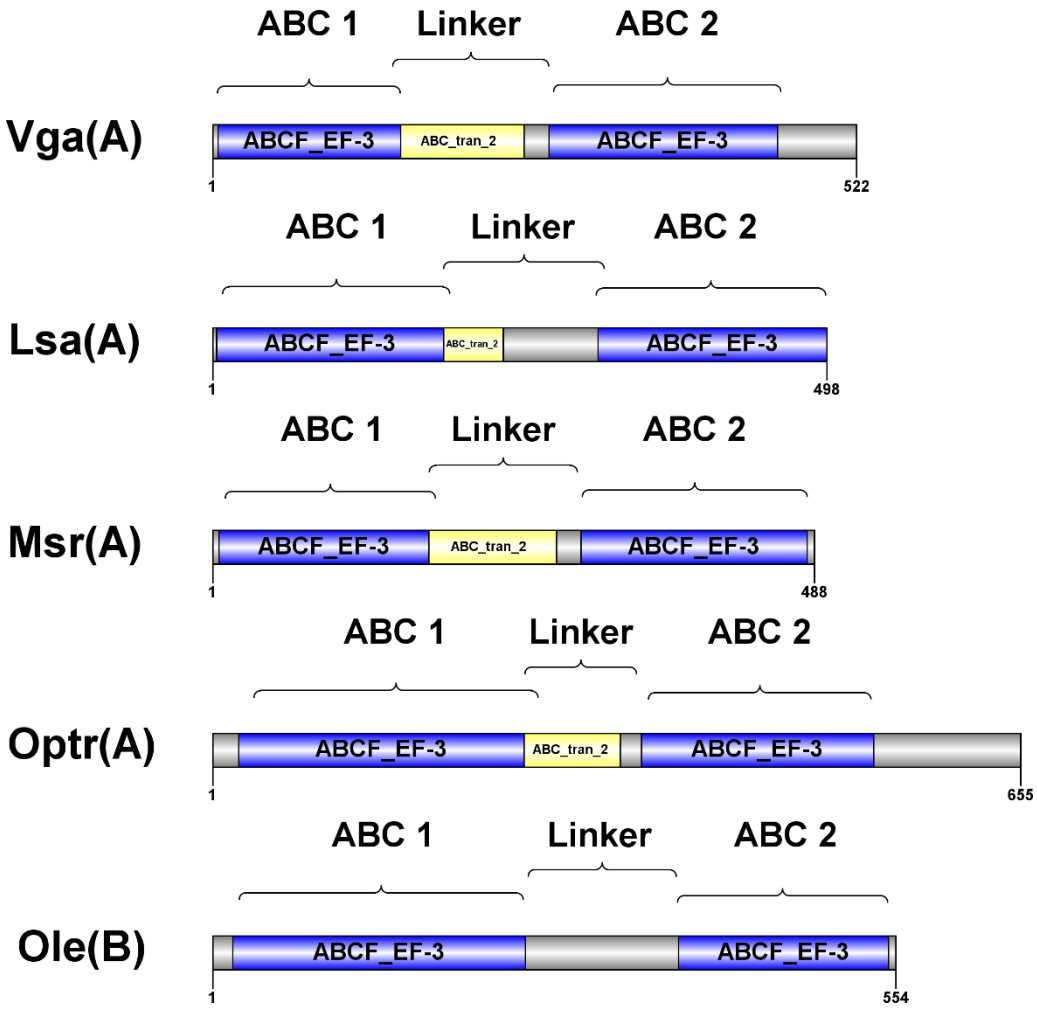


Figure 1.8. Predicted domains of representative members of five ARE ABC-F subgroups. Domains were assigned using the CDD: NCBI's conserved domain database (Marchler-Bauer *et al*., 2015).

1.5 Genetic environment and clinical prevalence of ARE ABC-F resistance determinants

Several studies have attempted to document the genetic environment of ARE ABC-F genes in clinical strains of staphylococci and enterococci. From the resulting data, it is clear that ARE ABC-F determinants are found in a diverse array of genetic environments; with *optrA, vga-*, *lsa-* and *msr*-type genes frequently located within mobile genetic elements, such as plasmids and transposons, or integrated into the chromosome where they may be coupled with factors to facilitate their mobilisation (Ross *et al*., 1990, Singh *et al*., 2002, Novotna and Janata, 2006, Gentry *et al*., 2008, Schwendener and Perreten, 2011, Hauschild et al., 2012, Tesse *et al*., 2013, Hot *et al*., 2014, Wang *et al*., 2015, Li et al. 2016). Although the majority of ARE ABC-F determinants found in Gram-positive pathogens confer acquired resistance, three genes, *lsa(A)*, *msr(C)* and *sal(A)*, are chromosomally encoded and thought to be intrinsic resistance determinants of *E. faecalis, E. faecium and Staphylococcus sciuri* respectively (Singh, Weinstock and Murray 2002; Hot, Berthet and Chesneau 2014; Singh, Malathum and Murray 2001). As such, *lsa(A)* is responsible for the innate resistance of *E. faecalis* to antibiotics of the lincosamide and streptogramin A classes, *sal(A)* confers the same resistance phenotype in *S. sciuri*, and *msr(C)* confers macrolide and streptogramin B resistance in *E. faecium*, although there is some debate as to whether the gene an intrinsic to all *E. faecium* isolates (Werner, Hildebrandt and Witte 2001).

The prevalence of ARE ABC-F determinants in clinical strains of staphylococci and enterococci exhibiting resistance to antibiotics targeting the large ribosomal subunit has been documented in several reports. For macrolide and streptogramin B antibiotics, it is evident that although ribosomal methylation is the predominant mechanism of resistance to these drugs, *msr*-type determinants, particularly *msr(A)*, can also be contributing factors (Gatermann, Koschinski and Friedrich 2007). The frequency at which *msr(A)* is found as the

sole resistance determinant in strains exhibiting the MS_B phenotype varies between <10% (Nawaz *et al.* 2000) to 100% (Eady *et al.* 1993) amongst different hospitals and geographical areas . However, from the available data, it can be roughly estimated that *msr(A)* is the sole determinant underlying around a tenth of strains exhibiting the MS_B phenotype (Martineau *et al.* 2000; Szczuka *et al.* 2016; Aktas *et al.* 2007; Gatermann, Koschinski and Friedrich 2007; Eady *et al.* 1993). Equivalent information regarding the clinical prevalence of *vga*-type determinants is lacking, however a study in which a large staphylococcal strain collection (5,676 strains) was screened for retapamulin resistance found that although the incidence of resistance was low (~1%), the *vga(A)* and *vga(A)v* genes were the sole resistance genes associated with this phenotype (Gentry *et al.* 2008). Similarly, as the *optrA* gene has only been recently identified, only one study has investigated its prevalence in human clinical isolates. This screen of 595 clinical enterococcal isolates from a Chinese hospital detected *optrA* in 2% of strains (Wang *et al.* 2015). A concurrent screen of 290 enterococcal strains isolated from animals in a variety of locations within China found *optrA* to be present in 15.9% of strains (Wang *et al.* 2015). It is also of note that *optrA* has been found co-resident with the ribosomal methylase *cfr* on a plasmid isolated from Staphylococcus sciuri; a particularly worrying development given that *optrA* confers resistance to tedezolid, the second commercially available oxazolidinone, which ordinarily shows activity against staphylococcal strains expressing *cfr* (Li *et al.* 2016).

1.6 The mechanism of ARE ABC-F mediated resistance

The mechanism by which the ARE ABC-F proteins mediate antibiotic resistance has been a subject of long-standing controversy, with two competing hypotheses having each attracted considerable support; antibiotic efflux and ribosomal protection (Ross *et al*., 1990, Reynolds *et al*., 2003, Kerr *et al*., 2005, Chesneau *et al*., 2005, Kerr, 2004, Nunez-Samudio and Chesneau, 2013, Lenart *et al*., 2015). The efflux hypothesis posits that ARE ABC-F proteins associate with as-yet-unidentified TMDs to form a functional efflux complex capable of

exporting antibiotics out of the cell, whilst the ribosomal protection hypothesis suggests that these resistance proteins act instead to reduce the accessibility or affinity of the antibiotic binding sites in the 50S subunit, thereby directly protecting the translational machinery from antibiotic-mediated inhibition (Reynolds *et al*., 2003, Kerr *et al*., 2005, Reynolds, 2005, Dorrian, 2009) (Figure 1.9). Evidence in support of each hypothesis is discussed below.

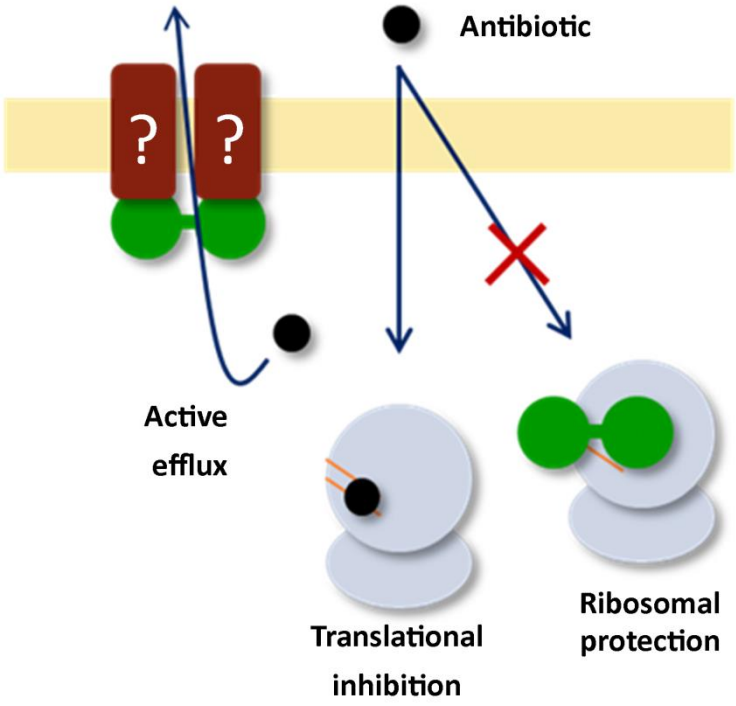


Figure 1.9. Potential mechanisms of ARE ABC-F mediated resistance. In the absence of the resistance determinant translation is inhibited by the antibiotic, arresting growth of the bacterial cell. ARE ABC-F proteins (green) are proposed to rescue translation from inhibition either by recruiting an unknown membrane-spanning protein to promote active efflux of the antibiotic (left), or, by displacing the antibiotic from its ribosomal binding site (right). Figure adapted from (Kerr *et al*., 2005).

1.6.1 Evidence in support of a mechanism of ARE ABC-F mediated resistance driven by efflux

Early studies of the first ARE ABC-F resistance determinant to be characterised, *msr(A)*, showed that expression of the *msr(A)* gene was associated with reduced intracellular accumulation of radiolabelled erythromycin in *S. aureus* (Ross *et al*., 1990) and was not associated with any erythromycin inactivating enzyme activity (Ross *et al*., 1989). Erythromycin accumulation experiments showed an initial uptake phase, followed by a decrease in the level of intracellular drug, which was abolished by addition of arsenate and strongly inhibited by dinitrophenol, leading to the hypothesis that Msr(A) functions in tandem with unidentified TMDs to drive ATP-dependent drug efflux. Subsequently, a similar study was conducted using the *vga(A)* variant *vga(A)*$_{LC}$, which like msr(A) was shown to mediate ATP-dependent reduced accumulation of its target antibiotic, radio-labelled lincomycin, when expressed in *S. haemolyticus* (Novotna and Janata, 2006).

Interpretation of the data generated by these accumulation studies is complicated by the observation that decreased accumulation of ribosomally active antibiotics can also result from protection of their target site. Addition of an excess of unlabelled erythromycin, or a streptogramin B antibiotic (which has an overlapping binding site to erythromycin), has been shown to result in decreased accumulation of radiolabelled erythromycin in *S. aureus* (Reynolds *et al*., 2003, Barre *et al*., 1986). Furthermore, the presence of the *erm(B)* ribosomal methylase has been shown to confer decreased erythromycin accumulation in *S. pyogenes* (Canton *et al*., 2005). This phenomenon is thought to occur due to the fact that erythromycin passes through the cell membrane by passive diffusion and accumulates in cells because it bind to ribosomes, which act as a “sink” and thereby maintain the concentration gradient (Barre *et al*., 1986, Capobianco and Goldman, 1990). Protection of the erythromycin

ribosomal binding site by a competing antibiotic or through methylation would abolish this gradient and thereby negate the driving force for import (Kerr *et al*., 2005).

A further observation that has been proposed as evidence for efflux can be found is studies of the sub-cellular localisation of ARE ABC-F proteins. Investigation of Vga(A) localisation though western blotting of cell fractions generated by centrifugation has shown that in clinical strains of *Staphylococcus epidermidis* the protein is solely present in the membrane fraction (Chesneau *et al*., 2005). Similarly, expression of *vga(A)* from the *vegII* promoter in a laboratory strain of *S. epidermidis* primarily resulted in membrane localisation of Vga(A), with around 20% of the protein present in the cytosol (Chesneau *et al*., 2005). A similar analysis of the cellular localisation of a maltose-binding protein tagged fusion of the N-terminal domain of Ole(B), an ARE ABC-F protein that mediates self-protection in the oleandomycin producer *Streptomyces anitbioticus*, showed the protein to be present in both the membrane and cytosolic fractions (Olano *et al*., 1995). Membrane localisation of multiple ARE ABC-F proteins appears to support the efflux hypothesis.

In order for ARE ABC-F proteins function to drive efflux they must co-opt TMDs. In classical ABC-transport proteins it is the TMDs that confer substrate specificity (Dassa and Bouige, 2001). However, if ARE ABC-F proteins are “hijacking” existing TMDs then a requirement is that they either confers substrate specificity themselves or alter the specificity of pre-existing TMDs. Work by Jacquet *et al*. investigated the ATPase activity of Vga(A) and, after demonstrating that ATPase activity was essential for biological function, found that pristinamycin IIA inhibited ATPase activity in a non-competitive manner, suggesting a direct interaction between the protein and antibiotic (Jacquet *et al*., 2008).

More recently, an investigation into the cellular localisation of *msr(D)*, a streptococcal macrolide resistance determinant, was proposed to provide the first example of an ARE ABC-F protein working in tandem with an efflux transporter. In streptococci, the *msr(D)* gene is

co-transcribed with the *mef(E)* MFS macrolide efflux transporter from a conserved operon found within large chromosomal elements that resemble defective transposons (Santagati *et al*., 2000, Gay and Stephens, 2001, Del Grosso *et al*., 2002). Through the use of *E. coli* strains expressing GFP-tagged Msr(D) and Mef(E) the sub-cellular localisation of each protein was investigated. Expression of GFP-tagged Msr(D) alone showed the protein was homogeneously distributed throughout the cytoplasm and expression of GFP-tagged Mef(E) alone showed several fluorescence spots throughout the bacterium. However, co-expression of both resistance determinants resulted in localisation of Mef(E) at the poles of the cell. Pull down assays were subsequently used to show an interaction of Msr(D) and Mef(E), leading to the conclusion that Msr(D) might act as an enhancer of macrolide efflux mediated by the MFS Mef(E) (Nunez-Samudio and Chesneau, 2013).

1.6.2 Evidence in support of a mechanism of ARE ABC-F mediated resistance through ribosomal protection

There is currently a lack of direct experimental evidence in support of ARE ABC-F mediated ribosomal protection. However, indirect evidence in support of this hypothesis can be found in the apparent correlation between resistance phenotypes of ARE ABC-F proteins and the binding sites of their target antibiotics within the 50S subunit. For example, the *msr*-type determinants mediate resistance to macrolide and streptogramin B antibiotics, which bind close to the peptide exit tunnel within the 50S subunit (Hansen *et al*., 2002, Tu *et al*., 2005). Whereas *vga*- and *lsa*- type determinants give resistance to streptogramin A, lincosamide, and pleuromutilin drugs, which bind to overlapping sites within the PTC (Harms *et al*., 2004, Tu *et al*., 2005, Davidovich *et al*., 2007). The apparent requirement of ARE ABC-F resistance phenotypes for an overlap in ribosomal binding site, rather than common chemical composition of their target drugs, suggests that these proteins mediate their effects while their target drugs are ribosomally associated.

Further indirect support for ribosomal protection can be found through analysis of the functions of members of the ABC-F sub-family not involved in antibiotic resistance. These proteins have been shown to function in non-transport processes, including regulation of translation. Three of these translational regulators have been shown to be associated with ribosomes. In fungi, the highly conserved ABC-F protein eEF3 binds to the ribosome and promotes release of tRNA from the E site. In eukaryotes and archaea the ABC-F protein ABCE1 acts in concert with both initiation factors and release factors to facilitate recycling of ribosomes, rescue of stalled ribosomes and subsequent promotion of initiation. In bacteria, the ABC-F protein EttA preferentially binds to the 70S ribosome with fMet-tRNA at the P-site and regulates translation in response to changes in the cellular ATP: ADP ratio. Thus, ABC-F proteins from all domains of life have been shown to interact with the ribosome and influence translation.

1.7 Objectives

This study was undertaken due to the lack of a coherent explanation for the mechanism governing antibiotic resistance mediated by ABC-F proteins. The primary objective of this work was therefore to provide a definitive answer to the efflux vs protection question, or to identify any other plausible mechanistic explanations. In order to achieve this goal, studies of ARE ABC-F proteins were required. The first step in this process was the optimisation of conditions for the heterologous expression and subsequent purification of members of the ARE ABC-F subfamily. Secondly, appropriate assays were established with which to distinguish between efflux and ribosomal protection and screening for conditions to promote crystallisation of ARE ABC-F proteins was performed. In tandem with biochemical approaches using purified protein, complimentary methods using DNA manipulation and bacteriology were used to further investigate ARE ABC-F resistance phenotypes. Finally, the data derived from this study was assessed in the context of the wider scientific literature to

re-evaluate the hypotheses for the ARE ABC-F resistance mechanism and an appropriate model has been proposed.

2. Materials and Methods

2.1 Materials

2.1.1 Bacterial strains, plasmids, and growth media

Strains of bacteria used in this study are listed in Table 2.1. Plasmids used during this study are listed in Table 2.2. Routine culture of *E. coli* strains used Luria-Bertrani (LBB) broth and (LBA) agar (Sigma-Aldrich, Poole, UK). *S. aureus* and *E. faecalis* strains were propagated using Mueller-Hinton broth (MHB) and agar (MHA) (Oxoid, Basingstoke, UK) unless otherwise stated. For long-term maintenance bacterial strains were stored as saturated cultures supplemented with 16% (v/v) glycerol at -80°C. Purified plasmid DNA was stored at -20°C.

2.1.2 Chemicals and antibiotics

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. All solutions were prepared using deionised water, and either heat-sterilised (121°C for 20 min) or filter-sterilised (0.22 µm filters Merck Millipore, Darmstadt, Germany). Antibiotics used in this study are listed in Table 2.3, along with related solvents and suppliers. Radiolabelled ^{3}H-lincomycin was from Quotient Bio Research (Nottingham, UK).

Table 2.1. Bacterial strains used in this study

Organism	Strain	Comments	Reference / Source
Enterococcus faecalis	ATCC 29212	Source of *lsa(A)* gene	Kim *et al*., (2012) ATCC
Enterococcus faecium	E1679	Source of *msr(C)* gene	Gift from W. Schaik (Department of Medical Microbiology, University Medical Center Utrecht).
Staphylococcus aureus	RN4220	Restriction deficient derivative of *S. aureus* 8325-4. Used for routine cloning and antibiotic susceptibility testing.	Fairweather *et al*., (1983)
	CYL557	RN4220 containing the accessory plasmid pLL2787 encoding the φ11 *int* gene.	Luong and Lee, (2007)
Escherichia coli	DH5α	For routine cloning procedures. Genotype: *fhuA2 lac*(del)U169 *phoA glnV44* Φ80' *lacZ*(del)M15 *gyrA96 recA1 relA1 endA1 thi*-1 *hsdR17*	Invitrogen (Paisley, UK)
	XL10-Gold	For use during site directed mutagenesis. Genotype: *endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte* Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *tetR* F'[*proAB lacI*qZΔM15 Tn10(*TetR Amy CmR*)]	Agilent Technologies (Cheshire, UK)
	BL21 (λDE3) Gold	For expression of genes from the T7 promoter. Genotype: F-*ompT gal dcm lon hsdSB*(rB- mB-) λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*])	Agilent Technologies
	BL21-CodonPlus (λDE3) RIL	For expression of genes from the T7 promoter. Designed to enhance expression of genes with a high AT-content. Genotype: B F- *ompT hsdS*(rB- mB-) *dcm+ Tetr gal* λ(DE3) *endA Hte* [*argU ileY leuW Camr*]	Agilent Technologies
	CopyCutter EPI400	Used to maintain plasmid pC19Saluc at low-copy number, can be induced to higher copy number for improved plasmid yield. Genotype: F-*mcrA*Δ(*mrr-hsd*RMS-*mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX*74 *recA1 endA1 araD139* Δ(ara,leu)7697 *galU galK* λ– *rpsL nupG tonA* Δ*pcnB dhfr*.	Epicentre (Madison, WI, USA)

Table 2.2. Plasmids used during this study.

Vector	Comments	Reference / Source
pEPSA5	*S. aureus* / *E. coli* shuttle vector. For expression of genes in *S. aureus* from pT5X xylose-inducible promoter. ampicillin^{ER}, chloramphenicol^{SR}	Forsyth *et al*., (2002) Elitra Pharmaceuticals, (San Diego, CA, USA)
pLL39	Single copy integration vector for integration at the L54a attP or φ11 attP sites on the *S. aureus* chromosome. spectinomycinl^{ER}, tetracycline^{SR}	Luong and Lee, (2007) Gift from C.Y. Lee (University of Arkansas for Medical Sciences, USA)
pIVEX2.3d	Optimised for expression in *in vitro* T7 T/T systems, also used for expression in *E. coli*. Encodes a non-cleavable C-terminal 6-His tag. ampicillin^{ER}	Roge and Betton, (2005) 5 PRIME (Düsseldorf , Germany)
pDIA17	Encodes *lacI* gene. Co-transformed with pIVEX2.3d to improve transformation efficiency. chloramphenicol^{ER}	Roge and Betton, (2005) Gift from J.M. Betton (Inst Pasteur, Paris, France)
pET28a-Tev	Modified pET28a expression vector encoding an N-Terminal 10xHis-tag followed by a Tobacco Etch Virus (TEV) protease recognition site. Vector used to allow affinity purification. kanamycin^{ER}	Novagen
pET28a-SUMO	Modified pET28a expression vector encoding N-Terminal 6xHis and SUMO (type 3) tags followed by a recognition site for U1p protease. Vector used to improve protein solubility allow affinity purification. kanamycin^{ER}	Novagen
pET28a-MAL	Modified pET28a expression vector encoding an N-Terminal 6xHis and maltose binding protein (MBP) tags followed by a PreScission protease cleavage site. Vector used to improve protein solubility allow affinity purification. kanamycin^{ER}	Novagen
pET28a-GST	Modified pET28a expression vector encoding N-Terminal 6xHis and Glutathione S-transferase (GST) tags followed by a PreScission protease cleavage site. Vector used to improve protein solubility and allow affinity purification. kanamycin^{ER}	Novagen
pBEST	Contains firefly luciferase (*luc)* gene under the control of the *E. coli tac* promoter. ampicillin^{ER}	Promega (Madison, WI, USA)
pBEST19*Sa*luc	Contains *luc* gene under the control of the strong staphylococcal Cap1A promoter and a staphylococcal origin of replication. ampicillin^{ER}, chloramphenicol^{SR}	Acquired from J.K. Hobbs (University of Leeds, unpublished work)
pBSSC12	Source for *vga(E)*	Schwendener and Perreten, (2011) Gift from V. Perreten (University of Berne, Switzerland)

* ^{ER} denotes resistance phenotype in *E. coli.* ^{SR} denotes resistance phenotype in *S. aureus*

Table 2.3. Antibiotics used in this study

Compound	Solvent	Source
Ampicillin	Water	Sigma-Aldrich (Poole, UK)
Blasticidin S	Water	Merck Chemicals (Nottingham, UK)
Chloramphenicol	50% (v/v)Ethanol	Sigma-Aldrich
Erythromycin	50% (v/v) Ethanol	Sigma-Aldrich
Fluorphenicol	50% (v/v) Ethanol	Sigma-Aldrich
Kanamycin	Water	Sigma-Aldrich
Leucomycin	DMSO	Santa Cruz Biotechnology (Dallas, TX, USA)
Lincomycin	Water	Sigma-Aldrich
Linezolid	DMSO	Sigma-Aldrich
Puromycin	Water	Sigma-Aldrich
Retapamulin	DMSO	Gift from GlaxoSmithKline (Brentford, UK)
Sparsomycin	DMSO	Gift from E. Cundliffe (University of Leicester, UK)
Spiramycin	DMSO	Santa Cruz Biotechnology
Tetracycline	Water	Sigma-Aldrich
Tiamulin	DMSO	Sigma-Aldrich
Tylosin	Water	Santa Cruz Biotechnology
Virginiamycin M	DMSO	Sigma-Aldrich

2.2 Molecular biology techniques

2.2.1 Determination of DNA concentration

DNA was quantified spectrophotometrically by reading absorbance at 260 nm using a P300 nanophotometer (Implem, Munich, Germany). The purity of DNA samples was assessed by determining the ratios of absorbance at 260 nm / 280 nm and 260 nm / 230 nm (Sambrook *et al*., 2001).

2.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* DH5α and BL21 strains using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, Limburg, Netherlands) according to manufacturer's instructions. The same kit was also used for extracting plasmid DNA from *S. aureus*. However, in order to degrade staphylococcal cell walls buffer P1 was supplemented with 100 µg/ml recombinant lysostaphin (affinity purified in our laboratory) and the P1-cell suspension was incubated at 37°C for 10 min before proceeding to alkaline lysis.

In order to isolate large quantities of plasmid pC19SA5 for use in *in vitro* T/T assays (2.5.2) 100 ml of LBB containing 100 µg/ml ampicillin was inoculated with a single colony of *E. coli* CopyCutter EPI400 carrying the pC19SA5 plasmid and grown overnight at 37°C with vigorous aeration. The following day, 500 ml of LBB containing 100 µg/ml ampicillin and 500 µl CopyCutter Induction Solution (Epicentre, Madison, WI, USA) was inoculated with 56 ml of saturated overnight culture and grown for 4 h. Cells were harvested by centrifugation (5000 *x g*, 15 min) and then processed using a HiSpeed Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions.

2.2.3 Isolation of genomic DNA

The PurElute Bacterial Genomic Kit (Edge BioSystems, Gaithersburg, MD, USA) was used to isolate chromosomal DNA from *E. faecalis* and *E. faecium* according to manufacturer's instructions. This kit was also used to isolate chromosomal DNA from *S. aureus*; however,

prior to the initial cell lysis step, spheroplast buffer was supplemented with lysostaphin and incubated as detailed in Section 2.2.2.

