Exploring the potential of Affimer artificial antibodies as antibacterial agents

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Antibacterial agents are important drugs for human health, providing a major advance in the control of bacterial infections. However, the effectiveness of conventional antibacterial drugs is increasingly being eroded owing to the emergence of antibacterial resistance, and the problem has been exacerbated by a prolonged void in development of new agents. As a result, multidrug-resistant and pandrug-resistant bacterial strains are now commonly encountered, raising the threat of a "post-antibiotic era". This has created renewed interest in exploring alternative strategies to antibacterial drug discovery to enhance the pipeline of effective therapies against bacterial pathogens, and (where possible) rejuvenating the activity of existing compounds against which resistance already exists.

In this study, a novel strategy for discovering potential new antibacterial agents - as well as restoring the antibacterial action of ones that are already in use - was investigated. This strategy involves the use of novel, recently developed artificial binding proteins, named Affimers, to generate inhibitors of essential bacterial proteins, and of proteins involved in antibiotic resistance. *Staphylococcus aureus* was used as a model organism in this study, and the focus was on inhibiting multiple target proteins with a single Affimer to restrict the development of resistance against selected molecules. Single Affimers were selected against two penicillin binding proteins (PBPs), and two proteins that mediate resistance against antibiotic fusidic acid (FA), namely, FusB and FusC (FusB-type proteins). Antistaphylococcal dual-targeted Affimer and FusB-type FA resistance-inhibiting Affimers were successfully isolated and characterised.

Table of contents

List of Tables

List of Figures

Abbreviations

The following abbreviations have been used throughout this thesis;

AMEs	aminoglycoside modifying enzymes
BICINE	N,n-Bis (2-Hydroxyethyl) glycine
CFU	Colony forming unit
C	Celcius
Cv	column volumes
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
Ef-G	Elongation factor G
FA	fusidic acid
FQs	fluoroquinolones
g	gravity
g	gram
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITC	Isothermal titration calorimetry
Kd	Dissociation constant
kJ	kilojoules
kDa	kilodaltons
LBA	Luria-Bertrani agar
LBB	Luria-Bertrani broth
M	molar
mAbs	Monoclonal antibodies
MDR	multidrug-resistant
MGEs	mobile genetic elements
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus aureus*
mg	milligram

MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	Minimum inhibitory concentration
ml	milliliter
mM	millimolar
MWCO	molecular weight cut off
NaCl	sodium chloride
NaH2PO4	soduim dihydrogen phosphate
NEB	New England Biolabs
Ni-NTA	nickel-nitrilotriacetic
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
pH	potential hydrogen
PBP2	Penicillin binding protein 2
PBP2a	Penicillin binding protein 2a
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl suplhate polyacrylamide gel electrophoresis
TAE	tris base, acetic acid, EDTA.
Tm	melting temperature
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride
TEV	Tobacco Etch Virus
TSA	tryptone soya agar
TSB	tryptone soya broth
v / v	volume per volume
w / v	weight per volume
ΔG	free energy
ΔH	enthalpy
ΔG	entropy
µg	microgram
µl	microlitres
µM	micromolar

1. Introduction

1.1 Why we are in need of new antibacterial treatments?

Antibiotics are an essential part of modern medicine, providing not only the primary means of treating bacterial infection, but enabling a wide range of medical applications (e.g. surgical intervention, organ transplants) that would otherwise not be possible (Coates and Hu, 2007). However, bacterial resistance to antibiotics is increasing, affecting every antibiotic class introduced into clinical practice (Davies and Davies, 2010). The problem of resistance is epitomized by the emergence of multidrug resistant “ESKAPE” organisms (*Enterococcus spp*., *Stapylococcus aureus*, *Klebsiella spp*., *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*.) (Boucher et al., 2009). Indeed, in the case of some Gram-negative bacteria, such as *Escherichia coli*, almost all strains have become resistant not only to ampicillin, but even to the second and third generation cephalosporins, by producing extended spectrum β-lactamases (ESBLs) (Nicolas-Chanoine et al., 2008, Coque et al., 2008). Furthermore, many resistant bacteria such as methicillin-resistant *S. aureus* (MRSA) (Chambers, 2001) (Stevenson et al., 2005) and extended-spectrum β-lactamase (ESBL)–producing *E. coli*, (Pitout et al., 2004) (Woodford et al., 2004) are causing problems within the community and in healthcare institutions. Infections caused by multidrug-resistant (MDR) organisms are associated with a higher mortality rate compared to those caused by susceptible bacteria and impose a major

economic burden, estimated at over 20 billion dollars per year in the US alone (Cosgrove, 2006) (DiazGranados et al., 2005) (Sydnor and Perl, 2011).