2.2.4 Concentration of DNA

DNA was concentrated by ethanol precipitation. Briefly, a 1/10 volume of 3M sodium acetate (pH 5.2) was added to one volume of DNA before addition of 2-3 volumes of 100% (v/v) ethanol. The resulting suspension was placed at -80°C for 1 h and subsequently centrifuged at 30 000 *x g* for 30 min at 4°C. Supernatant was carefully removed from the resultant DNA pellet. The pellet was washed in 2-3 volumes of 70% (v/v) ethanol and centrifuged at 30 000 *x g* for 10 min at 4°C. Finally, the supernatant was carefully removed and the DNA pellet was air-dried for 5 min and resuspended in an appropriate volume of 10 mM Tris-Cl (pH 8.5).

2.2.5 Polymerase chain reaction

Oligonucleotide primers were obtained from Eurofins MWG operon (Ebersberg, Germany), diluted to 100 pmol/µl in 10 mM Tris-Cl (pH 8.5), and stored at -20°C. Nucleotides were from Promega (Madison, WI, USA). PCRs were performed in a Techne TC-3000 thermal cycler (Bibby Scientific, Staffordshire, UK) using Q5 High-Fidelity DNA Polymerase (New England Biolabs [NEB], Ipswich, Massachusetts, USA). Q5 High-Fidelity DNA Polymerase catalysed PCRs were carried out according to manufacturer's instructions and reaction conditions were optimised where appropriate. Primer melting temperatures and corresponding PCR annealing temperatures were calculated using the "NEB-Tm calculator" at http://tmcalculator.neb.com/ (last accessed 27-07-2015). Oligonucleotide primers used for PCR during this study are listed in Table 2.4.

Table 2.4. Oligonucleotide primers used in this study. Restriction sites and sequences complementary to the pLL39 vector used for Gibson assembly are italicised and lower case, codons targeted for mutagenesis are underlined, and expression signals (promoters and ribosome binding sites) are shown in bold.

Designation	Description	Sequence (5'-3')
VgaA_E105Q_fwd	For mutagenesis of catalytic glutamine in N-terminal ABC	AAAAATCCAGAACTGCTATTAGCAGATCAGC
		CAACAACTAACTTAGATAATAAC
VgaA_E105Q_rev		GTTATTATCTAAGTTAGTTGTTGGCTGATCT
		GCTAATAGCAGTTCTGGATTTTT
VgaA_K219T_fwd	For mutagenesis of the *vgaA* interdomain linker	AAGTATGGCTTTGCTCCCGTTATTTTGCCTTC
		AGATAAACTTAAGTTTTTCG
VgaA_K219T_rev		CGAAAAACTTAAGTTTATCTGAAGGCAAAAT
		AACGGGAGCAAAGCCATACTT
VgaA_cap1a39_fwd	For introduction *of vga(A)* into plasmid pLL39 under control of the *cap1A* promoter	*agcttagatctaatcgaattcgagctcggtacc*CAGA
		GT**TTGCAA**AATATACAGGGGATTATA**TATA**
		ATGGAAAACAAGAAAGGAAAAT**AGGAGG**T
		TTATATGGCAAAAATAATGTTAGA
VgaA_cap1a39_rev		*tgtaggtaataaaaaagcttgcatgcctgcaggtcgac*
		*tctagaggat*TTATTTATCCAAATTTCTTTTTC
		A
VgaA_pEPSA5_fwd	For introduction of *vga(A)* into plasmid pEPSA5	GCTA*gagctc*AT**AAGAGG**ATGAGAAAATATG
		GCAAAAATAATGTTAGAGGGACT
VgaA_pEPSA5_rev		GACTCggatccTTATTTATCCAAATTTCTTTTTT
		CAT
Cfr_pEPSA5_fwd	For introduction of *cfr* into plasmid pEPSA5	GCAAC*gagctc*ATAGGAGGATCAGAAAATAT
		GAACTTTAACAACAAAACGAAATATGG
Cfr_pEPSA5_rev		GCAAC*ggatcc*TTACTGGGAGTTCTGATAGTT
		ACC
VgaA_pIVEX_fwd	For introduction of *vgaA* into plasmid piVEX2.3d	CT*ccatgg*CAAAAATAATAGAGGGAC
VgaA_pIVEX_rev		CT*gggccc*TTTATCCAAATTTCTTTTTTC
VgaA_28a_fwd	For introduction of *vga(A)* into pET28a based vectors	GCGCTAGGATCCATGGCAAAAATAATGTTA
		GAGG
VgaA_28a_rev		CGACTA*AAGCTT*TTATTTATCCAAATTTCTTT
		TTTCAT
VgaAv_28a_fwd	For introduction of *vga(A)v* into pET28a based vectors	GCGCTA*ggatcc*ATGAAAATATTGTTAGAGGC
		TCTTC
VgaAv_28a_rev		CGACTA*ctcgag*TCAATTATCTAAATTTCTTTT
		CTCGT

Table 2.4 continued. Oligonucleotide primers used in this study.

VgaC_28a_fwd	For introduction of *vga(C)* into pET28a based vectors	GCGCTA*ggatcc*ATGGTTTTACTAGAGGC
VgaC_28a_rev		CGACTA*aagctt*TTACTCCTTTAACTTACTTTTT
		T
VgaE_28a_fwd	For introduction of *vga(E)* into pET28a based vectors	GCGCTA*ggatcc*
		ATGTTATTATTTGAAGGTACAT
VgaE_28a_rev		CGACTA*aagctt*TTATAGTTTTTTAGTCAGTTC
		TT
MsrA_28a_fwd	For introduction of *msr(A)* into pET28a based vectors	GCGCTA*ggatcc*ATGGAACAATACACCATCAA
		AT
MsrA_28a_rev		CGACTA*aagctt*TTAGGTGATGTCGTGCAG
MsrC_28a_fwd	For introduction of *msr(C)* into pET28a based vectors	gcgcta*ggatcc*ATGGAAAATTTAGCAGTAAAT
		ATAAC
MsrC_28a_rev		CGACTA*gagctc*TTAAAAATTTCTCGTAAGTA
		CTTTTTTTA
LsaA_28a_fwd	For introduction of *lsa(A)* into pET28a based vectors	GCGCTA*ggatcc*ATGTCGAAAATTGAACTAAA
		ACAAC
LsaA_28a_rev		CGACTA*aagctt*TTATGATTTCAAGACAATTTT
		TTTATCTGT

2.2.6 Colony PCR

Colony PCR was performed using GoTaq Green Master Mix (Promega). A colony PCR primer stock was prepared by diluting forward and reverse primers to 2.5 pmol/µl in a volume of 200 µl. Single colonies were suspended in 20 µl phosphate buffered saline (PBS). Reactions were composed of 12 µl GoTaq Green Master Mix, 10 µl colony PCR primer stock (1 µM) and 2 µl PBS-colony suspension. Reactions underwent an initial denaturation at 95°C for 10 min, followed by 18 cycles of 30 s denaturation at 95°C, 30 s annealing at 50°C and 1 min/kb extension at 72°C. Reactions were held at 4°C before analysis by agarose gel electrophoresis (Section 2.2.8).

2.2.7 Site-directed mutagenesis

The Agilent Technologies Quick Change Lightning site-directed mutagenesis kit was used according to manufacturer's guidelines. Mutagenised plasmids were transformed into ultra-competent cloning strain *E. coli* XL1-Gold (Table 2.1). The plasmid DNA of putative mutants was extracted (Section 2.2.2) and mutations were confirmed by DNA sequencing (Section 2.2.14).

2.2.8 Agarose gel electrophoresis

Agarose gels (30 ml) were composed of between 0.5% and 1.2% (w/v) agarose in TAE buffer containing 3 µl 10 000X SYBR Safe DNA stain (Life technologies, Carlsbad, CA, USA). Agarose content was tailored to give optimum resolution for the DNA fragments undergoing analysis. DNA samples were mixed with 6x loading dye (Promega) in a 6:1 ratio and loaded into the gel. Electrophoresis was typically performed at 90 V for 28 min in TAE buffer. For quantification of purified ribosomal RNA, denaturing agarose gels were composed of 1% agarose (w/v) in TBE buffer containing 1M urea and SYBR-Gold DNA stain (Life Technologies). Electrophoresis of rRNA was performed at 90 V for 60 min in TBE buffer.

2.2.9 Restriction digests

Restriction enzymes and appropriate buffers were from NEB. Preparative digests of PCR products and plasmids were performed in a reaction volume of 50 µl. Typically, DNA was digested simultaneously with two restriction enzymes in reactions comprising 5 µl CutSmart buffer, 2 µg DNA and 800 units of each restriction enzyme. Reactions were incubated at 37°C for 3 h and digested DNA was gel or column purified (Section 2.2.10).

2.2.10 Purification of DNA

DNA amplified by PCR reactions was purified using the QIAquick PCR Purification Kit (Qiagen). In order to remove enzymes and exchange buffers following restriction digests, digested and undigested DNA was separated by electrophoresis (Section 2.2.8), the relevant band was

excised from the agarose gel and DNA was extracted from the gel using the QIAquick Gel Extraction Kit according to manufacturer's instructions (Qiagen).

2.2.11 DNA Ligation

DNA ligations were performed using the NEB Quick Ligation Kit according to manufacturer's guidelines. Reactions typically contained 50 ng of digested plasmid and a 3 molar excess of digested insert. Ligation reactions were incubated at 25°C for 10 min.

2.2.12 Transformation of *E. coli* strains

Chemically competent *E. coli* strains were prepared according to the Inoue method (Sambrook *et al*., 1989). For transformation, cells were thawed on ice and 1-10ng of DNA was added. The cell-DNA suspension was incubated on ice for 30 min, heat-shocked at 42°C for 30 s and allowed to recover on ice for 2 min. SOC media (Hanahan, 1983) was added to a final volume of 1 ml and the resulting culture was grown at 37°C for 1 h with vigorous aeration. Finally, volumes of culture were plated onto LBA containing appropriate antibiotics for selection and incubated at 37°C overnight. If the cells were transformed with a ligation reaction, resulting colonies were screened for the presence and correct orientation of a ligated insert by colony PCR (Section 2.2.6).

2.2.13 Transformation of *S. aureus* strains

Electrocompetent strains of *S. aureus* were prepared according to a method previously defined by Monk *et al.* (Monk *et al*., 2012). For electroporation, cells were thawed on ice for 5 min before being centrifuged (10 000 × g for 1 min) and resuspended in 50 µl of 10% glycerol (v/v) and 500 mM sucrose (filter sterilized). Plasmid DNA (1-50 ng for pEPSA5, 1-5 µg for pLL39) was added to the cells, the DNA-cell suspension was transferred to a 1-mm electroporation cuvette (Geneflow, Elmhurst, UK), and pulsed at 21 kV/cm, 100 Ω, and 25 µF. TSB supplemented with 500 mM sucrose (filter sterilized) was immediately added to a final volume of 1 ml and the resulting culture was incubated at 37°C for 1 h before plating

onto MHA containing appropriate antibiotics for selection. Plates were incubated at 37°C overnight.

2.2.14 DNA sequencing

DNA sequencing was used to confirm the correct orientation and sequence of cloned inserts following ligation (Section 2.2.11), and the introduction of point mutations following site directed mutagenesis (Section 2.2.7). Sequencing was performed by Beckman-Coulter Genomics (MA, USA) by Sanger sequencing (Sanger *et al*., 1977).

2.3 Determination of susceptibilities to antibacterial agents

2.3.1 Standardised susceptibility testing

MICs of antibacterial agents against various *S. aureus* strains were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill *et al*., 2012), by exposing bacteria to serial two-fold dilutions of antibiotic agents in MHB. *S. aureus* strains carrying the pEPSA5 plasmid (Table 2.2) used MHB supplemented with 2% (w/v) d-xylose to induce expression of resistance genes from the pT5Xpromoter.

2.3 Expression of recombinant proteins

2.3.1 Auto-induction media

Media for the production of proteins using the auto-induction method was prepared as previously described (Studier, 2005).

2.3.2 Small-scale expression trials

Prior to large scale production of proteins, expression levels of recombinant ARE ABC-F genes were assessed in 10 ml cultures. A single-colony from a freshly transformed *E. coli* BL21(DE3λ) strain was used to inoculate 10 ml of auto-induction media in a 50 ml sterile Falcon tube. This culture was grown at 25°C with vigorous aeration for 1-2 d. An aliquot of 1 ml was removed and centrifuged at 10 000 *x g* 2 min to harvest cells. The resultant pellet was

resuspended in 200 µl lysis buffer (see Table 2.4, Page 52 for buffer composition) and subjected to three cycles of rapid freezing at -80°C followed by thawing at 4°C. In order to separate the insoluble and soluble fractions, lysate was centrifuged at 30 000 *x g* at 4°C for 10 min. The supernatant corresponding to the soluble fraction was added in a 1:1 ratio to 2x sodium dodecyl sulphate (SDS) loading buffer (Sambrook *et al*., 2001). The pellet, corresponding to the insoluble fraction, was dissolved in 300 µl 8M urea solution and added in a 1:1 ratio to 2x SDS loading buffer. Samples were analysed by SDS-PAGE (Section 2.3.4).

2.3.3 Preparative scale expression of recombinant proteins

FusB was expressed as previously described (Cox *et al*., 2012). For large-scale expression of Vga(A) and Lsa(A), a single colony of BL21 (DE3) Gold containing pIVEX2.3d-*vga(A)* or BL21 (DE3) CodonPlus RIL containing pET28aSUMO-*lsa(A)* was used to inoculate 400 ml of sterile auto-induction media in 2 L baffled flasks. The cultures were incubated at 25°C (for *vgaA* expression) or 18°C (for *lsaA* expression) for 3 (*vgaA*) or 4 (*lsaA*) d with vigorous aeration. Cells were harvested by centrifugation (6 000 *x g*, 20 min), washed with PBS, weighed, and stored as pellets at -80°C.

2.3.4 SDS-PAGE

Protein samples containing SDS-PAGE loading buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue, 200 mM DTT and 0.125 M TrisHCl [pH6.8]) (Sambrook *et al*., 2001) were denatured by heating to 95°C for 5 min prior to separation by SDS-PAGE. Electrophoresis using pre-cast 4-20% (w/v) gradient polyacrylamide gels (Expedeon, Cambridge, UK) in TEO-Tricine buffer was performed at 175 V for 35 min. When improved resolution was required, acrylamide gels were cast with stacking and resolving layers of 4% (w/v) and 14% (w/v) acrylamide/bis-acrylamide (Severn Biotech, Kidderminster, UK) respectively and run in electrophoresis buffer (0.1% (w/v) SDS, 25 mM Tris base, 192 mM glycine) at 12 W for 3-4 h. Gels were stained by soaking in Coomassie stain: Coomassie

Brilliant Blue R-250 (150 mg/l), in 25 % (v/v) methanol, 10% (v/v) acetic acid and de-stained by soaking in de-stain solution: 40% (v/v) methanol, 10% (v/v) acetic acid. Alternatively, if SDS-PAGE results were required quickly, cells were stained with InstantBlue (Expedeon).

2.4 Purification of recombinant proteins

2.4.1 Preparation of buffer solutions

Buffers were prepared as detailed in Table 2.4. All buffers to be used with the ÄKTA purifier system (GE Healthcare, Buckinghamshire, UK) were degassed and filtered (0.22 µM filters, Merck Millipore) prior to use.

Table 2.4. Buffers used during purification of Vga(A) and Lsa(A)

Buffer	Composition
Lysis	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, 10 mM imidazole, 1 mM $MgCl_2$
VgaA-IMAC-W	50 mM NaH_2PO_4, pH 8.0; 2 M NaCl, 20 mM imidazole, 10% (v /v) glycerol, 1 mM $MgCl_2$
VgaA-IMAC-E	50 mM NaH_2PO_4 pH 8.0, 2 M NaCl, 250 mM imidazole, 10% (v/v) glycerol, 1 mM $MgCl_2$
VgaA-GF	50mM HEPES, pH 7.4; 2M NaCl, 1mM DTT, 1 mM $MgCl_2$
VgaA-IEX	50 mM HEPES, pH 7.4; 50 mM NaCl, 1 mM DTT
LsaA-IMAC-W	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol
LsaA-IMAC-E	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, 250 mMimidazole, 10% (v/v) glycerol
LsaA-IMAC-S	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, 40 mM imidazole, 10% (v/v) glycerol
Storage buffer*	50 mM HEPES, pH 7.4; 300 mM NaCl, 1 mM DTT

* Additionally used for LsaA gel filtration

2.4.2 Preparative scale lysis of *E. coli* cells

Cell pellets were thawed on ice and resuspended in 3 ml Lysis buffer (Table 2.4) per-g wet-cell weight. Cell suspensions were incubated with 7000 U chicken egg-white lysozyme (7000 U/ mg, Sigma-Aldrich), one EDTA-free protease inhibitor tablet / cell pellet (Roche, Basel, Switzerland) and 17 units Basemuncher endonuclease/ ml of suspension (Expedeon) at 4°C for 30 min and lysed by sonication (20s on / 40s off, 50% amplitude, large probe). During sonication samples were packed with ice and

checked regularly to prevent heating. Lysates were clarified by centrifugation (30,000 *x g*, 20 min) and kept on ice for all subsequent purification steps.

2.4.3 Purification of Vga(A)

Purification of Vga(A) was optimised as described in Chapter 3. The optimised protocol for Vga(A) purification using immobilized metal ion affinity chromatography (IMAC), gel filtration (GF) and ion exchange (IEX) is detailed below.

Cleared lysate was equilibrated with 3 mL Ni-NTA agarose (Expedeon) for 15 min and subsequently loaded into a 25-mL free-flow gravity column (GeneFlow). Unbound protein was allowed to flow through the column before washing with 10-column volumes of Vga-IMAC-W buffer (Table 2.4). Bound protein was eluted with 5 ml VgaA-IMAC-E buffer (Table 2.4) and dialysed overnight into Vga-GF buffer (Table 2.4) using SnakeSkin Dialysis Tubing (Life Technologies, 10 kDa MWCO). Dialysed protein was loaded onto a Superdex 200 (16/60) column (GE Healthcare) pre-equilibrated with VgaA-GF buffer. Fractions corresponding to Vga(A) were collected and concentrated to a volume <5 ml with Pierce Protein Concentrators (Life technologies). Once concentrated, Vga(A) was exchanged into VgaA-IEX buffer (Table 2.4) using five HiTrap Desalting columns (GE Healthcare) connected in series and subsequently loaded onto a 6 ml resource S column (GE Healthcare) for purification by cation-exchange. The resource S column was eluted with a 50 mM-1M NaCl linear gradient. Fractions corresponding to Vga(A) were collected, concentrated, exchanged into Storage buffer, and stored at -80 ^{o}C.

2.4.4 Purification of Lsa(A)

Purification of Lsa(A) by IMAC was performed as detailed in Section 2.4.3 with the following buffer substitutions (Table 2.4); LsaA-IMAC-W for VgaA-IMAC-W; LsaA-IMAC-E for VgaA-IMAC-E. Following IMAC, the eluate was dialysed (Section 2.4.3) against LsaA-IMAC-S buffer (Table 2.4) in the presence of U1p SUMO protease. After dialysis, the sample was re-applied to the Ni-TNA column in order to separate the cleaved 6-His-SUMO tag, which bound the column, from Lsa(A), which was eluted in the

flow through. Once the flow-through had been collected, it was concentrated to a volume < 5ml (Section 2.4.3) and loaded onto a Superdex 200 (16/60) column (GE Healthcare) pre-equilibrated with Storage buffer. Fractions corresponding to Lsa(A) were collected, concentrated, and stored at –80°C.

2.4.5 Purification of FusB

FusB was purified by IMAC and gel filtration as previously described (Cox *et al*., 2012).

2.4.6 Determination of protein concentration

Recombinant protein concentration was determined by measurement of UV absorbance at 280 nm using a P300 nanophotometer (Implem). Levels of contamination of protein samples with nucleic acid were assessed using the same instrument to measure absorbance at 260 nm and calculating the 260/280 ratio. Extinction coefficients were derived using the ProtParam tool on the ExPASy proteomics server at http://web.expasy.org/protparam/ (last accessed 27-07-2015).

The Bradford assay (Bradford, 1976) was used to determine the concentration of protein present in S30 fractions (Section 2.5.1). Sample concentrations were determined relative to a bovine γ-globulin standard curve. Bradford reagent was from Bio-Rad (Hercules, CA, USA).

2.5 *In vitro* transcription-translation assays

2.5.1 Preparation of S30 extracts

Staphylococcal S30 extract was prepared from *S. aureus* RN4220 in accordance with the protocol of Murray *et al*. (Murray *et al*., 2001) with limited modifications, as detailed below.

Cells were grown in Brain-Heart Infusion (BHI) broth to saturation, harvested by centrifugation (6000 *x g*, 20 min, 4°C), and washed successively in ice-cold buffer A (10 mM Tris-acetate pH8, 14 mM magnesium acetate, 1 mM DTT) containing 1 M KCl and cold buffer A containing 50 mM KCl. Cell pellets were weighed and frozen at -80°C. Subsequent steps were undertaken within 3 d, performed at 4°C unless otherwise stated, and kept strictly RNase free. Cell pellets were thawed on ice and resuspended in 2 ml buffer B (10 mM Tris-acetate pH8, 20 mM magnesium acetate, 50 mM KCl, 1 mM DTT) / g wet-

cell weight plus 15 U/ml RNasein (Invitrogen). Cell suspensions were transferred to SS34 centrifuge tubes and lysostaphin (Sigma-Aldrich) was added to a final concentration of 20 mg/ml. The suspension was incubated for between 30-60 min at 37°C, until it became visibly viscous. The lysed cells were fractionated by centrifugation (30 000 *x g*, 30 min, 4°C) and the resulting supernatant was dialysed using Float-A-Lyzer G2 dialysis tubing (3500 MWCO) (Spectrum Laboratories, Breda, Netherlands) against buffer A supplemented with 60 mM potassium acetate. Dialysis was performed for 1 h and repeated three times. Samples were concentrated to 10mg/ml using polyethylene glycol 8000, flash frozen using liquid nitrogen and stored at –80°C. Protein concentration was determined using the Bradford assay (Section 2.4.6).

E. coli S30 extract was purchased from Promega (*E. coli* T7 S30 Extract System for Circular DNA, L1130).

2.5.2 *In vitro* transcription-translation assays

The ability of Vga(A) and Lsa(A) to protect translation was investigated using the S30 extracts described in Section 2.5.1. An optimised quantity of S30 extract was added to a reaction mixture of amino acids (Promega), S30 pre-mix (Promega) and 1 µg p194SAluc/pBESTluc (Table 2.2). Antibiotics and purified proteins were added in accordance with assay requirements. Reactions were incubated for 1 h at 37°C and the level of transcription/translation was quantified by the addition of a saturating concentration of luciferase assay reagent (Promega) and subsequent measurement of luminescence using a FluoStar Optima plate reader (BMG labtech, Ortenberg, Germany). Concentrations of Virginiamycin M, fusidic acid and lincomycin were optimised to give 90% inhibition (IC_{90}) compared to a drug free, exogenous protein free, control.

2.6 Assays of lincomycin binding to staphylococcal ribosomes

2.6.1 Purification of staphylococcal ribosomes

Staphylococcal ribosomes were purified using an l-cysteine Sulfolink (Thermo Scientific, Waltham, MA, USA) column and ultracentrifugation as previously described (Maguire *et al*., 2008).

An l-cysteine Sulfolink column suitable for use with an ÄKTA purifier system (GE Healthcare) was prepared as follows. A total of 100 mL of a 50% (v/v) slurry of Sulfolink coupling gel was centrifuged (500 *x g*, 5 min), and the storage buffer carefully decanted. The gel was washed three times with 200 ml of Sulfolink coupling buffer (50 mM Tris, 5 mM EDTA-Na; pH 8.5) using the centrifuge-decant method. Next, 100 ml of a 50 mM solution of l-cysteine in coupling buffer was added and the slurry gently shaken for 1 h at 25°C. Residual l-cysteine was removed by centrifuge-decant to afford l-cystein Sulfolink resin. A 50% (v/v) slurry of l-cystein Sulfolink resin was produced by addition of dH_2O and the resin was packed into a XK 16/20 column (GE Healthcare) according to the protocol detailed in Column Packing the Movie (GE Healthcare). The resulting column was stored in 20% (v/v) ethanol and reused multiple times.

Staphylococcal cell pellets for extraction of ribosomes were prepared and stored as detailed in Section 2.5.1. Pellets were thawed on ice and resuspended in 2 ml of Ribosome Lysis buffer (10 mM Tris acetate, pH 8.0; 20 mM magnesium acetate, 1 mM DTT) supplemented with 60 mM NH_4Cl/ g of cell wet-weight. Cell suspensions were transferred to SS34 centrifuge tubes and lysostaphin (Sigma-Aldrich) was added to a final concentration of 20 mg/ml. The suspension was incubated for between 30-60 min at 37°C, until the cell pellet began to become visibly viscous. The lysed pellet was fractionated by centrifugation (30 000 *x g*, 30 min, 4°C) and the resulting supernatant was loaded onto an l-cystein Sulfolink column pre-equilibrated with ribosome lysis buffer using repeated applications of a 10ml superloop (GE Healthcare). The column was eluted using a 0-100% gradient (10 column volumes) of lysis buffer supplemented with 1 M NH_4Cl. Eluted fractions containing protein were collected and subjected to ultracentrifugation (100 000 *x g*, 16 h at 4°C using a SW32Ti rotor and

Optima L-80 XP Ultracentrifuge (Beckman Coulter, Brea, CA, USA) to obtain ribosome pellets. Finally pellets were resuspended in Ribosome Lysis buffer and stored at –80°C. Ribosome concentration was determined as detailed in Section 2.6.2.

2.6.2 Quantification of staphylococcal ribosomes

For quantification of rRNA, serial dilutions of purified ribosomes (Section 2.6.1) were run on a denaturing agarose gel (Section 2.2.8) alongside serial dilutions of RiboRuler High Range RNA Ladder (Life technologies). Alternatively, ribosomal protein was quantified using SDS-PAGE to separate erial dilutions of purified ribosomes and of bovine serum albumin (BSA). Images of gels were captured with the use of a GeneGenius UV transilluminator (Syngene, Cambridge, UK) and analysed by 2D densitometry using AIDA software (Raytest, Straubenhardt, Germany). Ribosome concentration was determined through comparison of 16S rRNA with a standard curve calibrated with RiboRuler and comparison of ribosomal protein L3 with a BSA calibrated standard curve. Results obtained from quantification of rRNA and ribosomal protein were averaged.

2.6.3 Lincomycin Ribosome binding assays

The ability of LsaA to protect the ribosome from lincomycin binding was assessed using a modification of a previously described tetracycline binding assay (Wilson, 2014). Ribosomes (500 nM) were pre-incubated in 50 µl reactions with LsaA, BSA or unlabelled lincomycin (concentrations described in Section 4.4.4) in assay buffer (10 mM Tris, pH 7.5; 60 mM KCl, 10 mM NH_4Cl, 300 mM NaCl, 6 mM $MgCl_2$, 0.1 mM ATP) at 37°C. After 10 min, ^{3}H-lincomycin (1 µM) was added, the reactions were incubated a further 10 min, vacuum-filtered through a 0.45 µm nitrocellulose filter and washed twice with 200 µl of ice-cold wash buffer (50 mM Tris-Cl, 50 mM KCl, 20 mM magnesium acetate) to remove unbound ^{3}H-lincomycin. Ribosome-associated ^{3}H-lincomycin was then quantified by scintillation counting. The ability of Lsa(A) to dissociate pre-bound lincomycin was investigated using the same assay conditions, however ^{3}H-lincomycin was pre-incubated with ribosomes for 10 min at 37°C prior

to the addition of LsaA, BSA or unlabelled lincomycin, incubation for a further 10 min at 37°C, washing, and scintillation counting.

2.7 Pull down assays

Polyhistidine pull-down assays were performed to establish putative interactions of Vga(A), or Lsa(A), with intracellular components. Assays were performed as follows. Aliquots of 50 µl Ni-NTA agarose resin were equilibrated in lysis buffer (Table 2.4, 50 mM KH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole) and 100 µg purified VgaA or LsaA was immobilised by incubation with resin for 15 min at 4°C. Subsequently, immobilised protein was incubated with samples containing potential binding partners for 30 min at 37°C or 4°C. These samples were as follows: (i) S30 fraction derived from *S. aureus* RN4220 (Section 2.5.1), (ii) S30 fraction derived from *E. coli* (Section 2.5.1), and (iii) purified staphylococcal ribosomes (Section 2.6.1). Incubation was in the presence of 2 µg/ml virginiamycin M with 100 µM ATP or 100 µM AMP-PNP (Roche) as detailed in Section 4.4.5. Samples were applied to Pierce Micro-Spin Columns and centrifuged (100 *x g*, 1 min) to remove unbound protein. Columns were repeatedly washed (3 x 20 CV, 50 mM KH_2PO_4, pH 8.0; 300 mM NaCl, 20 mM imidazole) to remove non-specifically bound protein. SDS-PAGE loading buffer (Section 2.3.4) supplemented with 100 mM EDTA was used to elute the column and samples were examined by SDS-PAGE. Bands corresponding to putative binding partners were excised from the gel and analysed by peptide-mass fingerprinting (Section 2.8).

2.8 Peptide-mass fingerprinting

Bands excised from SDS-PAGE gels were de-stained and subjected to tryptic digest. The resulting peptides were separated by liquid chromatography and identified by MALDI-TOF mass-spectrometry. De-staining, digest and LC-MS was carried out by Dr James Ault (Mass Spectrometry Facility, University of Leeds).

2.9 Screening for protein crystallisation conditions

Crystal screens from Hampton Research (California, USA; Crystal Screen I/II, Index, Salt RX), Molecular Dimensions (Florida, USA; Midas, Morpheus), and Rigaku Reagents (Washington, USA; Wizard I/II) were assessed for their ability to confer conditions suitable for crystallisation. Conditions were tested in sitting drops (0.4 µl drops) using the vapour diffusion reagent (60 µl reservoir volume) in the presence or absence of 100 µM AMP-PNP. Screens were set up in 3 drop, 96-well plates (Hampton Research, CA, USA) using a Formulatrix NT8 robot (Formulatrix, MA, USA) and incubated at 4°C or 25°C in a Rock Imager 1000 (Formulatrix). Crystal growth was examined after one day and then monitored at regular intervals up to two months.

3. Expression and purification of the ARE ABC-F proteins Vga(A) and Lsa(A)

3.1 Abstract

Efforts to determine the mechanism underpinning ABC-F-mediated antibiotic resistance would be facilitated by the production of homogenously purified ARE ABC-F proteins suitable for functional and structural studies. To date, only one ARE ABC-F has been purified in its native form; Vga(A). Here, an optimised method for purification of Vga(A) is described. Additionally, conditions conducive to the soluble expression and subsequent purification of a second ARE ABC-F protein, Lsa(A), are outlined. Attempts to crystallise the two proteins in order to pursue structural studies were unsuccessful, possibly due to proteolytic degradation observed even at low temperatures. However, optimised methodology for the homogenous purification of Vga(A) and Lsa(A) provides a platform from which to launch further structural and mechanistic investigations into the ARE ABC-F protein subfamily.

3.2 Introduction

To date, functional studies of ARE ABC-F determinants have concentrated on three proteins; Msr(A), Vga(A)and Ole(B), of which only Vga(A) and a fragment Ole(B) have previously been heterologously expressed and purified. The v*ga(A)* resistance determinant is located on mobile genetic elements and mediates acquired resistance to streptogramin A and lincosamide antibiotics (with variants also conferring pleuromutilin resistance) (Lenart *et al*., 2015, Novotna and Janata, 2006)) in several species of staphylococci (Allignet *et al*., 1992, Novotna and Janata, 2006, Lozano *et al*., 2012), whilst, the chromosomally encoded *ole(B)* gene confers self-protection in the oleandomycin producer, *Streptomyces antibioticus* (Olano *et al*., 1995). Functional studies of Ole(B) used only a fragment of the full-length protein, the N-terminal ABC domain, which was fused to the *E. coli* maltose binding protein (MBP) to promote solubility (Olano *et al*., 1995). In contrast, although Vga(A) has also been

expressed as an MBP-fusion (Chesneau *et al*., 2005), a Vga(A) construct modified solely by the addition of a C-terminal hexa-histidine tag that does not alter the proteins biological activity, has also been expressed and purified (Jacquet *et al*., 2008). Using purified protein, the ATPase activity of Vga(A) and the N-terminal domain of Ole(B) have been thoroughly characterised. However, functional studies of these two ARE ABC-F proteins have not provided a definitive explanation of the mechanism by which they mediate antibiotic resistance. Additionally, to date, no structural information is available for any member of the ARE ABC-F subfamily.

3.3 Aims

Work presented in this chapter provides optimised conditions for heterologous expression and purification of proteins belonging to the ARE ABC-F subfamily. Optimisation of a previously described method for production of Vga(A) is described. Conditions conducive to the production of soluble ARE ABC-F proteins other than Vga(A) were assessed and methods for purification of resulting soluble proteins was developed. Attempts to find conditions to promote crystallisation of ARE ABC-F proteins are outlined.

3.4 Results

3.4.1 Expression of Vga(A)

The optimisation of conditions conducive to the production of soluble protein permits subsequent purification steps to be conducted under native conditions and removes any requirement for denaturation and refolding of the target protein. Previous work had reported low-level production of soluble of Vga(A) with a C-terminal hexa-histidine tag when the *vga(A)* gene was expressed from the IPTG-inducible pIVEX2.3d vector in *E. coli* BL21 (DE3λ) Gold (Jacquet *et al*., 2008). The pIVEX plasmids are a somewhat unusual choice of vector for performing *E. coli*-based protein production given that they are primarily optimized for *in vitro* production of proteins. However, as pIVEX2.3d had been successfully

used to produce soluble Vga(A), the pIVEX2.3d-*vga(A)* plasmid was constructed with the intention of optimising Vga(A) production (G. Cox, unpublished work). Auto-induction is an alternative method for production of proteins using the T7-system that has been shown to increase bio-mass and target-protein yield in comparison to IPTG induction (Studier, 2005). I therefore sought to establish whether the Vga(A) protein could be produced using auto-induction of the pIVEX2.3d-*vga(A)* plasmid in *E. coli* BL21 (DE3) Gold (Jacquet *et al*., 2008). The pIVEX2.3d-*vgaA* plasmid was transformed into *E. coli* BL21 (DE3) Gold and subjected to small-scale auto-induction expression trials. Expression trials of *vga(A)* were performed alongside the ARE ABC-F gene *msr(A)* and tetracycline-resistance determinant *tet(M),* both of which had been codon optimised for expression in *E. coli*, synthesised, and cloned into pET28a by Genscript (New Jersey, USA), prior to transformation into *E. coli* BL21 (DE3λ) Gold.

Initial expression trials tested the ability of two auto-induction media of differing composition (2ZYM 2X Lac and 8ZYM 4X Lac) to induce protein production when incubated for 2 d at 25°C. 8ZYM 4X Lac is designed to permit growth to a higher cell-density than 2ZYM 2X Lac as it contains double the amount of glucose, lactose and glycerol, and four-times the amount of yeast extract and tryptone, than 2ZYM 2XLac. Both media produced a small amount of soluble Vga(A) (60.2kDa) that was amenable to purification using a small-scale Ni-NTA spin-column (Figure 3.1 A and B). The richer of the two media, 8ZYM 4X Lac, produced high-levels of insoluble Vga(A), Tet(M) (72.5 kDa), and Msr(A) (55.8 kDa). In an effort to increase the fraction of soluble protein generated using 8ZYM 4X Lac media, the incubation temperature was lowered to 18^{o}C and growth time extended to 3-days, however, this did not produce detectable amounts of protein (Figure 3.1 C).

As auto-induction with 8ZYM 4X Lac was able to produce soluble Vga(A), expression was scaled up and purification was attempted (Section 3.4.2). Expression of *msr(A)* was investigated further at a later point (Section 3.4.5). The *tet(M)* resistance determinant was not investigated further.

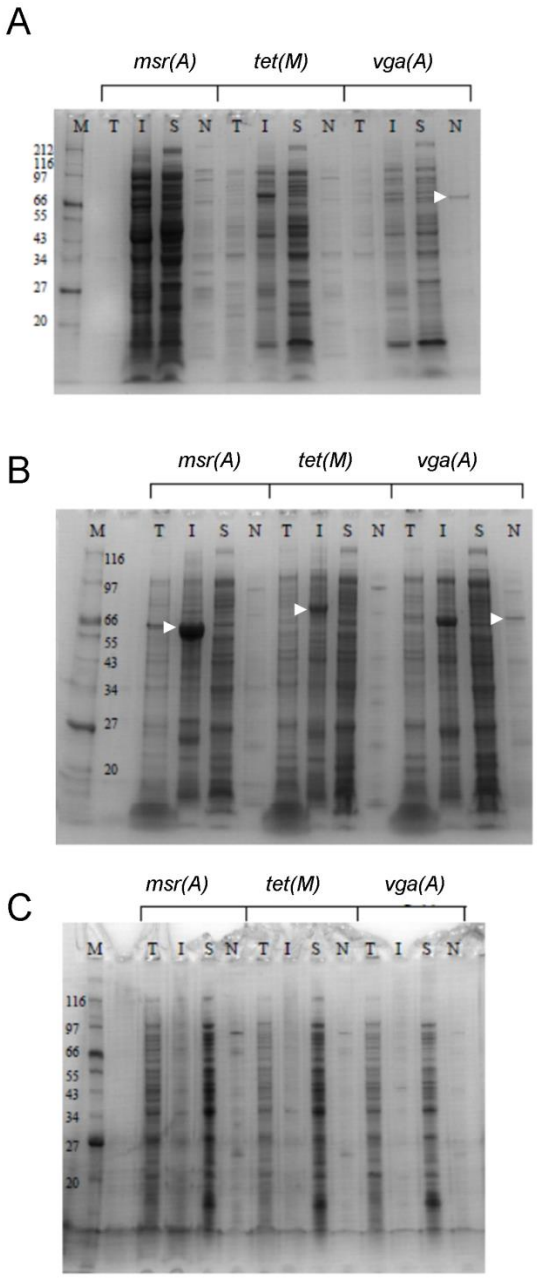


Figure 3.1. Small scale expression trials of *vga(A)* varying auto-induction media and temperature. (A) 2 ZYM 2X Lac at 25^{o}C for 2days. **(B)** 8 ZYM 4X Lac at 25^{o}C for 2days. **(C)** 8ZYm 4X Lac at 18^{o}C for 3days.

T - total cellular protein, I – Insoluble fraction, S – Soluble fraction, N – Eluate from Nickel-NTA mini-spin-column. M – Protein marker (Broad Range (2-212 kDa), New England Biolabs) showing molecular weight (Mw) in kDa.

Molecular weights:- Msr(A) 55.8 kDa, Tet(M) 72.5 kDa, Vga(A) 60.2kDa.

3.4.2 Initial strategy for Vga(A) purification

Initial efforts to purify Vga(A) utilised a three-step strategy; capture of Vga(A) using immobilised metal ion affinity chromatography (IMAC), followed by ion-exchange (IE) and gel filtration (GF) chromatography. Conditions for IMAC were optimised through evaluation of two resins, nickel-nitrilotriacetic acid (Ni-NTA) agarose (Expedeon) and TALON cobalt Affinity Resin (Clontech, CA, USA), washed with increasing concentrations of imidazole (Figure 3.2 A and B). A greater imidazole concentration was required to elute Vga(A) from Ni-NTA resin than cobalt resin, suggesting that Ni-NTA exhibited higher affinity for Vga(A) (Figure 3.2 A vs. B). Ni-NTA agarose was therefore used for IMAC throughout this study in tandem with 20 mM imidazole in all IMAC wash buffers. Application of cleared auto-induced cell lysate to Ni-NTA agarose repeatedly showed that the most prominent band found within the eluted fraction migrated as if it had a similar molecular weight to that expected of Vga(A) (Figure 3.2 C). Analysis of this band by peptide mass fingerprinting (PMF) confirmed its identity as Vga(A) (Personal communication, J. Ault, Biomolecular Mass Spectrometry Facility, University of Leeds).

Repeated attempts to further purify Vga(A) by cation-exchange were unsuccessful. After buffer exchange, the protein failed to bind the resource S IE column and was present in the flow through (Figure 3.2 D). Subsequent application of the flow through to an S200 GF column did not give a single peak corresponding to the molecular weight of Vga(A) (Figure 3.2 E). Analysis of the fractions by SDS-PAGE revealed Vga(A) to be eluted in the void volume and present in fractions corresponding to a molecular weight higher than 60.2 kDa, suggesting aggregation of the protein (Figure 3.2 F).

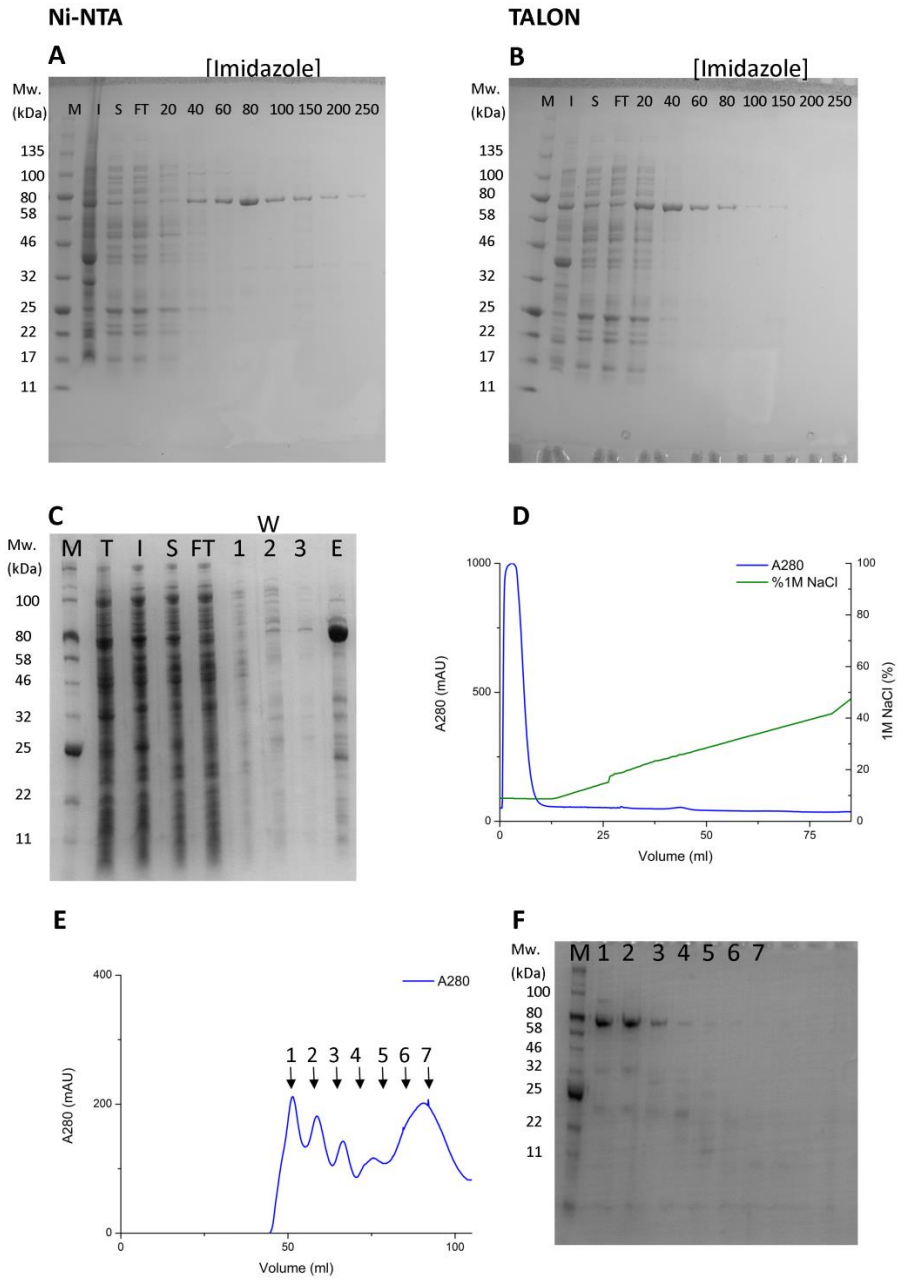


Figure 3.2. Initial purification of Vga(A). SDS-PAGE showing optimisation of imidazole concentration for Vga(A) purification by IMAC using **(A)** Ni-NTA agarose and **(B)** TALON cobalt resin. **(C)** SDS-PAGE analysis of fractions generated by purification of an auto-induced, cleared, cell-lysate using Ni-NTA agarose, washed with the imidazole concentration as optimised in A. **(D)** Chromatogram resulting from application of Ni-NTA IMAC eluate from C to a resource S IE column. **(E)** Chromatogram resulting from application of flow through from Resource S described in D to an S75 GF column. **(F)** SDS-PAGE analysis of fractions 1-7 resulting from GF column described in E.

Abbreviations used to annotate the SDS-PAGE gel images are as follows; M denotes protein marker (Broad Range 2-212 kDa, New England Biolabs); I, insoluble cell fraction; S, soluble cell fraction; FT, flow through; W, wash; and E, eluate. The blue trace on the presented chromatograms represents absorbance at 280 nm, whereas the green trace indicates a 10-500 mM NaCl gradient.

3.4.3 Removal of nucleic acid during Vga(A) purification

Jacquet *et al*.(Jacquet *et al*., 2008) reported that removal of nucleic acid contamination through incubation of the cell lysate with Benzonase nuclease (150 units / g wet-cell weight) (Merck Millipore) and protamine sulphate (0.8 mg / g wet-cell weight g wet-cell weight) (Sigma Aldrich) for one hour at room temperature, followed by an ultracentrifugation step (200 000 *x g* for 1 h) to precipitate protamine sulphate bound nucleic acid, improved the efficiency of the IE purification step (Jacquet *et al*., 2008). The purification protocol was modified to reflect these changes. As I observed auto-induction to produce a cell pellet of around 5x the wet-weight of a pellet resulting from an IPTG-induced culture of equivalent volume, the relative amounts Benzonase and protamine sulphate were adjusted accordingly. The cell lysate of the pellet resulting from 400 ml of auto-induced cleared culture was treated with Benzonase and protamine sulphate, then purified by IMAC. Following buffer exchange, the IMAC eluate was again applied to the resource S IE column (Figure 3.3 A), yielding a single eluted peak. Analysis of the fractions from IMAC, desalting and IE showed the eluted peak to correspond to Vga(A) (Figure 3.3 B), Purification using this methodology yielded 1.5 mg of purified protein (3.75 mg / litre culture); however, this amount was insufficient for screening of crystallisation conditions. Consequently, purification was scaled up to process 2.8 litres of auto-induced cultured and repeated; however, attempts to purify Vga(A) from larger culture volumes repeatedly failed to yield protein that was amenable to purification by IE (Figure 3.3 C). Protein produced using scale up through reversion to IPTG-induction also gave no eluted peak for IE (Figure 3.3 D).

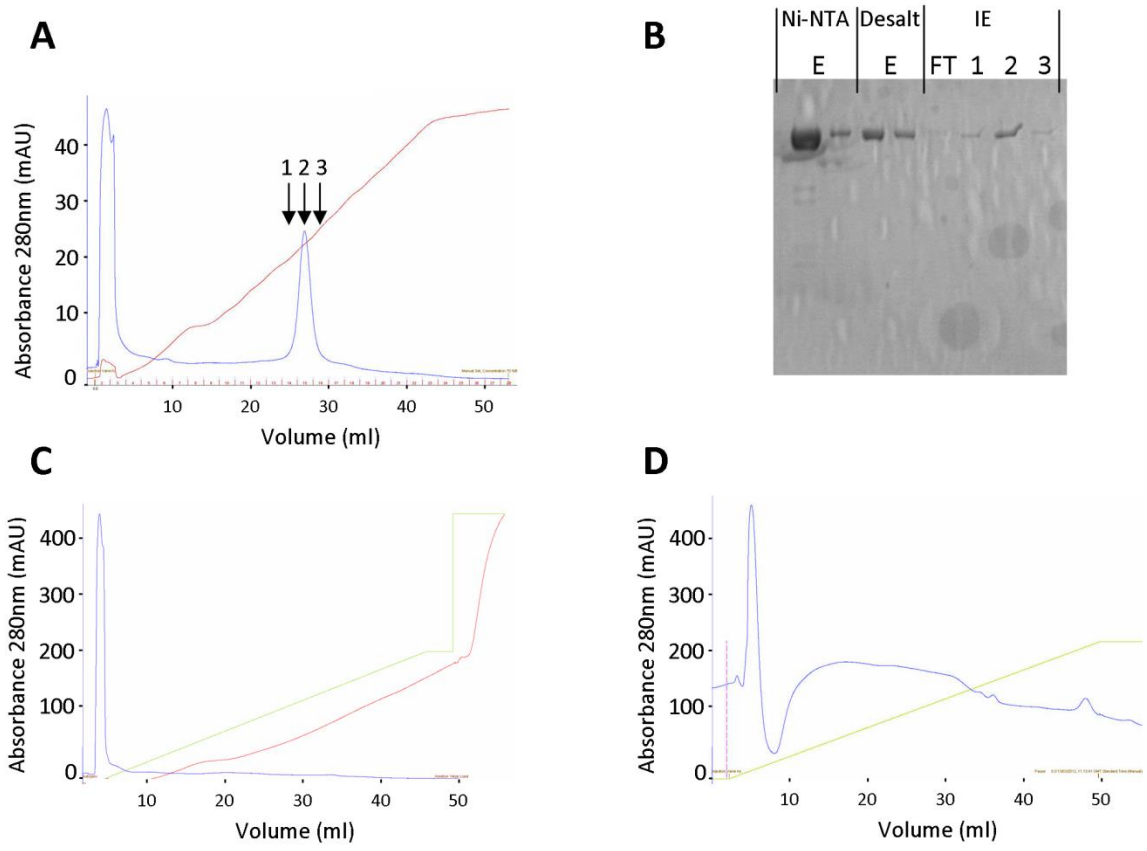


Figure 3.3. Purification of Vga(A) using the method described in Jacquet *et al.* (Jacquet *et al.*, 2008).

(A) Chromatogram showing peaks resulting from application of IMAC eluate resulting from a small scale purification using 400ml auto-induced culture to a resource S IE column. **(B)** SDS-PAGE analysis of fractions from the same small-scale purification. Ni-NTA E denotes the eluted fractions from IMAC, Desalt E; the same sample following desalting using a HiTrap desalting column, IE FT 1, 2, and 3 are the fractions resulting from the IE column shown in A. Chromatograms **(C)** and **(D)** show Resource S IE purification of protein resulting from IMAC eluates generated from 1.2 L of auto-induced culture, and 2.4 L IPTG-induced culture, respectively.

The blue trace on the presented chromatograms represents absorbance at 280 nm, whereas the red and green traces indicate 10-500 mM NaCl gradients.

After numerous attempts, it became clear that I was not able to consistently replicate the methodology developed by Jacquet *et al.* to remove contaminating nucleic acid from preparations of Vga(A). I therefore performed a series of purifications under different conditions and assessed their efficacy for removal of contaminating nucleic acid by monitoring the ratio of absorbance at 260 nm to absorbance at 280 nm (A260/280). Protein with an A260/280 ratio <0.7 was deemed free of contaminating nucleic acids (Ariza *et al*., 2013). Results are summarised in Table 3.1.

Monitoring the A260/280 ratio showed that although recapitulation of the conditions developed by *Jacquet et al*. caused a drop in A260/280, it did not produce protein free of nucleic acid (Table 3.1 condition B vs A). This explains the previously observed failure of desalted IMAC eluate to bind a cation-exchange column and subsequent elution of Vga(A) in the void volume during GF. Substitution of a large amount of DNase I and RNase A for Benzonase nuclease and increasing the incubation temperature to 37°C produced no alteration in A260/280 compared to the original method (Table 3.1 condition C vs B). However, the inclusion of 2 M sodium chloride in IMAC wash and elution buffers produced protein free of nucleic acid following IMAC (Table 3.1 condition C vs D). Unfortunately protein subjected to these conditions was prone to aggregation and could not be purified further. As increasing the sodium chloride concentration in IMAC buffers resulted in a decrease in A260/280, I included 2M sodium chloride in the GF buffer and removed initial steps to digest and precipitate nucleic acid IMAC (Table 3.1 condition E). This methodology was successful, removing nucleic acid across both purification steps and yielding protein with an A260/280 <0.7 following GF. As a result this method was subsequently used for routine purification of Vga(A) (Section 2.4.3). A final condition that used streptomycin sulphate for precipitation of nucleic acid in place of protamine sulphate also yielded Vga(A) free of nucleic acid (Table 3.1 condition F), however, due to the risk of residual streptomycin interfering with subsequent *in vitro* transcription-translation assays (4.4.3), this method was not used for routine purification of Vga(A) during this study.

Table3.1. Optimisation of conditions for removal of contaminating nucleic acid from preparations of Vga(A). With the exception of condition B, buffers were; Lysis; 50 mM NaH_2PO_4 (pH 8.0), 20 mM NaCl, 1 mM $MgCl_2$, 10 mM imidazole; IMAC Wash, 50 mM NaH_2PO_4 (pH 8.0), 20 mM imidazole, supplemented with NaCl as described; IMAC Elution, 50 mM NaH_2PO_4 (pH 8.0), 250 mM imidazole, supplemented with NaCl as described; GF, 50 mM HEPES pH 7.4, 1 mM DTT, supplemented with NaCl as described.
Buffers for condition B were from Jacquet *et al.* (Jacquet *et al*., 2008); Lysis, 50 mM NaH_2PO_4 (pH 7.0), 20 mM NaCl, 1 mM $MgCl_2$, 1 mM imidazole, and 0.5% (v / v) Triton X-100. IMAC wash; 50 mM NaH_2PO_4, (pH 7.0), 400 mM NaCl, 20 mM imidazole; IMAC elution, 50 mM NaH_2PO_4, (pH 7.0), 400 mM NaCl, 250 mM imidazole; GF, 25 mM Tris-HCl pH 7.0, 150 mM NaCl, 7 mM β-mercaptoethanol

Condition		A260/280 of Ni-NTA eluate	A260/280 of GF eluate
A	· No steps taken to remove nucleic acid. · Ni-NTA eluate applied directly to S200 GF column.	1.9	1.8 Aggregate eluted in void volume
B	· Incubation of cell lysate at room temperature (RT) for 30 min with Benzonase (150 units / g wet-cell weight), addition of protamine sulphate (0.8 mg / g wet-cell weight) and further incubation at RT for 30 min. · Centrifugation at 200 000 *x g* for 1 h. · IMAC wash, IMAC elution and GF buffers supplemented with 300 mM NaCl. · Method from Jacquet *et al.* (Jacquet *et al*., 2008).	1.2	- Not assessed
C	· Incubation of cell lysate at 37°C with DNase I (600 units / g wet-cell weight)(Sigma-Aldrich) and RNase A (140 units / g wet-cell weight)(Qiagen) for 30 min followed by addition of protamine sulphate (0.8 mg / g wet-cell weight) and incubation at RT for 30 min. · Centrifugation at 200 000 *x g* for 1 h. · IMAC wash and elution buffers supplemented with 300 mM NaCl.	1.2	- Protein precipitated during dialysis.
D	· Treatment of cell lysate as described in condition C · Centrifugation at 30 000 *x g* for 30 min. · IMAC Wash and elution buffers supplemented with 2M NaCl.	0.60	- Protein precipitated during dialysis.
E	· Benzonase (25 units / wet-cell weight) added prior to cell lysis to reduce viscocity. · No incubation of cell lysate with nuclease or precipitant. · Centrifugation at 30 000 *x g* for 30 min. · IMAC wash, IMAC elution and GF buffers supplemented with 2M NaCl. · Ni-eluate dialysed into GF buffer overnight and applied to S200 GF column.	0.95	0.65
F	· Incubation of soluble fraction at 4°C with Benzonase (300 units / g wet-cell weight) for 30 min followed by addition of streptomycin sulphate (5 mg / g wet-cell pellet weight) and incubation at 4°C for 30 min. · Centrifugation at 30 000 *x g* for 30 min. · IMAC wash, IMAC elution and GF buffers supplemented with 2M NaCl. · Ni-eluate dialysed overnight and applied to S200 GF column.	0.68	0.68

3.4.4 An optimised method for Vga(A) purification

The development of methodology to consistently remove contaminating nucleic acid from preparations of Vga(A) allowed us to optimise a purification protocol to reliably produce homogenous protein. The protocol involved four chromatography steps; initial capture of Vga(A) from cleared cell-lysate by Ni-NTA IMAC (Figure 3.4 A), removal of residual contaminating nucleic acid by GF (Figure 3.4 B), desalting (Figure 3.4 C), and a final polishing step using cation-exchange (Figure 3.4 D). Analysis of chromatograms from GF performed under high ionic strength revealed separation of the IMAC eluate into two distinct peaks. The initial peak eluted in the void volume and absorbed strongly at 260 nm, likely corresponding to aggregates of nucleic acid (Figure 3.4 B). This peak also repeatedly contained a small amount of Vga(A) and a contaminating band of higher molecular weight (Figure 3.4 C, lanes 1, 2 and 3). The second peak eluted corresponds to Vga(A) free of bound nucleic acid. In order to exchange the GF eluate into a buffer with lower-ionic strength suitable for IE, fractions were concentrated and applied to desalting columns. During desalting, two peaks were routinely observed; an initial larger peak with an A260/280 ratio <0.7 and a second peak that absorbs more strongly at 260 nm. Although both peaks contained Vga(A) (Figure 3.4 F, lanes 7 and 8), only the initial peak was collected and further purified. At this point, although Vga(A) was close to homogeneity, I consistently observed feint bands of ~40 kDa. LC-MS analysis confirmed that these bands were the result of degradation of Vga(A) (Personal communication, J. Ault, University of Leeds). A final IE step was employed to purify Vga(A) away from degradation products. Chromatograms of the IE step showed a single peak, eluted at a sodium chloride concentration of ~ 300 mM, with a small tail. Analysis of the peak by SDS-PAGE revealed that IE was able to reduce contamination by degradation products, with full-length Vga(A) primarily eluting in the main peak (Figure 3.4 F, lanes 11, 12 and 13) and bands of a lower molecular weight predominantly eluting in the tail (Figure 3.4 F, lanes 14, 15 and 16). Homogenously purified Vga(A) was either used immediately for functional assays or stored at -80°C. This methodology resulted in yields of ~3mg of WT Vga(A) per litre of culture.

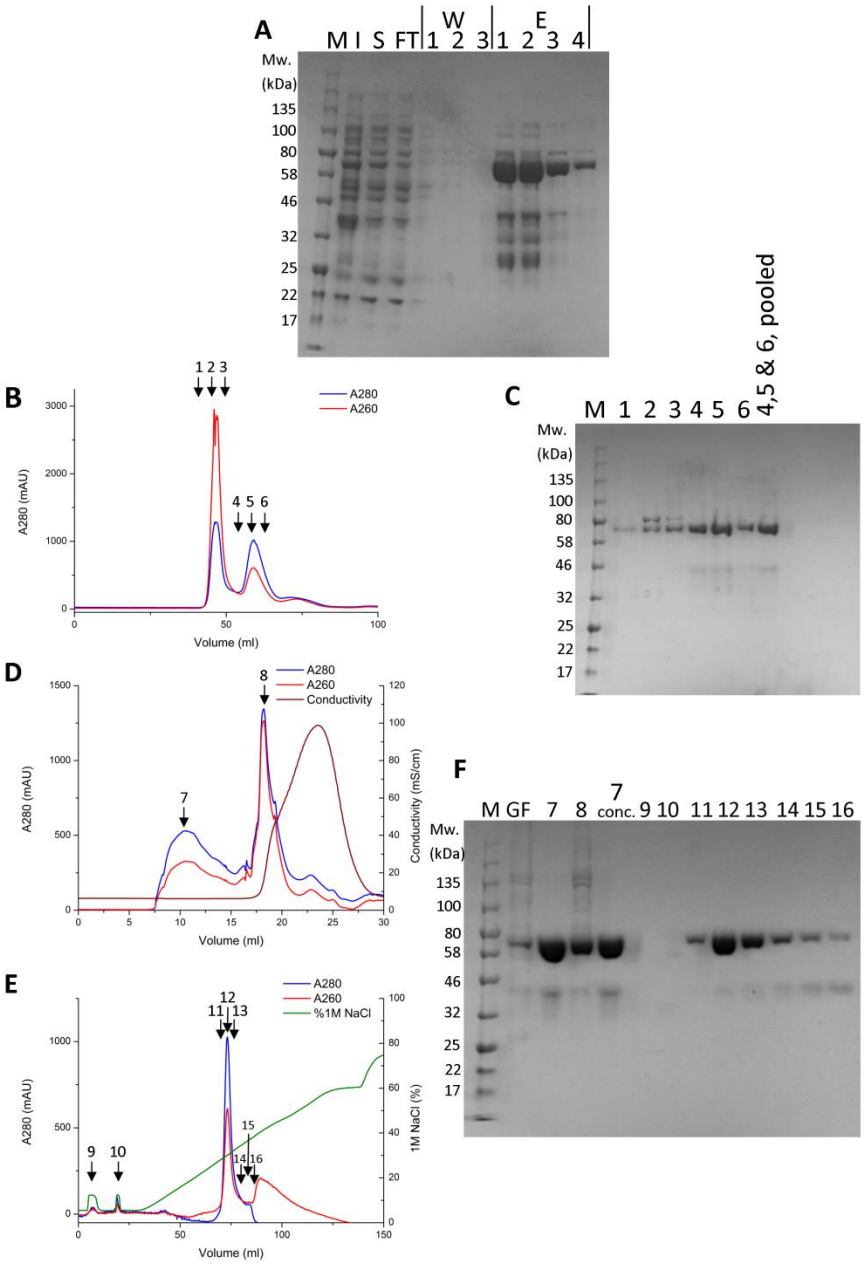


Figure 3.4. An optimised method for purification of Vga(A). Representative acrylamide gels and chromatograms. **(A)** SDS-PAGE showing Vga(A) capture from cleared cell lysate using IMAC and washing / elution with a buffer containing 2M NaCl. **(B) C**hromatogram showing application of the IMAC eluate to an S200 GF column **(C)** SDS-PAGE analysis of fractions from GF. Lane numbers correspond to labelled fractions in chromatogram B. **(D)** Chromatogram showing application of second GF peak to a HiTrap desalting column. **(E)** Chromatogram to show binding and elution of Vga(A) to a resource S IE column. **(F)** SDS-PAGE analysis of fractions from desalting and IE. Lane numbers correspond to labelled fractions in chromatograms D and E Abbreviations used to annotate the SDS-PAGE gel images are as follows; M denotes protein marker (Broad Range 2-212 kDa, New England Biolabs); conc., a protein that has been concentrated; I, insoluble cell fraction; S, soluble cell fraction; FT, flow through; W, wash; and E, eluate.

3.4.5 Expression of alternative ARE ABC-F proteins

In order to investigate the mechanism of ARE ABC-F proteins other than Vga(A), I sought to establish conditions for the production of alternative ARE ABC-F proteins in a soluble form. To this end, a panel of expression constructs was generated for the production of ARE ABC-F proteins fused to a variety of tags designed to enhance solubility and enable affinity purification. Specifically, small ubiquitin-like modifier (SUMO), maltose-binding protein (MBP), and glutathione S-transferase (GST) were chosen to be fused to the N-terminus of ARE ABC-F proteins. All three proteins have been reported to enhance solubility of the proteins to which they are fused. However, as it is not currently possibility to predict whether they will enhance solubility of the target protein, I chose to undertake an empirical approach and screen all three. Cloning was performed using a traditional restriction enzyme-based approach and the orientation, sequence, and frame of each insert was verified by DNA sequencing. Expression constructs generated during this study are shown in Table 3.2. Small scale expression trials were performed using 8ZYM 4X Lac auto-induction media and the *E. coli* strain BL21-CodonPlus(DE3λ)-RIL, which was chosen as it contains an accessory plasmid encoding tRNAs shown to enhance expression of heterologous proteins derived from organisms with AT-rich genomes, such as *S. aureus*.

Table 3.2. Constructs generated for the expression of ARE-ABC protein-tag fusions.

Plasmid	ARE ABC-F gene	Tag
pET28a-Tev	· *vga(A)* · *vga(A)v* · *vga(E)* · *msr(A)* · *msr(C)* · *lsa(A)*	Cleavable N-terminal 10-histidine
pET28a-SUMO	· *vga(A)* · *vga(A)v* · *vga(E)* · *msr(A)* · *msr(C)* · *lsa(A)*	Cleavable N-terminal 6-histidine and SUMO (Yeast SMT-3)
pET28a-GST	· *vga(A)* · *vga(A)v* · *msr(A)* · *msr(C)* · *lsa(A)*	Cleavable N-terminal 6-histidine and Glutathione S-transferase (GST)
pET28a-Mal	· *vga(A)* · *vga(E)* · *msr(A)* · *lsa(A)*	Cleavable N-terminal 6-histidine and maltose binding protein (MBP)
pIVEX2.3d	· *vga(A)v* · *vga(E)* · *msr(A)*	Non-cleavable C-terminal 6-histidine

Expression of ARE-ABC-F proteins as N- or C- terminally his-tagged, GST tagged, and MBP tagged, fusions resulted solely in the production of insoluble protein (Figure 3.5, A, C, D, E, black arrows). However, expression of both Vga(A) and Lsa(A) with a SUMO tag resulted in the production of soluble protein (Figure 3.5 B, white arrows). Analysis of the SDS-PAGE band through to correspond to SUMO-Lsa(A) confirmed its identity and as a result, expression of the pET28a-SUMO-*lsa(A)* construct was scaled up and a protocol for purification was developed.

3.4.6 Purification of Lsa(A)

Lsa(A) has a lower predicted pI than Vga(A) (5.77 vs. 9.06) and as a result purification of Lsa(A) was not complicated by a propensity to bind nucleic acid (Section 3.4.3). Lsa(A) was therefore amenable to purification using a simplified three-step strategy. Following initial capture and purification by IMAC using Ni-NTA agarose (Figure 3.6 A, page 76), 6-histidine and SUMO tags were cleaved, and the protein was reapplied to the IMAC column. Untagged Lsa(A) exhibited low affinity for Ni-NTA and was separated from the majority of cleaved histidine-SUMO tag using an imidazole gradient (Figure 3.7 B). Finally Lsa(A) was applied to an S200 GF column, where it eluted as a single peak (Figure 3.7 C) corresponding to homogenous Lsa(A) (Figure 3.7 D). Using this methodology, yields of ~12mg of Lsa(A) per litre of culture were obtained.

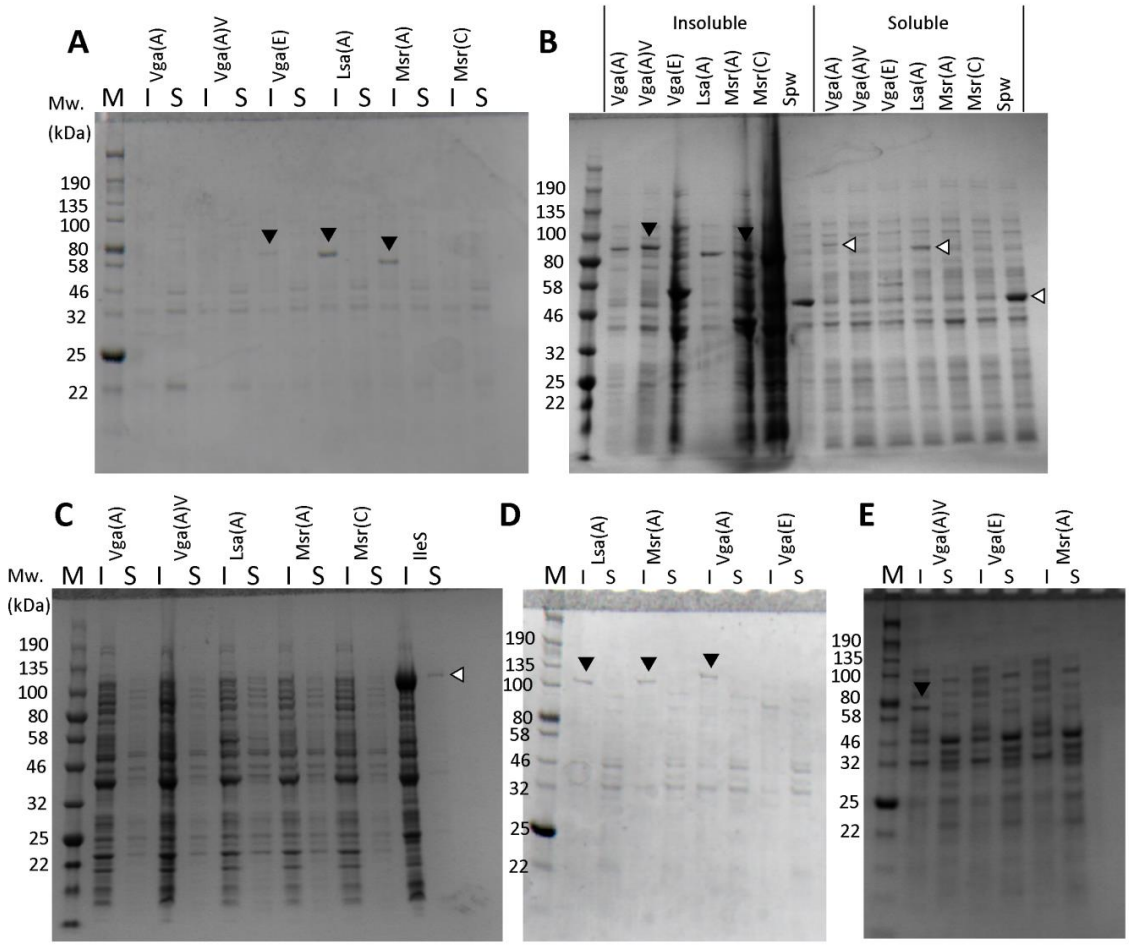


Figure 3.5. SDS-PAGE analysis of expression trials of ARE ABC-F proteins conducted using *E. coli* BL21 (DE3) RIL transformed with constructs based on vectors; (A) pET28a-Tev; **(B)** pET28a-SUMO; **(C)** pET28aGST; **(D)** pET28aMal; **(E)** pIVEX2.3d. Proteins overexpressed in the insoluble and soluble fractions are indicated by black and white arrows respectively. Isoleucile tRNA synthetase (IleS) and spectinoymcin 3' adenyltransferase (spw) were included as postivie controls for the production of soluble protein. Abbreviations used to annotate the SDS-PAGE gel images are as follows; M denotes protein marker (Broad Range 2-212 kDa, New England Biolabs); I, insoluble cell fraction; S, soluble cell fraction.

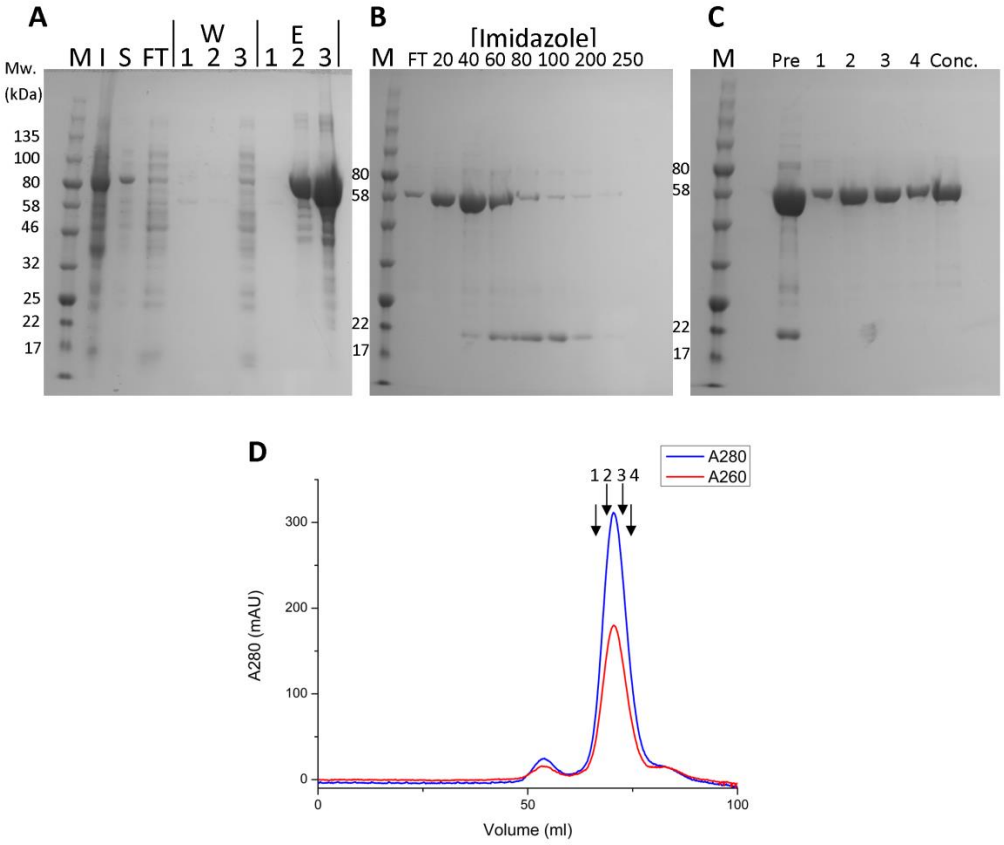


Figure 3.6. Purification of Lsa(A). Representative SDS-PAGE images showing analysis of (A) Initial capture using IMAC, (B) removal of cleaved 6-histidine-SUMO tag by IMAC (11 kDa), (C) gel filtration using an S200 column as shown in representative chromatogram D. Abbreviations used to annotate the SDS-PAGE gel images are as follows; M denotes protein marker (Broad Range 2-212 kDa, New England Biolabs); Pre, protein sample prior to loading onto specified column; I, insoluble cell fraction; S, soluble cell fraction; FT, flow through; W, wash; and E, eluate.

3.4.7 Screening for crystallisation conditions

Once methodology to purify Vga(A) and Lsa(A) to homogeneity was established, I attempted to find conditions for crystallisation of each protein with the intention of performing structural studies. Vga(A) and Lsa(A) were amenable to concentration in 50 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM DTT buffer to a maximum of 5 and 15 mg/ml respectively. Concentrated protein was used to set up eight commercially available crystallisation screens as detailed in Section 2.9. The protein concentration, incubation temperature, and ratio of crystallisation reagent to protein in each drop, was varied for each screen. Additionally, screens were established in the presence and absence of the non-hydrolysable ATP analogue AMP-PNP. During this study, no conditions conducive to crystallisation of Vga(A) or Lsa(A) were found. For both Vga(A) and Lsa(A), the majority of wells showed the formation of granular precipitates within three days of incubation, either at 4°C or 25°C. The addition of AMP-PNP did not appear to alter the rate at which the proteins precipitated and did not lead to formation of any needles, microcrystals or phase separation.

3.4.8 Degradation of Vga(A) and Lsa(A)

Throughout this study I repeatedly observed a loss of activity (assessed by the ability to protect transcription-translation assays from antibiotic inhibition, Section 4.4.3) following storage of Vga(A) and Lsa(A) at 4°C. Lsa(A) maintained its activity for ~4 days, whereas Vga(A) only remained active for ~2 days. As a result, all functional studies described in Chapter 4 were performed immediately following purification or using protein that had been stored at -80°C and thawed only once. Analyis of protein samples following storage at 4°C by SDS-PAGE revealed extensive degradation of the full-length proteins (Figure 3.7 A and B). Addition of neither protease inhibitors nor various compounds to the buffer during storage of Lsa(A) was able to prevent degradadation without causing precipitation of the protein (Figure 3.7 B). Analysis of degraded Vga(A) by SDS-PAGE (Figure 8A) revealed numerous protein fragments ranging in size from 25 to 40 kDa (Figure 3.7 A). As the N-terminal ABC, linker, and C-terminal

ABC domains of Vga(A) have masses of 17, 15 and 30 kDa respecitvely, the size range of fragments suggest non-specific cleavage within the linker, generating trucated protein composed of one ABC and differing lengths of linker. Peptide-mass fingerprinting analysis of the two distinct SDS-PAGE bands resulting from Lsa(A) degradation also revealed no distinct site of cleavage, with each band containing fragments corresponding to all three domains.

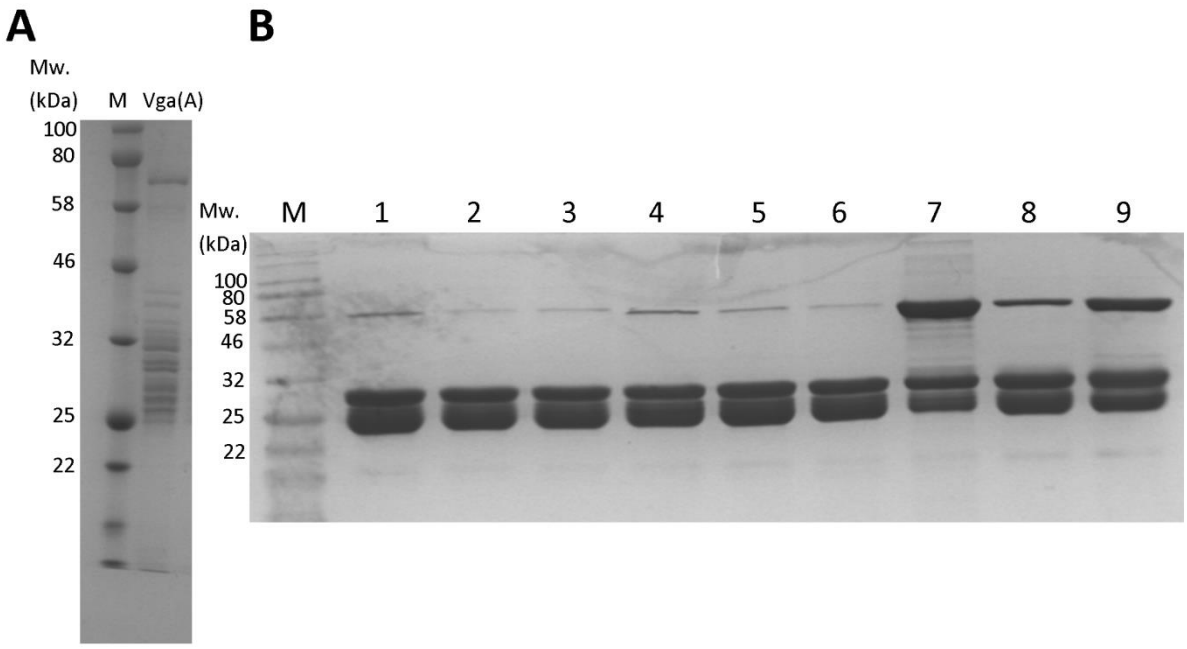


Figure 3.7. SDS-PAGE analysis of protein degradation. (A) Vga following 7 days storage at 4°C and (B) Lsa(A) following 5 days storage at 4°C. Several additives were screened for ability to protect Lsa(A) from degradation; 1, no additive; 2, 10% glycerol, 3, 0.1% (v / v) Tween 20; 4, 1mM $MgCl_2$; 5, cOmplete EDTA-free protease inhibitors (Roche); 6, 5mM Proteoloc protease inhibitors (Expedeon); 7, 5mM Proteoloc + EDTA (Expedeon); 8, 1mM EDTA; 9, 5% (v/v) EtOH. The final three conditions, 7, 8 and 9 caused precipitation of the protein.

3.4.9 Thermal stability of Vga(A)

In order to optimise buffer conditions for future work requiring Vga(A), I assessed the thermal stability of the protein in the presence of a range of additives. Measurement of intrinsic tryptophan fluorescence is an established technique used to follow protein unfolding, as the maximum emission wavelength of tryptophan differs depending on its environment (Eftink, 1994). Measurement of the ratio of fluorescence intensities at 350 nm and 330 nm during heating of the sample allows determination of the protein melting point (Tm, where half of the protein is folded and the other half is unfolded). The data revealed that Vga(A) with no additives was marginally stable at 37°C (Tm of 37.8°C, Figure 3.8, Table 3.3). Kosmotropes, glycerol and AMP-PNP increased the thermal stability of the protein, whereas chaotropes destabilised Vga(A) (Figure 3.8, Table 3.3). Of specific note was the addition of ammonium sulphate and glycerol, both of which increased Vga(A) thermal stability by more than 5°C. Future work requiring Vga(A) may benefit from inclusion of these additives in storage buffers.

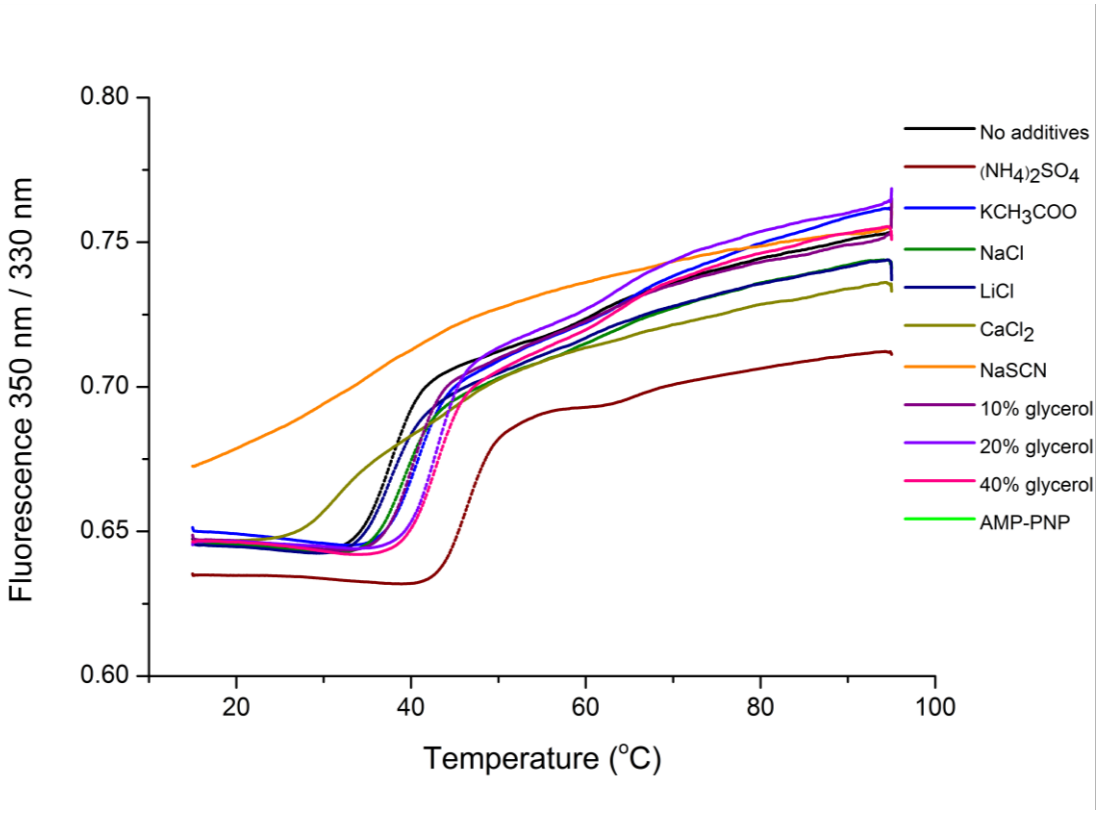


Figure 3.8. The effect of buffer additives on thermal stability of Vga(A). Vga(A) concentrated to 5 mg /ml (83 µM) was in GF buffer (50mM HEPES pH 7.4, 300 mM NaCl, 1 mM DTT), supplemented with additives to 500 mM, with the exception of AMP-PNP which was at 0.1 mM and glycerol (v/v) as shown below.

Table 3.3. Melting temperatures (Tm) of Vga(A) supplemented with a variety of buffer additives. Tms were determined from the maximum of the first derivative of data shown in in Figure 3.8.

Additive	Concentration	Tm (°C)
No additive	-	37.8
AMP-PNP	0.1 mM	39.3
$(NH_4)_2SO_4$,	500 mM	46.4
KCH_3COO	500 mM	40.7
NaCl	500 mM	39.3
LiCl	500 mM	37.7
$CaCl_2$	500 mM	31.2
NaSCN	500 mM	-
Glycerol	10% (v / v)	40.4
Glycerol	20% (v / v)	43
Glycerol	40% (v / v)	43

3.5 Discussion

An important step in attempts to elucidate the mechanism of ABC-F mediated antibiotic resistance is the production of homogenously purified proteins that are representative of the group. As Vga(A) is the best characterised of the ARE ABC-F proteins, I began this study with an attempt to replicate the expression and purification conditions previously described by Jacquet *et al*. (2008). However, despite numerous attempts, treatment of Vga(A) with Benzonase and protamine sulphate did not remove contaminating nucleic acid efficiently enough to facilitate reproducible purification of the protein. As a result, an alternative method of purification was developed, which uses high concentrations of sodium chloride to dissociate nucleic acid from Vga(A) and results in a marginally increased yield in comparison to that of Jacquet *et al.* (3 mg / l vs 1-2 mg / l). Similar methodology has previously been used in the purification of viral nucleocapsid proteins that specifically bind RNA (Ariza *et al*., 2013, Carter *et al*., 2012). However, the propensity of Vga(A) to bind nucleic acid throughout purification does not appear to be of functional significance. Although a ribosomal protection based resistance mechanism may involve interaction of Vga(A) directly with rRNA, purification of Lsa(A), which elicits a similar resistance phenotype to Vga(A) was not complicated by nucleic acid contamination. Instead, Vga(A) nucleic acid binding is likely non-specific and the result of its high isoelectric point (pI, predicted 9.06), a property not universally conserved amongst Vga-type determinants or ARE ABC-F proteins in general (Table 3.4).

Table 3.4. Predicted pIs of ARE ABC-F proteins found in non-producer organisms. The pI of each protein was predicted from its primary sequence using the ProtParam server (Wilkins *et al*., 1999).

ARE ABC-F	Predicted pI
Vga(A)	**9.06**
Vga(A)v	6.54
Vga(B)	8.89
Vga(C)	8.69
Vga(D)	7.26
Vga(E)	6.21
Msr(A)	6.05
Msr(C)	9.12
Msr(D)	6.28
Msr(E)	6.02
Lsa(A)	**5.77**
Lsa(B)	5.79
Lsa(C)	8.76
Lsa(E)	6.36

The optimised methodology for purification of Vga(A) and Lsa(A) described in this chapter yielded homogenously purified protein that was suitable for functional studies as outlined in Chapter 4. However, as there has been no structural determination of any ARE ABC-F protein to date, I attempted to find conditions for crystallisation of Vga(A) and Lsa(A) to permit structural studies. Despite conducting numerous screens under a variety of conditions, with or without ligand, no conditions conducive to crystallisation were found.

Recently, the structure of the bacterial ABC-F protein EttA was determined by X-ray crystallography and cryro-electron microscopy (cry-EM) (Boel *et al*., 2014, Chen *et al*., 2014), providing the first structural information regarding an ABC-F protein. EttA, which regulates bacterial translation in response to changes in the relative levels of cellular ATP and ADP, shares 21% and 26% amino acid identity with Vga(A) and Lsa(A) respectively. Functional insights into the ARE ABC-F proteins obtained from analysis of the EttA structure are outlined in chapter 4, however, EttA also serves to demonstrate that despite the presence of a flexible interdomain linker, ABC-F proteins do possess the structural uniformity required for formation of X-ray diffracting crystals, even in the absence of bound nucleotide. It is likely

therefore, that the inability to find conditions to promote crystallisation of Vga(A) and Lsa(A) during this study is the result of a factor other than intrinsic disorder of either protein. Degradation of both Vga(A) and Lsa(A) at 4°C provides a simple alternative explanation. Although crystal screens were established within a day of purification, in the absence of any crystallisation condition that could effectively stabilise the protein and prevent degradation, the protein sample would quickly become heterogeneous and unable to form crystals. The cause of protein degradation observed during this study is not clear, neither the addition of a variety of commercially available protease inhibitor cocktails during and after purification, nor purification under aseptic conditions, prevented degradation. It is of note that truncated fragments resulted from cleavage of the interdomain linker in both Vga(A) and Lsa(A). As structural studies of the ABC-F EttA have revealed inherent flexibility in the interdomain linker and as proteases preferentially bind and cleave flexible or unfolded domains (Fontana *et al*., 2004), it is likely that the degradation pattern of Vga(A) and Lsa(A) is the result of cleavage of the interdomain linker by trace amounts of contaminating protease that are not sufficiently inhibited by the protease inhibitors tested in this study.

Stabilisation of the globular structure of Vga(A) and Lsa(A) may decrease flexibility of the linker domain and thereby reduce proteolytic degradation. To this end, I assessed the thermal stability of Vga(A) under various conditions. At physiological temperatures purified Vga(A) was found to be partially unfolded, exhibiting a Tm of close to 38°C. Although examples of proteins that are unstructured in solution but are capable of mediating antibacterial resistance can be found, as exemplified by the heavy metal resistance determinants PcoE and SilE (Zimmermann *et al*., 2012, Gupta *et al*., 1999), the mechanisms by which ARE ABC-F proteins are proposed to act do not immediately suggest a role for transitions between a folded and unfolded state. It is therefore likely that the low Tm of Vga(A) is the result of sub-optimal buffer composition failing to adequately replicate the staphylococcal intracellular environment. As the thermal stability of Vga(A) was improved by

addition of kosmotropes and glycerol, future buffers for crystallographic studies of Vga(A) should be altered accordingly.

3.6 Conclusions

Work outlined in this chapter describes optimisation of methodology for the expression and purification of two ARE ABC-F proteins; Vga(A) and Lsa(A). Expression of Lsa(A) was facilitated through fusion of a solubility enhancing SUMO tag to the N-terminus of the protein and purification of Vga(A) was reliant upon successful removal of contaminating nucleic acid. Degradation of the two proteins was likely the result of proteolytic cleavage of the interdomain linker and may be the cause of a failure to identify conditions for crystallisation. The homogenously purified proteins generated using methods described in this chapter were used to conduct functional studies as described in Chapter 4.

4. Investigating the mechanism of ARE ABC-F mediated antibiotic resistance

4.1 Abstract

The mechanism by which ARE ABC-F proteins mediate resistance to several antibiotic classes that target the 50S subunit of the bacterial ribosome is not understood. However, two competing hypotheses exist; antibiotic efflux and ribosomal protection. Here, this mechanism was investigated using a combination of biochemical and bacteriological techniques. Using bacteriological assays, the resistance phenotypes mediated by ARE ABC-F proteins were found to be negated by protection of the ribosome through methylation and shown to correlate with the overlap of antibiotic ribosome binding sites. For the first time, *vga(A)* and *lsa(A)* were shown to mediate cross-resistance to select antibiotics of the macrolide class. Preparation of staphylococcal cell extracts suitable for transcription-translation (T/T) assays allowed the function of ARE ABC-F proteins Vga(A) and Lsa(A) to be assessed in the absence of a cell-membrane. Addition of Vga(A) and Lsa(A) to staphylococcal T/T reactions subject to antibiotic inhibition caused a drug specific, dose-dependent, rescue of translation. Several previously described Vga(A) and Lsa(A) conferred phenotypes were recapitulated in T/T assays, thereby verifying that rescue of translation observed *in vitro* is representative of the action of these proteins in whole cells. Finally, ribosome binding assays using radiolabelled lincomycin showed Lsa(A) to be capable of displacing the drug from staphylococcal ribosomes. Collectively, the experiments outlined in this chapter provide the first direct evidence to support a mechanism of ARE ABC-F resistance based on ribosomal protection.

4.2 Introduction

Two models to explain the action of ARE ABC-F proteins have been suggested (Kerr *et al*., 2005, Reynolds *et al*., 2003). In the first model, these proteins function as classical ABC transporters, associating with as yet unknown trans-membrane domains and use energy released by ATP hydrolysis to drive transport of antibiotics out of the cell. Alternatively, in the second model, ARE ABC-F proteins act to reduce the accessibility or affinity of the antibiotic binding sites within the 50S ribosomal subunit, thereby protecting the translational machinery. Experimental evidence permitting a definitive conclusion regarding the mechanism by which they mediate resistance is lacking; however, ARE ABC-F resistance determinants are most frequently cited as efflux proteins in the literature (Matsuoka *et al*., 1993, Olano *et al*., 1995, Chesneau *et al*., 2005, Nunez-Samudio and Chesneau, 2013).

The predominance of the efflux hypothesis perhaps stems from the characterisation of the first ARE ABC-F protein to be described; the macrolide and streptogramin B resistance determinant Msr(A) (Ross *et al*., 1989, Ross *et al*., 1990). The dual observations of Msr(A) homology to ABC-transporters and Msr(A) induced, ATP-dependent, decreased accumulation of erythromycin, understandably led to the conclusion that Msr(A) and subsequently other ARE ABC-F proteins mediate resistance through active efflux. However, the paper detailing this work included a cautionary note; that decreased accumulation of erythromycin could also result from ribosomal protection, a hypothesis that was later proven experimentally (Reynolds *et al*., 2003). More recently, as an increasing number of ARE ABC-F proteins have been described, a correlation between the resistance phenotypes conferred by ARE ABC-F determinants and the positions at which antibiotics bind within the 50S ribosomal subunit has become apparent (Kerr *et al*., 2005, Dorrian, 2009). This is evident when comparing vga- and lsa- type determinants, which confer resistance to antibiotics from structurally unrelated classes that bind overlapping sites within the peptidyl-transferase centre, with msr-type genes, which show specificity for antibiotics binding within the peptide

exit tunnel. This observation, coupled with the fact that several ABC-F proteins not involved in antibiotic resistance have recently been shown to interact directly with the ribosome or with ribosomally-associated proteins (Kerr, 2004), has made the hypothesis that that ARE ABC-F proteins mediate ribosomal protection increasingly plausible.

Methodology for the characterisation of antibiotic resistance proteins that act to protect translation has been previously established. For example, the mechanism underlying horizontally-transmissible fusidic acid resistance was initially determined using staphylococcal T/T assays, which showed FusB-type proteins were able to restore translation in the presence of fusidic acid (O'Neill and Chopra, 2006). Subsequently, pull-down experiments using immobilised FusB and staphylococcal cell-lysates identified a direct interaction with elongation factor-G (EF-G), the target of fusidic acid (O'Neill and Chopra, 2006). Building on these findings, an X-ray crystallography based structural characterisation of two FusB-type proteins has been completed (Cox *et al*., 2012, Guo *et al*., 2012), the interaction with EF-G has been characterised (Cox *et al*., 2013), and an appreciation of the molecular basis of fusidic acid resistance mediated by FusB-type proteins now exists. Similarly, characterisation of the tetracycline ribosomal protection proteins (RPPs) began with cell-free protein synthesis assays that showed extracts derived from bacteria expressing the RPP Tet(M) were resistant to tetracycline inhibition, whilst extracts containing the tetracycline efflux protein, Tet(L), were not (Burdett, 1986). This mechanism was studied further using ribosome binding assays in which the ability of Tet(M) and Tet(O) to displace bound tetracycline was discovered (Trieber *et al*., 1998, Burdett, 1996), and, more recently, the structure of both Tet(M) and Tet(O) in complex with the *E. coli* ribosome has been determined using cryo-electron microscopy (cryo-EM) (Arenz *et al*., 2015, Li *et al*., 2013).

Due to the paucity of evidence in support of ARE ABC-F mediated antibiotic efflux, and the increasing amount of indirect evidence indicating a mechanism of ribosomal protection, I

aimed to use the established biochemical methodology outlined above, in-tandem with bacteriological assays to investigate ARE ABC-F resistance phenotypes, to provide a definitive explanation of the ARE ABC-F resistance mechanism.

4.3 Aims

Work described in this chapter aimed to determine the mechanism by which ARE ABC-F proteins mediate antibiotic resistance.

4.4 Results

4.4.1 Probing the correlation between ARE ABC-F resistance phenotypes and the binding sites of 50S targeted antibiotics.

In initial experiments using staphylococci expressing the Vga(A) protein, I sought preliminary support for a mechanism of resistance involving either ribosomal protection or efflux. Previous studies have noted a correlation between the resistance phenotypes mediated by ARE ABC-F proteins and the extent of overlap between binding sites of protein synthesis inhibitors within the 50S subunit (Reynolds *et al*., 2003, Kerr *et al*., 2005). A testable prediction is that, if ARE ABC-F proteins do mediate resistance through ribosomal protection, they would likely offer cross-resistance to further classes of structurally-unrelated antibiotic that bind the ribosome in close proximity to their target antibiotics.

In order to test this hypothesis, the ARE ABC-F genes *msr(A), lsa(A),* and *vga(A),* were cloned using the *S. aureus / E. coli* shuttle vector pEPSA5 (Forsyth *et al*., 2002) and transferred into *S. aureus* RN4220. Whilst this study was in progress, a paper detailing a mutational characterisation of the Vga(A) interdomain linker was published (Lenart *et al*., 2015), in which an expanded spectrum mutant of Vga(A), Vga(A)$_{K219T}$, was characterised. As Vga(A)$_{K219T}$ conferred increased levels of resistance to lincosamides and pleuromutilins, it follows that it may exert an effect on a larger area of the PTC than wild-type Vga(A) and therefore provide

an increased chance of finding previously undescribed cross-resistance. Consequently, pEPSA5-*vga(A)* was subjected to site-directed mutagenesis to generate pEPSA5-*vga(A)*$_{K219T}$ and following confirmation of the mutation by sequencing, pEPSA5-*vga(A)*$_{K219T}$ was included in the screen. Subsequently, the MICs of numerous protein synthesis inhibitors were determined against the four constructed strains and RN4220 containing only the pEPSA5 vector (Table 4.1).

As previously described, strains expressing *vga(A)* showed cross-resistance to streptogramin A (64 fold, virginiamycin M), lincosamide (8 fold lincomycin) and pleuromutilin (4 fold retapamulin) antibiotics. Strains expressing *vga(A)*$_{K219}$ exhibited resistance to the same classes, but gained the ability to confer tiamulin resistance (512 fold) and showed elevated levels of resistance to lincomycin (LNC, 128 fold) and retapamulin (1024-fold). Similarly, expression of *lsa(A)* conferred resistance to virginiamycin M (VGM, 64 fold), LNC (64 fold), retapamulin (128 fold), and tiamulin (512 fold). In contrast, of the compounds tested, *msr(A)* only conferred resistance to erythromycin (256 fold). No cross-resistance to, fluorphenicol, blasticidin S, sparsomycin, or the 16-membered macrolides tylosin and spiramycin, was observed amongst any of the tested strains. However, expression of *vga(A)* was shown to elicit reduced susceptibility to the 16-member macrolides leucomycin A1 (4 fold) and carboymycin (4 fold), antibiotics with binding sites predicted to partially overlap that of VGM (Di Giambattista *et al*., 1987). In contrast, *lsa(A)* mediated reduced susceptibility to carbomycin (4 fold), but not leucomycin A1. To my knowledge this is the first report of a vga-type or lsa-type resistance determinant mediating any degree of macrolide resistance.

Table 4.1. Activity of protein synthesis inhibitors that target the PTC and peptide exit tunnel against S. aureus RN4220 expressing the ARE ABC-F genes msr(A), lsa(A), *vga(A)* and *vga(A)*$_{K219T}$. All MICs were determined in the presence of 2% xylose in order to induce expression from the pT5X promoter of plasmid pEPSA5.

	MIC (µg/ml) against *S. aureus* RN4220 strain				
Antibacterial compound	pEPSA5 (no insert)	pEPSA5-*msr(A)*	pEPSA5-*lsa(A)*	pEPSA5-*vga(A)*	pEPSA5-*vga(A)*$_{K219T}$
Virginiamycin M	1	1	64	64	64
Lincomycin	0.25	0.125	8	2	32
Retapamulin	0.03125	0.03125	4	0.125	32
Tiamulin	0.25	0.125	128	0.125	64
Erythromycin	0.5	128	0.5	0.5	0.5
Linezolid	2	2	2	2	2
Puromycin	8	4	8	8	8
Blasticidin S	128	128	128	128	128
Sparsomycin	32	32	32	32	32
Fluorphenicol	4	4	4	4	4
Leucomycin A1	0.25	0.25	0.25	1	1
Carbomycin	0.5	0.5	1	2	2
Tylosin	0.5	0.5	0.5	0.5	0.5
Spiramycin	1	1	1	1	1

4.4.2 Function of *vga(A)* in cells with protected ribosomes

A common feature of antibiotic resistance mechanisms involving efflux is that, when co-resident in a bacterial cell with another determinant mediating resistance to the same antibacterial agent but which acts to protect the drug target, a synergistic or additive increase in resistance is observed. For example, *S. aureus* strains in which the tetracycline RPP, *tet(M),* and efflux pump, *tet(K),* are both present have been shown to engender higher tetracycline MICs than strains harbouring only one of these resistance determinants (Trzcinski *et al.*, 2000). By contrast, no such enhancement in antibiotic resistance may be observed when two resistance determinants, both of which act at the level of the drug target, co-exist in a bacterial cell. As exemplified by the fact that carriage of both an endogenous *fusA* gene possessing mutations resulting an production of a fusidic acid resistant EF-G protein and a gene encoding FusB does not lead to a synergistic interaction between the two resistance mechanisms. Instead the level of resistance in strains co-expressing these resistance mechanisms is determined by the protein giving highest protection (O'Neill and Chopra, 2006). It is therefore reasonable to assume that failure of Vga(A) to exhibit an additive or synergistic effect when co-resident in a cell with a ribosomal protection mechanism of antibiotic resistance would suggest that Vga(A) does not mediate resistance by efflux.

In order to establish a system in which this hypothesis could be assessed, I utilised the Cfr-ribosomal methylase. The *cfr* gene encodes an rRNA methyltransferase that methylates 23S rRNA at position 8 of adenine 2503 (*E. coli* numbering), thereby protecting ribosomes from binding of several antibiotic classes including those encompassed within the spectrum of activity of Vga(A) (Long *et al*., 2006). The *cfr* gene was cloned into plasmid pEPSA5 under control of the inducible *pT5X* promoter and both pEPSA5 and pEPSA5-*cfr* were transformed into *S. aureus* CYL557. The *vga(A)* gene was cloned into plasmid pLL39 under control of the strong staphylococcal *cap1a* promoter and transformed into *S aureus* strain CYL557,

whereupon it integrated into the chromosome. Integration was confirmed by PCR using a primer pair specific for *vga(A)* and the φ11 attP site on the *S. aureus* chromosome. Subsequently, pEPSA5 and pEPSA5-*cfr* were separately transformed into strain CYL557containing integrated *vga(A)*.

Susceptibility to virginiamycin M, and linezolid was determined for *S. aureus* RN4220 expressing v*ga(A)* alone, or v*ga(A)* and *cfr*. As expected, expression of *vga(A)* mediated resistance to virginiamycin M, but not to linezolid, whilst expression of *cfr* alone gave resistance to both drugs (Table 4.5). Co-expression of both resistance determinants did not confer a decrease in susceptibility for any of the drugs beyond that exhibited by the strain solely expressing *cfr*, establishing that co-expression of the two resistance proteins does not produce an additive or synergistic effect (Table 4.5), and further reinforcing the idea that resistance is more likely mediated through ribosomal protection than efflux.

Table 4.5. The effect of Cfr-mediated ribosomal methylation on *vga(A).*

	MIC (μg/ml) against *S. aureus* CYL557strain			
Antibacterial compound	pEPSA5 (no insert)	pLL39-*vgaA*	pEPSA5-*cfr*	pEPSA5-*cfr* +pLL39-*vgaA*
Virginiamycin M	1	64	128	128
Linezolid	2	2	8	8

4.4.3 The activity of Vga(A) and Lsa(A) in transcription-translation assays

Initial bacteriological work (Sections 4.4.1 and 4.4.2) was indicative of an ARE ABC-F mechanism based on ribosomal protection and prompted investigations to determine whether ARE ABC-F proteins could mediate antibiotic resistance in a system where efflux was not possible. This system was established through the generation of staphylococcal S30 extracts, and their use to perform T/T assays (Section 2.5). During T/T assays, plasmid pC19Saluc provided a template for the expression of firefly luciferase and subsequent

addition of luciferin allowed the level of translation to be assessed through measurements of luminescence. As I observed that different preparations of S30 extract showed varying levels of activity, all results described below are expressed as a percentage of an uninhibited positive control, run in parallel, which contained no exogenous proteins or antibiotics. Homogenously purified Vga(A) (Section 3.4.4) and Lsa(A) (Section 3.4.6) were introduced into inhibited T/T assays and the ability of these proteins to rescue translation from antibiotic inhibition was assessed.

Introduction of 4 µM purified Vga(A) into T/T assays inhibited with the IC_{90} of VGM resulted in a restoration of translational activity to 59% of the uninhibited control (Figure 4.1 A, columns 1, 3 and 5). This effect was Vga(A) specific, as neither addition of the fusidic acid resistance protein FusB (Section 2.4.5) nor heat-denatured Vga(A) were able to protect translation (Figure 4.1 A, columns 4 and 10). Furthermore, addition of 4 µM Vga(A) to T/T assays inhibited with the IC_{90} of fusidic acid did not rescue activity, demonstrating Vga(A)-mediated protection as specific to VGM (Figure 4.1 A, columns 8 and 9). Protection of translation from VGM by Vga(A) occurred in a dose-dependent manner between 1 and 4 µM Vga(A) (Figure 4.1 B). However, restoration was not complete and plateaued at 59% with no increase observed upon further addition of Vga(A) (Figure 4.1 B).

In order to investigate whether the ability to protect staphylococcal translation *in vitro* was a common property of ARE ABC-F proteins and not exclusive to Vga(A), the phylogenetically distant ARE ABC-F protein Lsa(A) (25 % amino acid identity) was titrated into staphylococcal S30 extracts inhibited with an IC_{90} of VGM (Figure 4.1 C) or LNC (Figure 4.1 D). As with Vga(A), rescue of translation was concentration dependent, but not complete, reaching a maximum of 49% and 68% for VGM and LNC inhibited reactions respectively (Figure 4.1 C and 1D). A lower concentration of Lsa(A) was required to restore translational activity in LNC inhibited reactions (Figure 4.1 D) than those knocked down with VGM (Figure 4.1 C). As with Vga(A), heat denaturation of Lsa(A) resulted in a loss of ability to protect translation from VGM (Figure 4.1 A, columns 3, 6, 7).

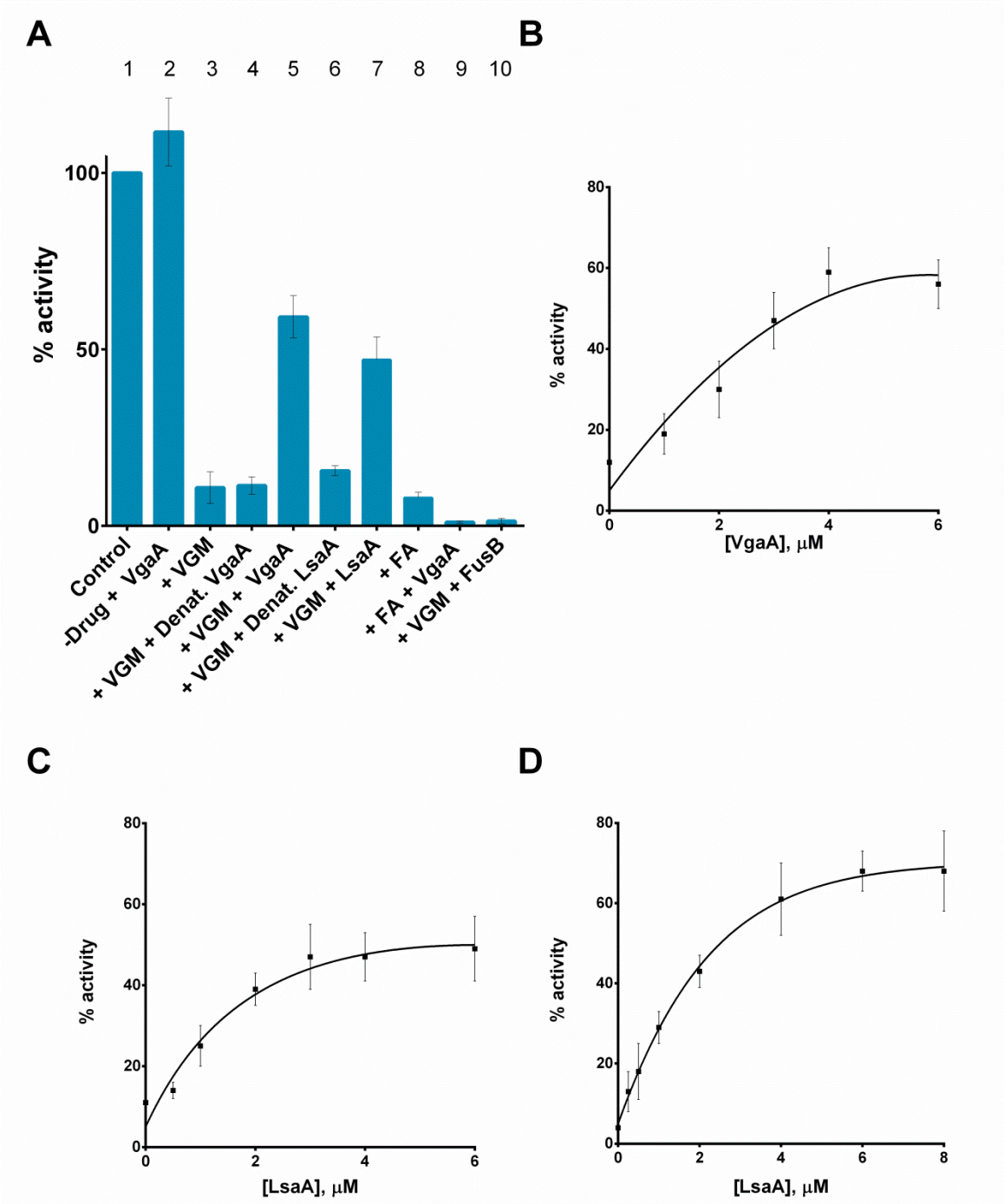


Figure 4.1. Vga(A) and Lsa(A) rescue staphylococcal translation from antibiotic inhibition. (A) Numbered columns indicate reactions containing the following: 1, control with no exogenous protein or antibiotic; 2, 4 µM Vga(A) but no antibiotic, 3; IC_{90} of virginiamycin M (VGM) with no exogenous protein; 4, IC_{90} of VGM with 4 µM heat-denatured Vga(A) (Denat.); 5, IC_{90} of VGM with 4 µM Vga(A); 6, IC_{90} of VGM with 4 µM heat-denatured Lsa(A); 7, IC_{90} of VGM with 4 µM Vga(A); 7, IC_{90} of VGM with 4 µM Lsa(A); 8, IC_{90} of fusidic acid (FA) with no exogenous protein; 9, IC_{90} of fusidic acid (FA) with 4 µM Vga(A); 10, , IC_{90} of VGM with 4 µM FusB. **(B)** The effect of Vga(A) (0–6 µM) on T/T inhibited by an IC_{90} of VGM. **(C)** The effect of Lsa(A) (0–6 µM) on T/T inhibited by an IC_{90} of VGM. **(D)** The effect of Lsa(A) (0–4 µM) on T/T inhibited by an IC_{90} of LNC. All data points derive from assays conducted at least in triplicate. All error bars correspond to standard deviations.

To provide confirmation that the observed ability of ARE ABC-F proteins to protect an *in vitro* translation assay from antibiotics reflects the activity of these proteins in whole cells, and to further explore this phenomenon of protection, I sought to recapitulate in the T/T assay several phenotypes that have been associated with these proteins in bacteria.

The Vga(A) protein is not functional in *E. coli*, failing to confer any reduction in susceptibility to streptogramin A antibiotics even when detectably overproduced in this bacterium (Jacquet *et al*., 2008). This result was mirrored in an *in vitro* T/T assay using *E. coli* S30 extract; addition of increasing concentrations of Vga(A) (Figure 4.2 A) or Lsa(A) (Figure 4.2 B) to maximum of 4 μM into T/T reactions inhibited with ≥IC_{90} of virginiamycin M produced no rescue of translation.

It has previously been demonstrated that substitution for glutamine of the catalytic glutamate residue following the Walker B motif in either nucleotide binding domain of Vga(A) results in a non-functional protein incapable of mediating resistance to virginiamycin M in cells of *Staphylococcus epidermidis* (Jacquet *et al*., 2008). I confirmed that this also holds true in *S. aureus*, with expression of $Vga(A)_{E105Q}$ in *S. aureus* RN4220 having no effect on virginiamycin M susceptibility (Figure 4.2 C). This same loss of ability of $Vga(A)_{E105Q}$ to mediate virginiamycin M resistance was also seen *in vitro*, with addition of up to 4 μM purified $Vga(A)_{E105Q}$ to a T/T assay employing *S. aureus* S30 extract producing no restoration of translational activity (Figure 4.2 D).

A single amino acid substitution ($K_{219}T$) in the linker region between the two nucleotide binding domains of Vga(A) has recently been reported to increase the level of phenotypic resistance to lincosamides from low-level (4-fold) to high-level (64-fold) (Figure 4.2 E) (Lenart *et al*., 2015). This shift in resistance profile was successfully recapitulated in the *S. aureus* T/T assay; addition of purified $Vga(A)_{K219T}$ to a T/T reaction inhibited with lincomycin resulted in restoration translation activity to ~30% of the uninhibited control, whilst 4 μM wild-type Vga(A) did not protect translation against lincomycin (Figure 4.2 F).

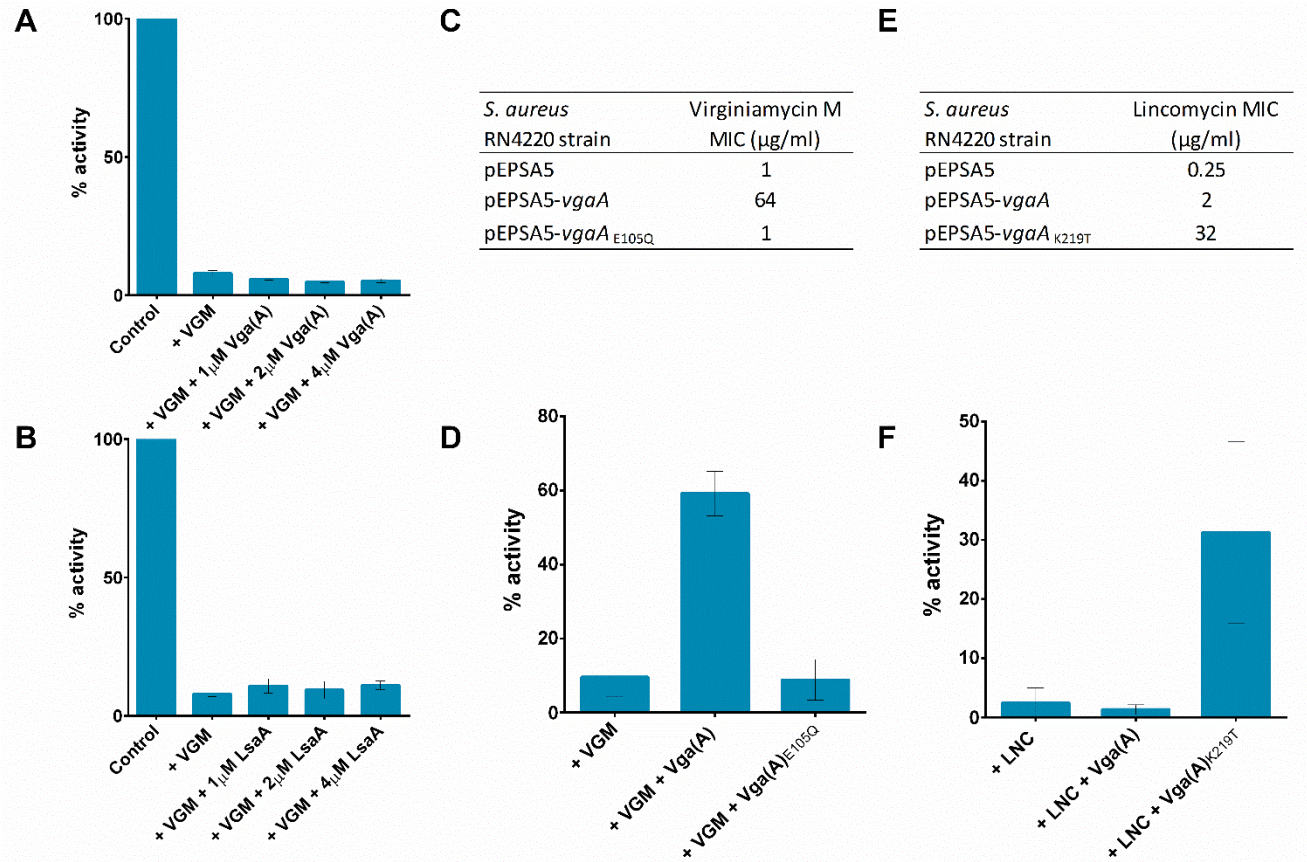


Figure 4.2. T/T assay results reconstitute previously described phenotypes. Addition of increasing concentrations of exogenous **(A)** Vga(A) or **(B)** Lsa(A) to a coliform T/T reaction containing an IC_{90} of VGM. **(C)** Table showing MIC values of VGM against *S. aureus* RN4220 expressing wild-type *vga(A),* an ATPase deficient *vga(A)*$_{E105Q}$ mutant, or transformed with the pEPSA5 plasmid only. **(D)** Addition of exogenous Vga(A)$_{E105Q}$, or wild-type Vga(A), to a staphylococcal T/T reaction containing an IC_{90} of VGM. **(E)** Table showing MIC values of lincomycin against *S. aureus* RN4220 expressing wild-type *vga(A),* an expanded spectrum *vga(A)*$_{K219T}$ mutant, or transformed with the pEPSA5 plasmid only. **(F)** Addition of the exogenous Vga(A)$_{K219T}$, or wild-type Vga(A), to a staphylococcal T/T reaction inhibited with a >IC_{90} of LNC. Data points derive from assays conducted at least in triplicate.

4.4.4 The activity of Lsa(A) in ribosome binding assays

As both Vga(A) and Lsa(A) were shown to protect staphylococcal translation from antibiotic inhibition *in vitro*, it follows that these proteins may function by preventing the binding of antibiotics to the ribosome, or by causing dissociation of the drug once it has bound. As previous binding assays to investigate the function of tetracycline RPPs had used radiolabelled drug to follow binding, and as the neither VGM or LNC are intrinsically fluorescent attempts were made to procure radiolabelled VGM and LNC. As radiolabelled VGM was not commercially available, the ability of purified Lsa(A) to mediate changes in binding of tritium-labelled lincomycin (^{3}H-LNC) to ribosomes was assessed. In order to establish an assay suitable to investigate this hypothesis, staphylococcal ribosomes were purified (Section 2.6.1) and quantified through comparisons of ribosomal protein L2 and 16S rRNA to known quantities of BSA (Figure 4.3 A) and RNA ladder (Figure 4.3 B). All binding assays were performed using a 2x molar excess of ^{3}H-LNC to ribosomes in the presence of ATP. As I observed that radioactivity of ^{3}H-LNC decreased over time, all results described below are expressed as a percentage of a positive control containing ribosomes and ^{3}H-LNC, but no exogenous protein or unlabelled drug.

Pre-incubation of staphylococcal ribosomes with between 1:1 and 8:1 Lsa(A) : ribosome molar ratios caused a dose-dependent decrease in ribosome associated ^{3}H-LNC, before reaching a plateau past which addition of Lsa(A) at did not cause further reduction in binding (Figure 4.4 A [i], page 99). Addition of an 8-fold excess of Lsa(A) (0.5 µM ribosomes, 4 µM LNC) resulted in the largest decrease in LNC binding, to 43% of the level observed in Lsa(A) free ribosomes (Figure 4.4 A [ii]). In comparison, pre-incubation of ribosomes with 4 µM BSA or 4 µM denatured Lsa(A) did not cause any reduction in levels of ribosomally associated ^{3}H-LNC (Figure 4.4 A [ii]), whereas addition of a 50-fold excess of unlabelled LNC resulted in almost complete removal of ^{3}H-LNC binding (Figure 4.4 A [ii]).

In order to test whether Lsa(A) might displace bound LNC, ^{3}H-LNC was pre-incubated with ribosomes prior to the addition of 4 µM Lsa(A). Subsequent addition of Lsa(A) caused dissociation of ^{3}H-LNC, resulting in a 73% reduction in the amount of ribosomally associated drug in comparison to the protein-free control (Figure 4.4 B). BSA had no effect on the release of ^{3}H-LNC from ribosomes and ^{3}H-LNC was exchanged with unlabelled LNC, resulting in almost complete removal of the radiolabelled compound from the ribosome (Figure 4.4 B).

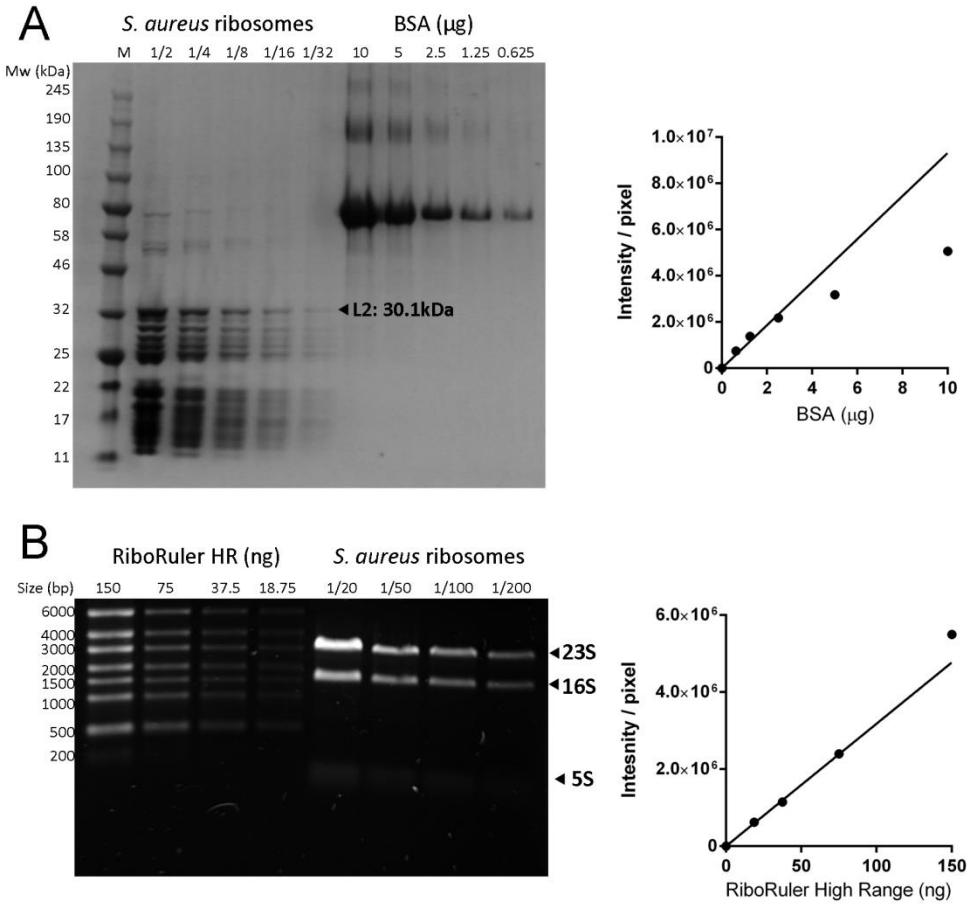


Figure 4.3. Purification and quantification of staphylococcal ribosomes. Purified staphylococcal ribosomes were quantified through comparisons of ribosomal protein L2 with known quantities of BSA (**A),** and 16S rRNA with RNA ladder RiboRuler HR.

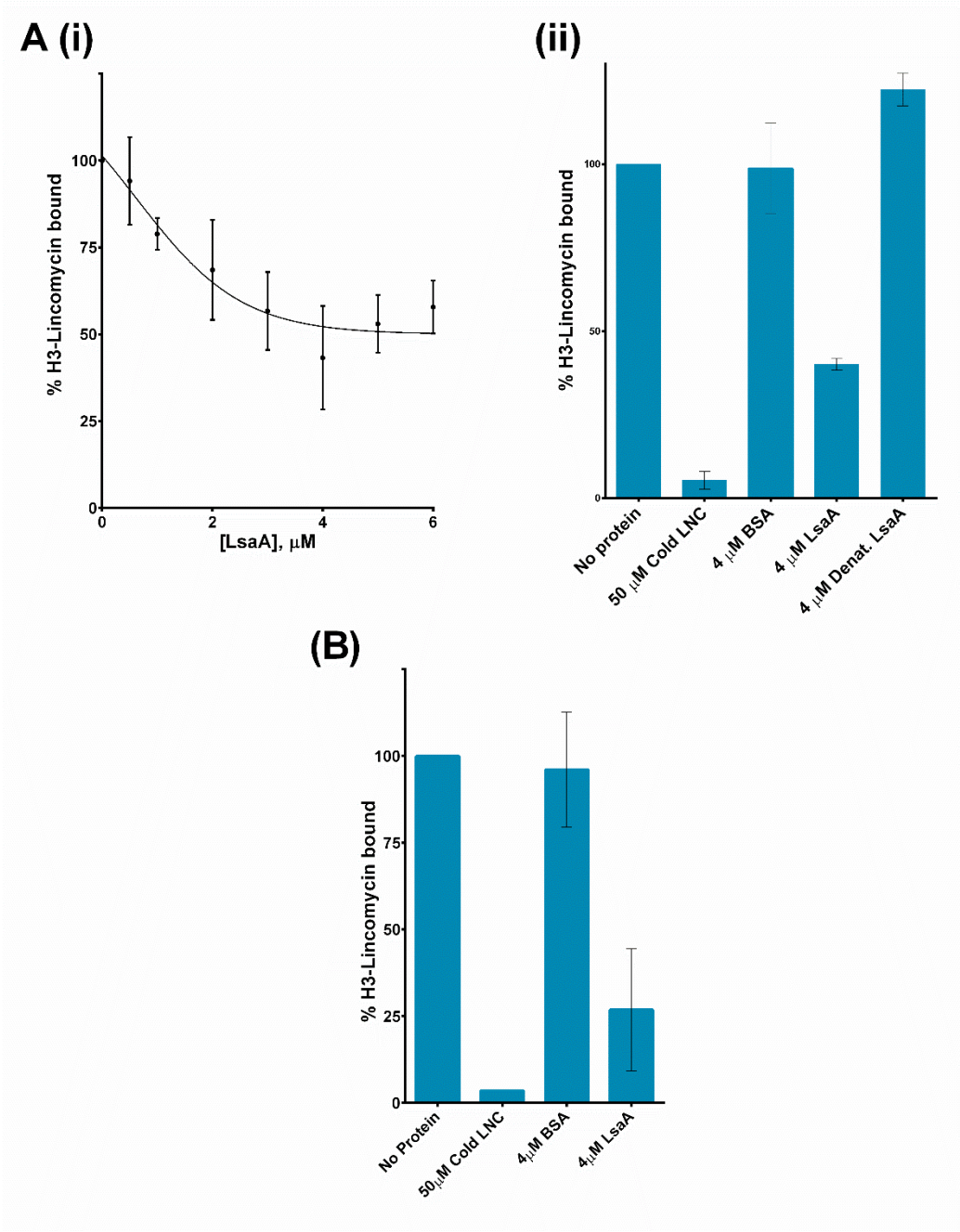


Figure 4.4. Lsa(A) protects ribosomes from ^{3}H-LNC binding and displaces pre-bound drug. **(Ai)** Pre-incubation of increasing concentrations of Lsa(A) with 0.5µM staphylococcal ribosomes. **(ii)** Pre-incubation of 0.5µM ribosomes with either; a 50x excess of unlabelled LNC (Cold LNC), 4 µM BSA, 4 µM Lsa(A), or 4 µM denatured Lsa(A). **(B)** Addition of either a 50x excess of unlabelled LNC, 4 µM Lsa(A), or 4 µM BSA to staphylococcal ribosomes that have been pre-incubated with LNC. Error bars correspond to standard deviations. Data points derive from assays conducted a minimum of three times.

4.4.5 Screening for protein: protein interactions involved in ARE ABC-F mediated resistance

As Vga(A) and Lsa(A) rescue *S. aureus,* but not *E. coli*, translation from antibiotic inhibition, I speculated that Vga(A) and Lsa(A) may directly interact with staphylococcal proteins to exert their protective effect, and that these proteins are either different or absent in *E. coli.* To examine this hypothesis, and to identify any putative binding partners of Vga(A) and Lsa(A), pull-down experiments were performed on cell lysates, using either Vga(A) or Lsa(A) as immobilised "bait". In order to reproduce conditions in which Vga(A) and Lsa(A) mediate protection, experiments were performed at 37°C in the presence of 1 µg / ml (1.9 µM) VGM. Previously, studies of the ABC-F protein EttA, which regulates translation in *E. coli* through a direct interaction with the ribosome, have shown that a double EttA mutant incapable of hydrolysing ATP mediates pull-down of *E. coli* ribosomes whilst wild type EttA does not (Chen *et al*., 2014). Given the homology between Vga(A) and Lsa(A) with EttA (25% and 24.5% amino acid identity respectively), I predicted that pull-down of ribosomes by ARE ABC-F proteins may also require the proteins to be trapped in an ATP-bound conformation. Therefore, I generated pIVEX2.3d-*vga(A)*$_{E105Q\text{-}E410Q}$ using site-directed mutagenesis. This construct encodes *vga(A)* with a glutamate to glutamine substitution of the catalytic glutamate in each ABC domain; a double mutation that permits ATP-binding, but not hydrolysis, in other ABC proteins (Chen *et al*., 2014, Orelle *et al*., 2003). However, expression from the pIVEX-*vga(A)*$_{E105Q\text{-}E410Q}$ construct did not produce soluble Vga(A)$_{E105Q\text{-}E410Q}$ under the conditions that had successfully been used to express WT Vga(A), Vga(A)$_{E105Q}$, or Vga(A)$_{K219T}$. As an alternative, I conducted pull down experiments using WT Vga(A) or Lsa(A) in the presence of ATP, or, the non-hydrolysable ATP analogue adenosine 5'-(β,γ-imido)triphosphate (AMP-PNP).

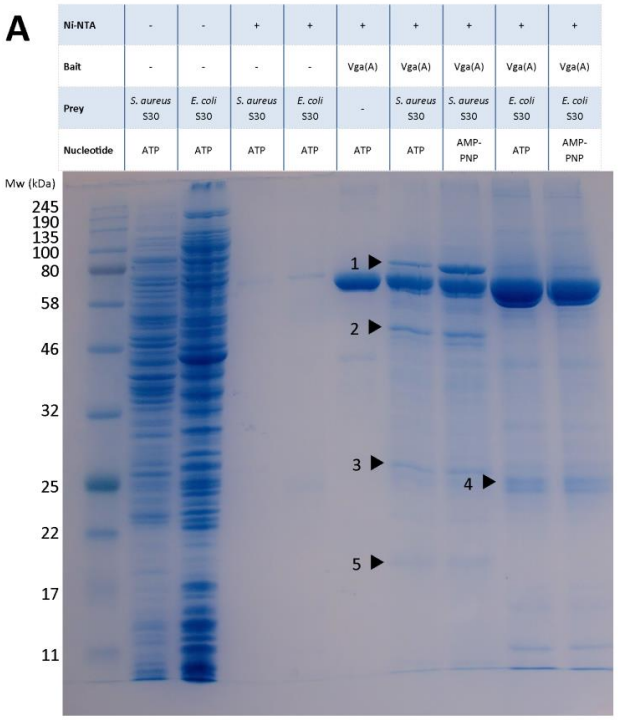
The use of his-Vga(A) and his-SUMO-Lsa(A) as immobilised bait and staphylococcal cell-extracts as prey resulted in pull-down of proteins that formed four distinct bands when

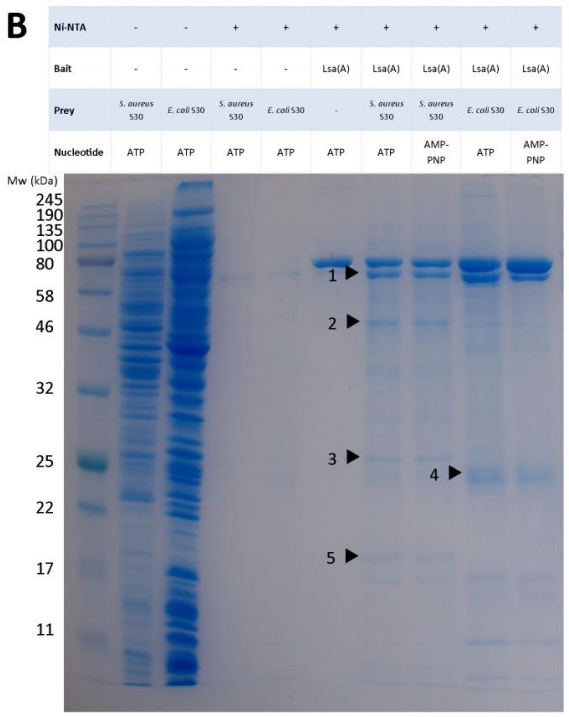
analysed by SDS-PAGE, none of which appeared in control experiments using *E. coli* cell lysates (Figure 4.5). These bands, plus an additional band present solely in pull-downs using *E. coli* cell-lysates, were excised and sent for identification by peptide mass fingerprinting. From each band, several proteins were identified (Personal communication, James Ault, University of Leeds) (Figure 4.5). Although analysis of SDS-PAGE bands though peptide fingerprinting and LC-MS does not give a direct measure of the proportion of each protein in a band, the percentage of coverage of each primary sequence by LC-MS identified peptides is indicative of prevalence of each protein in a sample (Personal communication, James Ault, University of Leeds). However, the identification of multiple proteins per band, and of the same protein in multiple bands (seemingly independent of molecular weight e.g. elongation factor Tu [EF-Tu], Pyruvate kinase, SlyD, Figure 4.5), complicates interpretation of the data arising from these pull-down experiments. It is therefore only possible to conclude from this data that Vga(A) and Lsa(A) appear to interact with proteins participating in a range of cellular processes, including translation, and that these interactions differ in *S. aureus* and *E. coli.* It is of note that a two-hybrid screen conducted in *E. coli* to investigate a possible interaction between Vga(A) and EF-Tu did not provide evidence of an interaction (Personal communication, Chris Randall, University Of Leeds).

As Lsa(A) has been shown here to displace LNC from the staphylococcal ribosome (Section 4.4.4) and ribosomal proteins were identified as putative binding partners for Vga(A) and Lsa(A) in both *S. aureus* and *E. coli* cell-lysates, a final set of pull-down experiments was conducted using purified staphylococcal ribosomes as prey and Lsa(A) or Vga(A) as bait. However, no pull-down of ribosomal proteins was observed (Figure 4.6, page 103).

Pull down assays using cell-lysates identified EF-Tu as a potential binding partner of Vga(A) and Lsa(A). As Ef-Tu is a ribosomally associated protein, the possibility of the protein co-purifying with ribosomes was assessed. A single band identified in preparations of

staphylococcal ribosomes that appeared to correspond to a protein with a similar molecular weight to Ef-Tu (black arrow, Figure 4.6) was sent for identification by peptide mass fingerprinting. However, the only protein detected in this band was Enolase (70% coverage, Personal communication, James Ault, University of Leeds), an enzyme associated with glycolysis and cell adhesion in *S. aureus* (Carneiro *et al*., 2004).





Band	ID	Coverage (%)	Mw (kDa)	Function
1	Pyruvate kinase	78	63102	Glycolysis / adhesion
	DnaK chaperone protein	56	66361	Protein Folding, Stress response
	Ef-Tu	52	43104	Translation
	Threonine tRNA ligase	39	74460	Translation
2	Ef-Tu	89	43104	Translation
	MurC	65	49188	Peptidoglycan biosynthesis
	3-phospohshikimate 1-carboxyvinyltransferase	58	47003	Amino acid biosynthesis
	3-oxoacyl-[acyl-carrier-protein] synthase 2	57	43739	Fatty acid biosynthesis
	Pyrimidine-nucleoside phosphorylase	54	46308	DNA biosynthesis
	Enolase	53	47117	Glycolysis / adhesion
	S-adenosylmethionine synthase	43	43641	S-Adenosyl methionine biosynthesis
	ATP-dependent Clp protease, ATP-binding subunit, ClpX	36	46298	Protein Folding, Stress response
	NAD specific glutamate dehydrogenase	36	45760	Nitrogen and Carbon metabolism
	Dihydrolipoyl dehydrogenase	35	49451	Component of pyruvate dehydrogenase complex
	Tyrosine t-RNA ligase	33	47598	Translation
3	Ef-Tu	85	43104	Translation
	30S ribosomal protein S3	56	24100	Translation
	L-threonine dehydratase catabolic TdcB	46	37306	Amino acid metabolism
	Glutamate racemase	42	29698	Peptidoglycan biosynthesis
	Thymidine kinase	39	22214	DNA biosynthesis
	Pyruvate kinase	34	63102	Glycolysis / adhesion
4	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	76	20853	Protein Folding, Stress response
	GTP cyclohydrolase 1	70	24831	Riboflavin biosynthesis
	50S ribosomal protein L4	63	22087	Translation
	30S ribosomal protein S4	46	23469	Translation
	50S ribosomal protein L3	44	22244	Translation
	3,4-dihydroxy-2-butanone 4-phosphate synthase	37	23353	Riboflavin biosynthesis
	Triose phosphate isomerase	37	26972	Glycolysis / adhesion
	Carbonic anhydrase 2	36	25097	Carbon homeostasis
	cAMP-activated global transcriptional regulator CRP	31	23640	Transcriptional regulation
5	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	67	20853	Protein Folding, Stress response

Figure 4.5. Pull down experiments using cell-lysates as prey to identify putative binding partners of Vga(A) and Lsa(A). SDS-PAGE images showing the results of immobilisation of **(A)** His-Vga(A) or **(B)** His-SUMO-Lsa(A) on Ni-NTA agarose and their subsequent use as bait to identify putative binding partners from staphylococcal and coliform S30 extracts. Assays were performed in the presence of 1µg / ml virginiamycin M and included either ATP or AMP-PNP. Control experiments assessed non-specific biding of proteins in the S30 extract to Ni-NTA agarose. Four bands specific to staphylococcal S30 extracts (1, 2, 3, 5) and one band specific to *E. coli* S30 extracts were sent for analysis by peptide mass fingerprinting, the results of which are shown on the right of the figure.

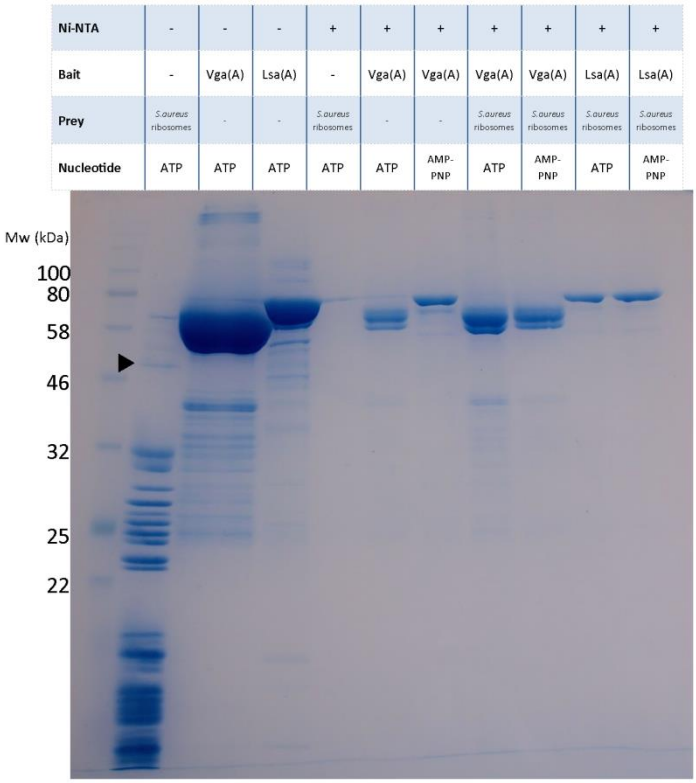


Figure 4.6. Pull down experiments using purified staphylococcal ribosomes as prey to identify putative binding partners of Vga(A) and Lsa(A). SDS-PAGE images showing the results of immobilisation of His-Vga(A) or His-SUMO-Lsa(A) on Ni-NTA agarose and their subsequent use as bait to identify putative binding partners from purified staphylococcal ribosomes. Assays were performed in the presence of 1µg / ml virginiamycin M and included either ATP or AMP-PNP. Control experiments assessed non-specific biding of ribosomal proteins to Ni-NTA agarose. A single band of around 48 kDa (black arrow) was sent for identification by peptide mass fingerprinting and was identified as Enolase (70% coverage).

4.5 Discussion

The mechanism by which ARE ABC-F proteins mediate antibiotic resistance has remained obscure since their discovery 25 years ago (Ross *et al*., 1990). Experiments described in this chapter were therefore designed to distinguish between the two proposed models of resistance. Previous studies using *in* vitro translation experiments to investigate tetracycline resistance in streptococci proved able to differentiate between functions of Tet(L), a tetracycline efflux protein, and Tet(M), a RPP (Burdett, 1986). Similar experiments conducted here found that Vga(A) and Lsa(A) mediated dose-dependent protection of a staphylococcal cell-free T/T system from inhibition by streptogramin A and lincosamide antibiotics, showing that these proteins confer resistance in a system where antibiotic efflux is not possible. The subsequent *in vitro* reconstitution of resistance phenotypes observed in whole cells indicate that results derived from the T/T assay are representative of the mechanism by which Vga(A) and Lsa(A) mediate antibiotic resistance in whole cells.

It is of note that neither protein restored translational activity to its uninhibited level. This result is in contrast to that seen for the tetracycline RPP Tet(M), which when added to *E. coli in vitro* translation assays has been shown to restore activity to uninhibited levels (Burdett, 1996). This may result from the proposed ability of Tet(M) to prevent rebinding of tetracycline by promoting alterations to the conformation of the drug binding site that persist following dissociation of Tet(M) from the ribosome (Connell *et al*., 2002, Connell *et al*., 2003b, Dönhöfer *et al*., 2012). In contrast, titration of the fusidic acid resistance protein FusB into staphylococcal fusidic acid inhibited T/T assays, resorted activity to a maximum of only 40% (O'Neill and Chopra, 2006). Fusidic acid inhibits protein synthesis by binding to ribosome associated EF-G and preventing its dissociation following GTP hydrolysis, leading to ribosomal stalling and cessation of translation (Bodley *et al*., 1969, Tanaka *et al*., 1968). FusB accelerates the release of EF-G from the ribosome and thereby overcomes the inhibitory effect of fusidic acid (Cox *et al*., 2012). As fusidic acid has a low affinity for non-

ribosome associated EF-G (Bodley *et al*., 1969), the drug is released on dissociation of EF-G from the ribosome and is free to re-bind Ribosome : EF-G : GDP complexes. FusB mediated fusidic acid resistance is therefore the result of FusB driving the equilibrium of fusidic acid binding to EF-G towards dissociation. Although it is difficult to extrapolate from an *in vitro* T/T assay to whole cells, where both Vga(A) and Lsa(A) function effectively, it is possible to speculate that the incomplete restoration of activity *in vitro* could indicate that these proteins mediate resistance in a similar manner to FusB, driving the equilibrium of ribosome : drug binding towards dissociation, rather than modifying the ribosome to prevent drug re-binding as proposed for tetracycline RPPs (Connell *et al*., 2003b). °

An alternative explanation for the dose-dependent restoration of translational activity observed in staphylococcal T/T assays is direct sequestration of the drug by Vga(A) and Lsa(A). Previous studies of Vga(A) and the N-terminal ABC domain of Ole(B) have proposed a direct interaction of the proteins with their target antibiotic (Buche *et al*., 1997, Jacquet *et al*., 2008), which would permit protection through sequestration (although a more recent analysis of Vga(A) binding to VGM and LNC using surface plasmon resonance failed to detect this interaction (Lenart *et al*., 2015)). However, neither Vga(A) or Lsa(A) rescued activity in coliform T/T assays performed using identical materials and methods to staphylococcal T/T assays. Nor did the protective effect mediated by Vga(A) and Lsa(A) in staphylococcal T/T assays continue indefinitely as more protein was titrated into the assay. It is therefore possible to rule out a sequestration based mechanism and conclude that protection is mediated by a specific interaction between Vga(A) or Lsa(A) with a component of the staphylococcal S30 extract.

Addition of Lsa(A) to staphylococcal ribosomes was shown to displace bound LNC. It follows that in order to drive displacement, Lsa(A) must interact directly with staphylococcal ribosomes or a co-purifying soluble factor. However, although several putative binding

partners involved in translation were amongst the proteins identified from pull-down experiments using staphylococcal S30 extract, comparable experiments using purified ribosomes did not suggest any Vga(A) or Lsa(A) mediated ribosomal binding. Due to time constraints, the specificity of the interactions found using cell extracts as bait could not be further investigated by repetition or through conducting pull down assays using immobilised putative binding partners as bait and cell extracts containing Vga(A) or Lsa(A) as prey. Similar pull down assays conducted to investigate the mechanism by which the ABC-F protein EttA regulates *E. coli* translation in response to relative cellular levels of ATP / ADP showed that only an EttA double mutant, able to bind but not hydrolyse ATP, captured ribosomes during pull-down experiments conducted *in vivo* (Boel *et al*., 2014). Equally, studies of Tet(M) and Tet(O) resistance determinants have shown that complexes amenable to purification by gel filtration only form between RPPs and *E. coli* ribosomes in the presence of the non-hydrolysable GTP analogous guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) and 5'-Guanylyl imidodiphosphate (GMP-PNP) (Trieber *et al*., 1998, Dantley *et al*., 1998). However, as addition of AMP-PNP to Vga(A) and Lsa(A) pull down assays did not reveal an interaction between the ARE ABC-F proteins and the ribosome, further work is required to explain the discrepancy between Lsa(A) mediated displacement of lincomycin from ribosomes and the inability to detect ribosome binding in pull-down assays. Due to constrains of time and material, a dissection of the effects of various adenosine nucleotides on the ability of Lsa(A) to displace LNC from the ribosome was not conducted during this study. Such an experiment may inform the discovery of conditions conducive to formation of a stable ribosome : ARE ABC-F complex. Additionally, investigation of complex formation through analytical gel filtration using untagged, Vga(A) and Lsa(A), free of any conformational restrictions imposed by immobilisation on Ni-NTA agarose may lead to an understanding of the conditions required to promote binding to ribosomes.

Indirect evidence suggestive of a direct interaction of ARE ABC-F proteins with the ribosome stems from the apparent correlation between the resistance phenotypes conferred by ARE ABC-F determinants and the degree of overlap of their target antibiotic binding sites within the 50S subunit. During this study, determination of the MICs of a panel of antibiotics that bind the 50S ribosomal subunit against *S. aureus* expressing representative genes from the vga-type, lsa-type and msr-type groups confirmed several previously reported resistance phenotypes.

The alignment of multiple crystal structures of ribosome : antibiotic complexes permits a structural representation of ARE ABC-F resistance phenotypes (Figure 4.7). Proteins belonging to the vga- and lsa- type groups confer resistance to antibiotics that overlap both the A-site and P-site (streptogramin As and pleuromutilins) or the A-site and entrance to the peptide exit tunnel (lincosamides) (Figure 4.7 B). In contrast, msr-type determinants confer resistance to 14- and 15-membererd ring macrolides, ketolides and type B streptogramins, all of which bind to overlapping sites in the nascent polypeptide exit tunnel (Figure 4.7 C). However, the *msr(A)* gene did not confer resistance to any of the 16-membered macrolides tested, a result also observed for *msr(C)* and *msr(D)* (Reynolds and Cove, 2005). Although the 14- and 16- membered macrolides bind the ribosome in an overlapping position, 16-membered macrolides are predicted to form an additional covalent bond with the ribosome, resulting in increased affinity for their binding site (Hansen *et al*., 2002), and perhaps explaining the lack of resistance to 16-member macrolides mediated by msr-type determinants.

The recently characterised *optrA* gene confers resistance to phenicols and oxazolidinones, which share an overlapping binding site at the ribosomal A-site (Figure 4.7 D). During this study, *vga(A)* and *lsa(A)* did not confer any detectable level of resistance to fluorphenicol or linezolid, equally, *optrA,* has been shown to confer no resistance to type A streptogramins,

lincosamides, or pleuromutilins (Wang *et al*., 2015). It is evident, that although phenicols and oxazolidinones, share a degree of overlap in their binding sites with pleuromutilins, lincosamides, and type A streptogramins, it is not sufficient for a single ARE ABC-F protein to confer cross-resistance to antibiotics of every class, as is the case with the ribosomal methylase Cfr (Long *et al*., 2006). An explanation of this discrepancy in resistance phenotypes may lie in the precise interactions of ARE ABC-F proteins with elements of the PTC, details of which could be obtained through structural studies.

No resistance to antibiotics binding in direct competition with tRNA within the PTC was observed amongst the ARE ABC-F determinants screened during this study (Figure 4.7 E). However, for the first time, *vga(A)* and *lsa(A)* genes were shown to confer decreased susceptibility to the 16-membered macrolides leucomycin A1 and carbomycin. A possible explanation of this phenotype can be gleaned from a detailed analysis of the ribosomal binding sites and structure-activity relationships of various macrolides. Macrolides are composed of a macrolactone ring of varying size to which differing numbers of sugars are attached (Figure 4.8 A). 14-membered macrolides, such as eryhtomycin, act by binding in the peptide exit tunnel and blocking the egress of nascent polypeptides (Schlunzen *et al*., 2001, Hansen *et al*., 2002, Tu *et al*., 2005). The 16-membered macrolides bind in an overlapping site to erythromycin and also block the peptide exit tunnel, however, in addition, these antibiotics inhibit peptidyl-transferase activity to various degrees (Omura *et al*., 1968, Haupt *et al*., 1976, Poulsen *et al*., 2000). The level of inhibition is determined by the extent to which the groups attached to the C5 position of the macrolactone ring protrude towards the A-site of the PTC. Erythromycin has a monosaccharide at this position and therefore does not inhibit peptidyltransferase activity, whereas spiramycin, tylosin, leucomycin A1, and carbomycin, possess disaccharides at this position and protrude further into the PTC, inhibiting peptidyltransferase activity (Hansen *et al*., 2002) (Figure 4.8 B). Leucomycin and carbomycin protrude furthest towards the A-site due to an isobutyrate extension of the

disaccharide. This results in greater degree of overlap with the binding site of type A streptogramins, the point at which Vga(A) and Lsa(A) appear to mediate displacement (Figure 4.8 B).

The information derived from a structural analysis of antibiotic binding sites within the PTC and peptide exit tunnel supports the notion that the resistance phenotypes of ARE ABC-F proteins are determined not by the chemical composition of their target antibiotics, but by the precise molecular details of antibiotic binding sites within the 50S ribosomal subunit. Coupled with the dual findings of protection of translation in staphylococcal T/T assays and Lsa(A) mediated displacement of lincomycin from the staphylococcal ribosome, these results support a mechanistic model in which ARE ABC-F proteins bind directly to ribosome and displace antibiotics from their binding sites. Such a model is discussed in the context of information available within the scientific literature in chapter 5.

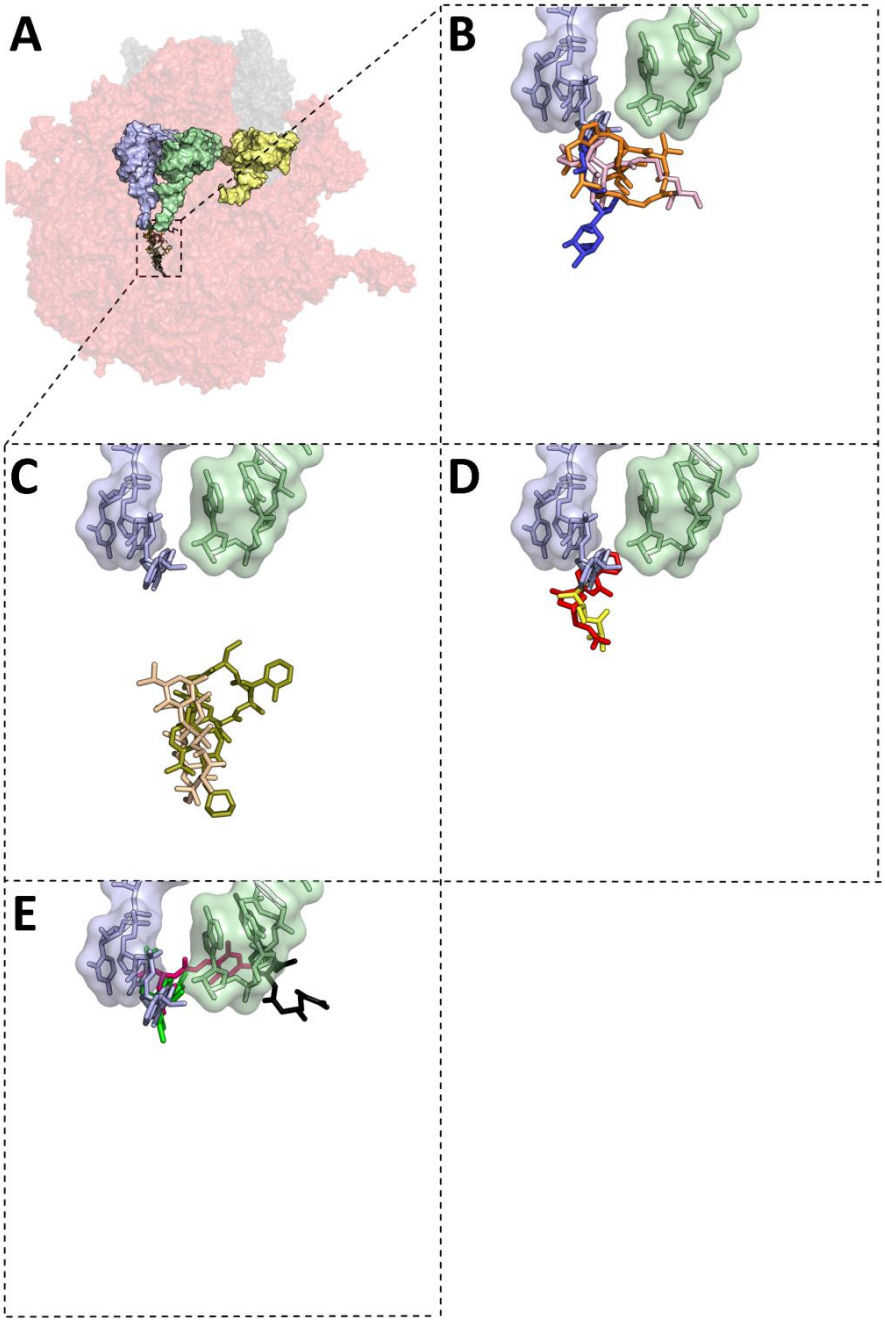


Figure 4.7. Binding sites of antibiotics within the peptidyl-transferase centre and peptide exit tunnel of the 50S subunit. (A) The *Thermus thermophilus* ribosome with tRNA occupying the A-site (blue), P-site (green) and E-site (yellow). The 50S and 30S subunits are shown in red and black respectively. Co-ordinates from PDB: 4V5C. **Magnified view of antibiotics at the peptidyl-transferase centre and peptide exit tunnel. (B)** Binding sites of antibiotics to which vga-type and lsa-type ARE ABC-F proteins confer resistance; dalfopristin (streptogramin A, orange, PDB: 1SM1), retapamulin (pleuromutilin, pink, PDB: 2OGO), and clindamycin (lincosamide, blue, PDB: 4V7V). **(C)** Binding sites of antibiotics to which msr-type determinants confer resistance; erythromycin (14-membered macrolide, light brown, PDB: 4V7U) and quinupristin (streptogramin B, dark yellow, PDB: 1SM1). **(D)** Binding sites of antibiotics to which OptrA confers resistance; chloramphenicol (phenicol, yellow, PDB: 4V7T) and linezolid (oxazolidinone, red, PDB: 3DLL). **(E)** Antibiotics to which no identified ARE ABC-F confers resistance; blasticidin S (black, PDB: 1K8A), puromycin (green, PDB: 1Q82) and sparsomycin (green, PDB: 1M90). Figure generated in PyMOL through alignment of co-ordinates for antibiotic:ribosome complexes with PDB: 4V5C.

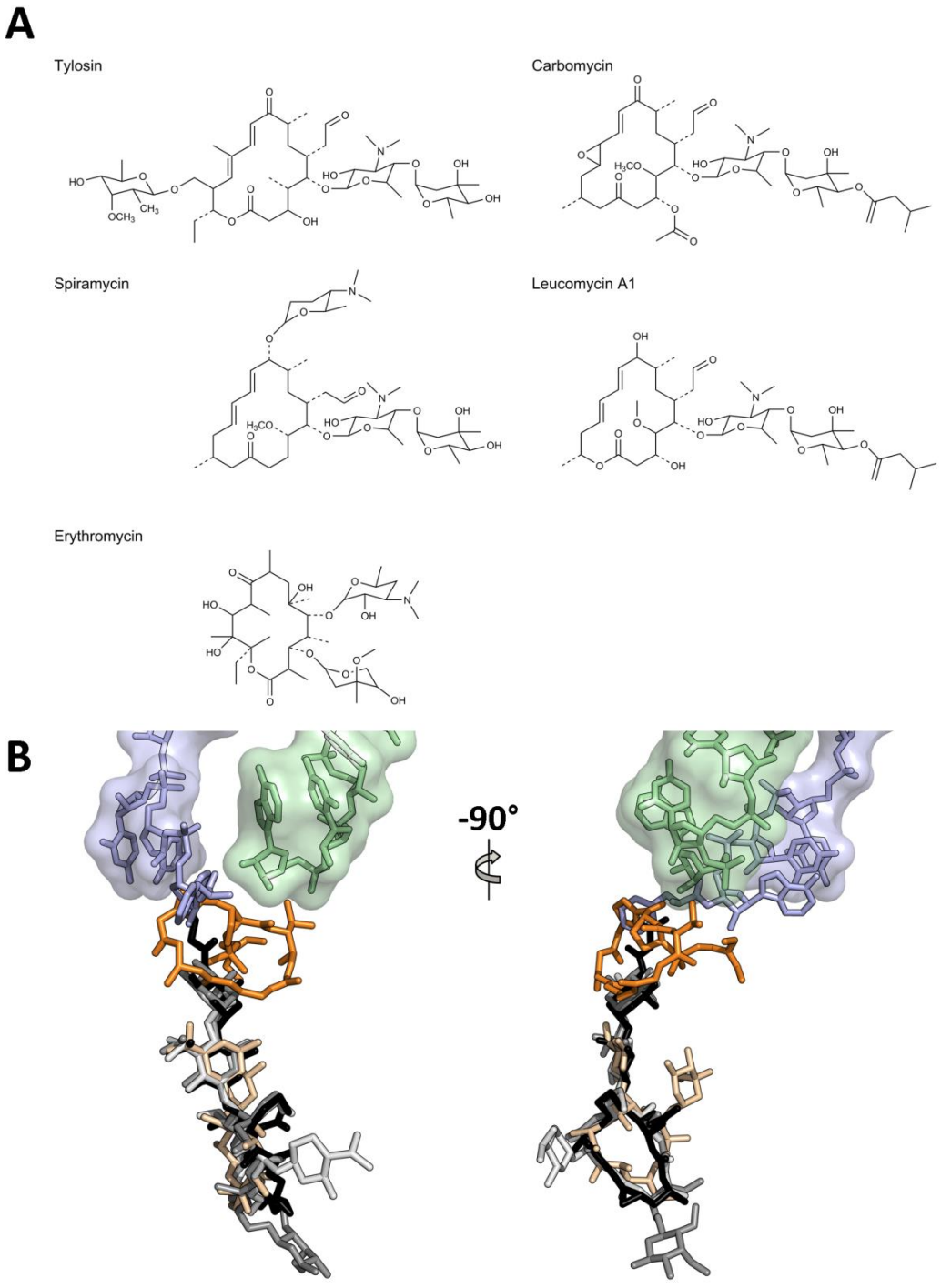


Figure 4.8. Macrolides that bind at the peptide exit tunnel of the 50S ribosomal subunit. (A) Chemical structures of macrolides used during this study **(B)** Binding sites of macrolide antibiotics; erythromycin (light brown), tylosin (grey, PDB:1KD1), spiramycin (white, PDB: 1KD1) and carbomycin (black, PDB: 1K8A). A transparent surface representation of tRNA occupying the A-site (blue), P-site (green) is shown (Co-ordinates from PDB: 4V5C). No co-ordinates describing the ribosomal binding site of leucomycin A1 are currently available.

4.6 Conclusions

The experiments described within this chapter provide direct and indirect evidence to support a mechanism of ARE ABC-F mediated resistance that acts at the level of translation, rather than through efflux. The observations of Lsa(A) mediated displacement of lincomycin from staphylococcal ribosomes and dependency of ARE ABC-F resistance phenotypes on the precise details of antibiotic binding sites, suggest a mechanism involving direct binding of the ribosome by ARE ABC-F proteins. However, further work will be required to confirm, and elucidate the molecular basis of, such an interaction. These results are discussed together with information from the scientific literature regarding the ARE ABC-F mechanism of action in Chapter 5.

5. Discussion

The experimental findings of this thesis, which are discussed in detail in Sections 3.5 and 4.5, provide strong support for a mechanism of ARE ABC-F mediated antibiotic resistance based on ribosomal protection. However, at present the prevailing view held by the scientific community is that ARE ABC-F proteins mediate antibiotic resistance through efflux (Wondrack *et al*., 1996, Matsuoka *et al*., 1993, Roberts *et al*., 1999, Chesneau *et al*., 2005, Jacquet *et al*., 2008, Nunez-Samudio and Chesneau, 2013). Here, the results obtained during this study are considered alongside information derived from the published scientific literature in order to re-evaluate the evidence proposed to support a mechanism of ARE ABC-F mediated efflux. In addition, the homology of ARE ABC-F proteins to other ABC proteins involved in translation is used to propose a model describing the details underlying the ribosomal protection mechanism. Finally, the plausibility of a blanket application of the ribosomal protection hypothesis to all proteins within the ARE ABC-F subfamily is discussed.

5.1 A re-evaluation of the evidence proposed in support of antibiotic efflux

The genesis of the efflux hypothesis lies in the 1990 study in which the deduced amino acid sequence of the first ARE ABC-F protein to be identified, Msr(A), was used to search an early protein sequence database developed at the University of Leeds (Bleasby and Wootton, 1990, Ross *et al*., 1990). This bioinformatics approach revealed a high degree of homology between Msr(A) and the ABC domains of ATP transport proteins, leading the authors to infer a mechanism of resistance through efflux and subsequently conduct transport experiments to assess the accumulation of erythromycin in strains expressing *msr(A)* (Ross *et al*., 1990). In the intervening 25 years, due to the accumulative body of work regarding ABC proteins and the dramatic expansion in the size of protein databases, it has become increasingly apparent that Msr(A) and other ARE ABC-F proteins exhibit the greatest degree of homology

not with the ABC domains of transporters, but with ABC proteins that participate in cellular processes other than transport (Kerr, 2004, Dorrian, 2009, Lenart *et al*., 2015).

Nevertheless, a cursory inspection of the transport experiments conducted during early studies of Msr(A) and, more recently, of Vga(A), leads even an informed reader down the road of antibiotic efflux. Only upon closer inspection do the pitfalls in these studies become clear. It has been consistently shown the expression of *msr(A)* and *vga(A)* results in an energy-dependent reduction in intracellular drug accumulation (Ross *et al*., 1990, Wondrack *et al*., 1996, Matsuoka *et al*., 1999, Novotna and Janata, 2006). However, experimental evidence that the expression of Erm-type methylases results in decreased accumulation of macrolides (Canton *et al*., 2005, Piatkowska *et al*., 2012), along with the observation that addition of an excess of an unlabelled macrolide or streptogramin B antibiotic to transport experiments using radiolabelled erythromycin also results in decreased accumulation (Barre *et al*., 1986, Reynolds *et al*., 2003), demonstrates that such accumulation experiments are inherently flawed: they are unable to distinguish between efflux and ribosomal protection.

Although decreased drug accumulation due to ARE ABC-F expression is the only direct experimental evidence proposed in support of the efflux hypothesis, studies in which ARE ABC-F determinants have consistently been found within the membrane fraction of lysed cells have frequently been cited as indirect corroborative evidence for resistance through efflux (Matsuoka *et al*., 1993, Olano *et al*., 1995, Chesneau *et al*., 2005, Nunez-Samudio and Chesneau, 2013). This observation suggests, that like the ABC domains of ABC transporters, ARE ABC-F proteins may be peripherally associated with the membrane. Furthermore, a study in which the presence of Vga(A) in ribosome preparations was assessed showed no co-localisation of Vga(A) with the L24 ribosomal protein in *S. epidermidis* and was interpreted as an absence of evidence for ribosomal protection (Chesneau *et al*., 2005). The significance of membrane localisation of ARE ABC-F proteins in the context of ribosomal protection

remains unclear. It is of note however, that a portion of the ARE ABC-F proteins assessed in these cellular localisation studies has repeatedly been found within the soluble fraction when the proteins are overproduced (Chesneau *et al*., 2005, Nunez-Samudio and Chesneau, 2013, Olano *et al*., 1995). Additionally, a fraction of cellular ribosomes has previously been shown to be membrane associated in *S. aureus* (Adler and Arvidson, 1984).

An important prediction of the efflux hypothesis is that in order for ARE ABC-F proteins to participate in efflux, they must associate with TMDs. Two studies have been undertaken in an effort to identify such TMDs in *S. aureus*. In the first such study, DNA sequence analysis of the *S. epidermidis* plasmid on which the *msr(A)* gene was originally identified (pUL5050) revealed a putative ABC transporter upstream of *msr(A)* that was proposed as a candidate to provide the missing TMDs (Ross *et al*., 1995). Two open reading frames were identified within this region; a single ABC domain (designated *stp*) and a single TMD (*smp*). These sequences were consistently found to be associated with *msr(A)* on staphylococcal plasmids, and are also present on the chromosome of erythromycin sensitive *S. aureus* (Ross *et al*., 1995). It was therefore suggested that an interaction between Msr(A) and this ABC transporter could explain the observation that *msr(A)* isolated from *S. epidermidis* was able to mediate resistance in *S. aureus* in the absence of any other plasmid encoded sequences (Ross *et al*., 1995). However, subsequent work showed that the *msr(A)* resistance phenotype was unaffected following inactivation of the endogenous *S. aureus* RN4220 *stp* and *smp* sequences through allelic replacement (Ross *et al*., 1996), establishing that these sequences are not required for Msr(A)-mediated resistance. The second study to search for Msr(A) associated TMDs utilised transposon mutagenesis of an *S. aureus* RN4220 strain containing a single chromosomal copy of *msr(A)*. Despite screening of over 3000 mutants for reduced susceptibility to macrolides, no such TMDs were identified (Reynolds, 2005).

Although efforts to identify TMDs of ABC transport proteins with which ARE ABC-F proteins may interact have not been successful, a recent study of the streptococcal macrolide resistance determinants *msr(D)* and *mef(E)* provided the first report of an interaction between an ARE ABC-F protein and a membrane-located MFS transporter (Nunez-Samudio and Chesneau, 2013). The use of fluorescently labelled Msr(D) and Mef(E) in a heterologous host, *E. coli,* allowed an analysis of the cellular localisation of the two proteins. In the absence of *msr(D)* expression, Mef(E) was distributed unevenly into several focal points throughout the cytoplasm, a result which was also observed when a catalytic mutant of Msr(D), $Msr(D)_{E434Q,}$ was co-expressed with *mef(E)*. However, when functional *msr(D)* and *mef(E)* genes were co-expressed, Mef(E) localised to the poles of the cell. Unlike *msr(A)* (Ross *et al*., 1990) or *vga(A)* (Chesneau *et al*., 2005), and contrary to a previous report regarding *msr(D)* (Reynolds, 2005), heterologous expression of *msr(D)* in a macrolide susceptible *E. coli* strain conferred erythromycin and azithromycin resistance (16-fold). By contrast, heterologous expression of *mef(E)* in *E. coli* resulted in only a two-fold increase in erythromycin MIC and no azithromycin resistance, compared with the high-level resistance (up to 200-fold) to both drugs observed in its native host, *S. pneumoniae* (Wierzbowski *et al*., 2005). Co-expression of both determinants had a synergistic effect, increasing levels of erythromycin and azithromycin resistance 32 fold. Subsequently, an *in vitro* pull-down assay using histidine tagged Msr(D) as bait detected an interaction between Msr(D) and Mef(E) through Western blotting.

From these observations the authors concluded that in the absence of Msr(D), the Mef(E) protein is predominantly aggregated. Co-expression of *msr(D)* is proposed to ensure the correct integration of Mef(E) into the cell membrane and Msr(D) is thought to enhance or hijack the Mef(E) pump, broadening its substrate specificity. Although the observation that expression of *msr(D)* alone conferred macrolide resistance was noted, a result previously described in *S. pneumoniae* (Daly *et al*., 2004) and *S. aureus* (Reynolds and Cove, 2005), the

authors did not speculate as to the mechanism underlying this phenotype. Analysis of the data arising from this study with the assumption that Msr(D) is acting to protect the ribosomes offers an alternative interpretation. The large decrease in the level of macrolide resistance conferred by *mef(E)* when expressed in *E. coli* in comparison to *S. pneumoniae*, together with the detection of several focal points throughout the cytoplasm, suggests that *mef(E)* is poorly expressed in *E. coli* in the presence of macrolide antibiotics and the Mef(E) protein is predominantly aggregated. Lifting of macrolide-mediated protein synthesis inhibition by co-expression of *msr(D)* may permit higher levels of *mef(E)* expression and correct folding of the transport protein, allowing membrane integration and promoting a synergistic effect between efflux and ribosomal protection. Although pull-down assays analysed through western blotting showed an interaction between Msr(D) and Mef(E), both determinants have previously been shown to function as independent resistance determinants in *S. pneumoniae* (Daly *et al*., 2004). In order to establish the specificity of this interaction it would be useful to see an SDS-PAGE analysis of the pull-down assays, as work outlined in Section 4.4.5 of this thesis showed Vga(A) and Lsa(A) to pull-down multiple proteins from cell-extracts, unfortunately no such analysis was included in the paper (Nunez-Samudio and Chesneau, 2013).

The *msr(D)* determinant was not included in the screen to obtain conditions for soluble ARE ABC-F proteins conducted during this study (Section 3.4.5); however, the use of *E. coli* expressing *msr(D)* to conduct pull-down assays suggests that this protein may be amenable to large scale purification. Future work in which homogenously purified Msr(D) could be tested for its ability to protect *E. coli* and *S. aureus* T/T assays would be beneficial. I predict such experiments would show Msr(D) to act in the same manner as Vga(A) and Lsa(A), and that any interaction with Mef(E) is secondary to the primary function of Msr(D) as a ribosomal protection protein.

5.2 Resistance through ribosomal protection

5.2.1 A re-evaluation of the lack of evidence for ribosomal protection

The work outlined in this thesis provides several lines of indirect and direct evidence that together offer support for a mechanism of resistance involving ribosomal protection. By contrast, previous studies in which attempts have been made to provide evidence of ribosomal protection have been unsuccessful. Possible explanations for these failures are provided below.

Previously, translation assays composed of centrifugally purified ribosomes, S150 fractions, and poly(A) transcripts, have shown no difference in the erythromycin sensitivity of assays derived from *msr(A)* expressing drug resistant strains and wild-type drug sensitive strains (Wondrack *et al*., 1996). Perhaps the key difference between these translation experiments and the T/T assays described in this thesis is the addition of exogenously purified protein. It is possible to speculate that due to the membrane association of Msr(A), the centrifugation steps required for purification of ribosomes and S100/150 fractions remove the majority of the ARE ABC-F protein from the system, resulting in sensitivity of these translation assays to the drug (Matsuoka *et al*., 1993, Wondrack *et al*., 1996). This poses questions regarding the effect of membrane localisation of the protein may have upon resistance mediated through protection *in vivo*. Is there sufficient protein within the cytoplasm to mediate ribosomal protection? Can results obtained through addition of exogenously purified protein to T/T assays be said to accurately depict events occurring in whole cells? The results described within Section 4.4.3 of this work are therefore of particular importance. The recapitulation of phenotypes observed in whole cells within the T/T assays allows conclusions drawn from these *in vitro* experiments to be extrapolated to events occurring *in vivo,* and eliminates the possibility that these results are merely an artefact of the T/T assay. The relationship between membrane localisation of ARE ABC-F proteins and their ability to mediate ribosomal

protection remains an open question, however, it is of note that when cellular localisation of GFP-tagged Msr(D) in *E. coli* was assessed *in vivo* the protein was seen to be evenly distributed throughout the cytoplasm, whereas within the same study, analysis of centrifugally derived cell fractions by western blotting showed that the protein was present in both the soluble and membrane factions (Nunez-Samudio and Chesneau, 2013). Although speculative, it is possible to suggest that in the case of ARE ABC-F proteins, results obtained from cellular fractionation may not necessarily reflect events in whole cells. This is in contrast to studies of tetracycline RPPs, in which ribosomes and S150 fractions isolated from strains expressing *tet(M)* have proven to be refractory to the inhibitory action of tetracycline (Burdett, 1986, Burdett, 1991). An *in vivo* assessment of the cellular localisation of a fluorescently labelled ARE ABC-F protein in its native host may provide useful insights with which to reconcile the ribosomal protection model and previous cellular fractionation studies.

5.2.2 A model for ribosomal protection mediated by ARE ABC-F proteins

The results described in this thesis, coupled with the lack of conclusive evidence in support of efflux, indicate that ARE ABC-F proteins mediate their effects at the level of translation, through displacement of the drug from its ribosomal binding site. The observation that Lsa(A) mediated displacement of lincomycin from a preparation purified staphylococcal ribosomes, in which ribosomally associated proteins such as EF-Tu did not appear to be present, suggests that ARE ABC-F proteins act directly upon the ribosome and mediate resistance in a manner analogous to the tetracycline RPPs.

The tetracycline RPPs show homology to the ribosome-associated GTPases EF-G and EF-Tu (Doyle *et al.* 1991), and bind the ribosome at the same position as these elongation factors (Li *et al*., 2013, Arenz *et al*., 2015). Analysis of the literature pertaining to ABC proteins not involved in transport yields three characterised proteins that have been shown to directly

bind the ribosome: ABCE1, a highly conserved protein that plays a role in ribosome recycling and rescue of stalled ribosomes in eukaryotes and archaea (Andersen *et al*., 2006); eEF3, a fungal protein that plays an essential role in elongation and recycling (Becker *et al*., 2012); and EttA a widely distributed bacterial protein involved in energy dependent regulation of translation (Boel *et al*., 2014, Chen *et al*., 2014). Of these proteins both eEF3 and EttA fall into the ABC-F subfamily, whereas ABCE1 belongs to the closely related ABC-E subfamily, a group of dual ABC domain proteins that also lacks TMDs. All three proteins contain tandemly repeated ABC domains separated by a linker. However, ABCE1 and eEF3 contain additional N-terminal HEAT repeat and iron-sulphur cluster (FeS) domains respectively, which are essential to their interaction with the ribosome (Andersen *et al*., 2006, Becker *et al*., 2012). Of these three proteins, the predicted domain architecture of ARE ABC-F determinants most resembles that of EttA (Figure 5.1 A).

EttA regulates translation in *E.coli* in response to changing cellular energy levels, specifically the ATP/ADP ratio of the cell (Boel *et al*., 2014, Chen *et al*., 2014). The protein is thought to contribute either to survival of the organism in stationary phase, or to aid its transition from stationary phase to logarithmic growth. In order to perform these functions, EttA binds to the ribosome at the E-site, bringing the L1 stalk and P-site, and modulates the conformation of the PTC through contracts with ribosomal proteins, rRNA and P-site fMet-tRNA (Figure 5.1 B [i] and [ii]) (Boel *et al*., 2014, Chen *et al*., 2014). It is currently thought that the ADP-bound form of EttA, which is predominant in stationary phase, binds to the vacant E site of ribosomes positioned at the start codon and inhibits translation. Whereas ATP-bound EttA, which is predominant during logarithmic growth, promotes formation of the first dipeptide bond, hydrolyzes ATP and dissociates from the E site (Boel *et al*., 2014, Chen *et al*., 2014). Although this model provides an initial framework for understanding EttA function, there is an open question as to exactly how ATP hydrolysis can promote the release of EttA from the ribosome, whereas ADP can stabilize EttA and cause translation inhibition.

The functional and structural characterisation of EttA (Boel *et al*., 2014, Chen *et al*., 2014) infomred the conclisions drawn during a recent study of Vga(A) conducted by Lenart *et al.* (2015). Using site-directed mutagenesis mapping of Vga(A), they demonstrate that the spectrum of antibiotic resistance mediated by the protein can be altered by mutations to an 8-residue variable region within the interdomain linker (Lenart *et al*., 2015). Lenart *et al.* point out that the PtIM of EttA is conserved in Vga(A), with an extension of 30 amino acids inserted at its centre, into which the 8-amino acid antibiotic specificity determining region falls. They therefore propose that the Vga(A) linker acts analogously to the EttA linker, but the extension allows further penetration towards the PTC, where it causes dissociation of its target drugs either directly, or through contacts with the P-site tRNA (Figure 5.1 B [ii]) (Lenart *et al*., 2015). This model of ribosomal protection fits with the data outlined in this study. However, it is important to note that EttA interaction with the ribosomal L1-stalk is mediated by a second functionally important structural motif, a 44 amino acid insertion between the Q-loop and signature motif of the N-terminal ABC domain referred to as the arm region, which is not present in ARE ABC-F proteins. This position is important for determining the protein : protein interactions in which ABC proteins participate (Dassa and Bouige, 2001, Davidson et al., 2008, Dassa, 2011), insertion of a chromodomain into the equivalent position of the C-terminal ABC domain of eEF3 mediates interaction with a different region of the ribosome (Andersen et al., 2006). Therefore although the interdomain linker of ARE ABC-F proteins may interact with the P-site tRNA in a similar manner to EttA, the position of ARE ABC-F ribosomal binding may not be identical.

EttA preferentially binds to ribosomes containing fmet-tRNA at the A-site and competes with deacetylated tRNA within the E-site for its binding position (Boel et al., 2014, Chen et al., 2014). Although 14-membered macrolide antibiotics such as erythromycin are unable to inhibit elongating ribosomes (Andersson and Kurland, 1987), at least one example of an antibiotic that inhibits the peptidyl-transferase reaction, lincomycin, exhibits inhibitory action against ribosomes in the post-translocation conformation.

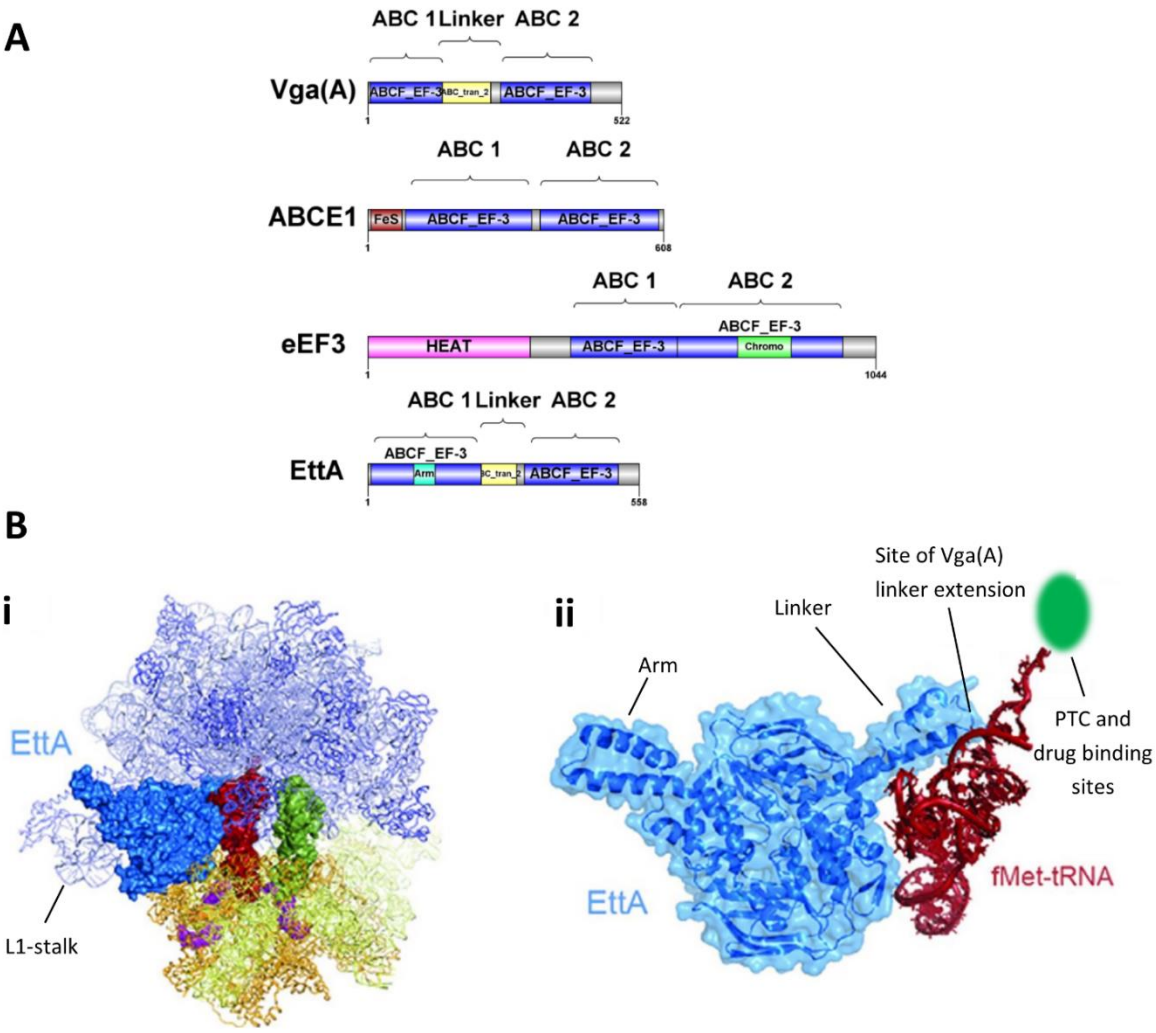


Figure 5.1. (A) Domain architectures of ABC proteins that directly bind the ribosome. A comparison between the ARE ABC-F subfamily, represented here by Vga(A), to other ABC proteins that directly bind the ribosome. Unlike the ARE ABC-F proteins ABCE1 and eEF3 lack an extended inter ABC linker and possess additional N-terminal domains important for their interaction with the ribosome. In contrast, like ARE ABC-F proteins, EttA is composed by two ABC domains separated by an extended interdomain linker, which is recognised as a ABC_tran_2 conserved domain. **(B) The interaction of EttA with the ribosome. (i)** EttA (blue) binds at the E-site of the 70S ribosome where it contacts the acceptor arm of the P-site tRNA (red) as shown in **(ii)**. ARE ABC-F proteins possess an extended linker domain which may protrude further towards the PTC (depicted as green circle) in order to displace 50S targeted drugs from their binding sites. Figure adapted from Starosta *et al.* (2014).

(Kallia-Raftopoulos *et al*., 1994). This poses the question of how an ARE ABC-F protein would mediate antibiotic displacement from an elongating ribosome. Although EttA preferentially binds pre-translocation ribosomes, *in vitro* peptide synthesis assays have shown the protein as capable of binding post-translocation complexes albeit at a lower affinity. The ribosomal E-site was shown not to stably retain deacetylated tRNA in these assays, providing an opportunity for the EttA to bind (Boel *et al*., 2014). Perhaps ARE ABC-F proteins act in a similar manner *in vivo*, binding the vacant E-site and promoting displacement of the antibiotic. Such a system would not prevent rebinding of the displaced antibiotic and would therefore require cyclic ARE ABC-F association, antibiotic displacement, and dissociation of the ARE ABC-F protein, processes likely to be driven by the different conformational states resulting from binding and hydrolysis of ATP. The plausibility of this model is dependent on peptide bond formation occurring at a faster rate than rebinding of the antibiotic. Further work is required in order to establish the precise details of such a model.

5.2.3 Do all ARE ABC-F proteins mediate ribosomal protection?

The work described in this thesis provides strong support for a mechanism of ribosomal protection for the Lsa-type and Vga-type ARE ABC-F proteins. Previously, the efflux hypothesis was derived predominantly from experiments concerning Msr(A), Vga(A) and Ole(B) and applied to all ARE ABC-F proteins. Is it equally appropriate to apply the ribosomal protection hypothesis to all members of the subfamily? The homology exhibited by ARE ABC-F proteins found in Gram-positive pathogens, coupled to the consistent correlation of resistance phenotypes amongst this group with the precise ribosomal binding sites of their target antibiotics, suggests that the ribosomal protection model can be applied to this entire group. Efforts to identify conditions conducive to the soluble expression of an Msr-determinant during this work were unsuccessful; however, work in which strains expressing Msr(D) have been used in pull down assays suggests that purification of this protein may be possible (Nunez-Samudio and Chesneau, 2013), analysis of this protein in the T/T assay may

further validate the application of the ribosomal protection hypothesis to all ARE ABC-F proteins found in Gram-positive pathogens.

Observations derived from experiments regarding ARE ABC-F proteins found in Gram-positive antibiotic producers makes application of the ribosomal protection model to this group of proteins more difficult. For example, deletion of either the N- or C-terimal ABC domains of the oleandomycin resistance determinant Ole(B) has been shown not to affect its ability to confer resistance (Olano *et al*., 1995). Similarly, macrolide resistance conferred by the LmrC lincomycin resistance determinant from *Streptomyces lincolnensis* expressed within the heterologous host *Lactococcus lactis* has been shown to occur independently of the proteins ability to bind ATP (Dorrian *et al*., 2011). Heterologous expression, purification, and analysis within a T/T assay of one or more of the ARE ABC-F determinants found within antibiotic producing bacteria would prove useful in an effort to determine whether all ARE ABC-F proteins mediate ribosomal protection.

5.3 Future work

An important step to enable further characterisation of the ribosomal protection model is the isolation of stable ARE ABC-F : ribosome complexes. In order for this to be achieved, an analysis of the relative effects of different adenosine nucleotides on Lsa(A) binding to ribosomes should be performed and examined through analytical gel filtration or surface plasmon resonance using an immobilised ARE-ABC-F protein. Similar experiments have previously been used to identify the requirements for binding of tetracycline RPPs to the ribosome (Dantley *et al*., 1998, Trieber *et al*., 1998). Other parameters that could be varied are salt concentration (as increasing ionic strength has been shown to cause dissociation of tetracycline RPPs from ribosomes (Burdett, 1991)) and magnesium concentration. Attempts to purify a Vga(A) double catalytic mutant(Vga(A)$_{E105Q,E410Q}$), that is capable of binding but not hydrolysing ATP, were not successful during this project. A similar mutant, EttA-EQ$_2$, was

used to obtain complexes of EttA bound to the ribosome, however, rather than attempting pull down assays using purified EttA-EQ_2 these complexes were isolated through an "*in vivo* pull-down" in which induction of a tightly regulated construct encoding EttA-EQ_2 followed by cell lysis and IMAC resulted in a one-step purification of the EttA-EQ_2 : ribosome complex (Chen *et al*., 2014). A similar experiment could be conducted in *S. aureus* using *vga(A)*$_{E105Q,E410Q}$ present in a tightly regulated vector such as pAJ96 (O'Neill *et al*., 2007).

The isolation of a stable ARE ABC-F : ribosome complex would permit structural studies of the complex using cryo-EM. Similar investigations have yielded structural information regarding the interactions of tetracycline RPPs and translation associated ABC proteins, including EttA, with the ribosome (Boel *et al*., 2014, Li *et al*., 2013, Arenz *et al*., 2015, Andersen *et al*., 2006, Becker *et al*., 2012). Further attempts to crystallise Vga(A) and Lsa(A) may benefit from the analysis of buffer components for stabilisation of Vga(A) described in Section 3.4.9, although it is clear that the problem of protein degradation will need to be solved in order to obtain a high-resolution structure of either protein. If such a structure was obtained, in tandem with information derived from a cryo-EM study of the protein : ribosome complex, this information could potentially prove useful in the rational modification of 50S targeted antibiotics to circumvent ARE ABC-F mediated resistance. Such work would translate the dissection of the fundamental biology underlying the resistance mechanism described within this thesis into useful intelligence in the ongoing fight against antibiotic resistance.

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