In addition to the rise in resistance, there is a severe lack of new antibiotics in development to combat the escalating threats of resistant pathogens (Lewis, 2013).This presents a global concern to both science and medicine, resulting in the World Health Organisation (WHO) declaring antibiotic resistance “a major threat to public health”, in its first global report on antibiotic resistance. If no action is taken towards development of new antibacterial therapies, it is predicted that antimicrobial resistance will cause 10 million deaths each year by 2050 (O'Neill, 2016).

1.2 Antibacterial resistance

It was already understood in the early years of the antibiotic era that bacteria could in some cases resist the growth-inhibitory and lethal actions of antibiotics, a situation that can compromise cure of infection in the patient (Abraham and Chain, 1940). Antibacterial resistance is ancient and it is the anticipated result of the interaction of many organisms with their environment. Most antimicrobial compounds are natural products, and, as such, co-resident bacteria have evolved mechanisms to overcome their action in order to stay alive. Therefore, these organisms are often considered to be “intrinsically” resistant to one or more antimicrobial compounds. In clinical settings, however, it is usually referred to the expression of “acquired resistance” in a bacterial population that was initially susceptible to the antimicrobial compound. As it will be discussed in the following section, the development of acquired resistance can be caused by mutations in chromosomal genes or acquisition of external genetic determinants of resistance

(by horizontal gene transfer), likely acquired from intrinsically resistant bacteria present in the environment (Munita and Arias, 2016). Many factors can contribute to the development of antibacterial resistance, epidemiological studies have demonstrated a direct association between antibiotic overuse and the emergence and distribution of resistant bacteria strains. Inaccurately prescribed antibiotics also contribute to the rise of resistant bacteria, subinhibitory and subtherapeutic antibiotic concentrations can promote the development of antibiotic resistance by supporting genetic alterations, such as changes in gene expression (Viswanathan, 2014). Another factor that contribute to the development and dissemination of antibacterial resistance is their extensive use in agriculture, which enables the transfer of resistant bacteria to human by farm animals (Bartlett et al., 2013). Extensive use of antibibacterial agents in health care and agriculture creates a strong and persistent selective pressure favouring the evolution of antibiotic-resistant strains, a phenomenon referred to as ‘use it and lose it’ (Hall, 2004). For this reason, there has been a considerable focus on eliminating, or at least controlling, antibacterial resistance once it has evolved. The most common strategy is to restrict using antibiotics, the assumption being that mutations conferring resistance impose a large fitness cost in the absence of the drug. Fitness can be defined here to be the rate of replication under usual environmental conditions and can be measured as the growth rate of the strain or population being considered. Sensitive genotypes that do not pay a cost of resistance should therefore replace resistant strains at a rate relative to the extent of the cost imposed by resistance (Johnsen et al., 2009). Resistance mutations may be expected to impart a fitness cost because they target important biological functions in the cell. For instance, resistance to fluoroquinolones in pseudomonads can cause reduced motility (Stickland et al., 2010), and

resistance to aminoglycosides can alter the structure of the ribosome and so interfere with basic cellular functions (Holberger and Hayes, 2009).

1.2.1 Genetic basis of antibacterial resistance

From an evolutionary perspective, bacteria have two major genetic routes by which they may become resistant to antibiotics. The first route is the so-called endogenous route, involving the alteration of their existing genetic material by mutational change. The second route - known as the exogenous route - involves the acquisition of resistance genes from other organisms through horizontal gene transfer (HGT) (Munita and Arias, 2016).

1.2.1.1 Mutational resistance

In this scenario, bacteria within an antibiotic susceptible population spontaneously accumulate one or more mutations in genes that in turn limit the antibacterial activity of the antibiotic, and hence enable the cell to survive in the presence of the drug. The antibiotic inhibits the antibiotic-susceptible population, leading to the resistant bacteria becoming predominant. Acquired resistance via mutation usually mediates resistance through one of the following mechanisms; (1) modification to the antimicrobial drug target, thereby reducing the affinity for the drug, (2) a reduction in drug accumulation, either as a result of reduced uptake or activation of efflux mechanisms to excrete the damaging molecule (Munita and Arias, 2016).

1.2.1.2 Horizontal Gene Transfer

HGT is defined as the acquisition of foreign DNA, and is often responsible for the development of antibiotic resistance. Most antibiotics in clinical use are-or derive

from-products that naturally exist in the environment (typically soil). Bacteria sharing the environment with these molecules carry intrinsic genetic determinants of resistance and there is strong evidence to suggest that this “environmental resistome” is a major source for antibiotic resistance genes in clinically relevant bacteria (Munita and Arias, 2016).

Typically, bacteria obtain external genetic material through three main routes, (1) transformation (uptake of naked DNA), (2) transduction (phage mediated), and (3) conjugation (bacterial “sex”). Transformation is arguably the simplest type of HGT, but only a few clinically relevant bacterial species are capable of “naturally” integrating naked DNA to acquire resistance (Manson et al., 2010b). Dissemination of resistance in the hospital environment is frequently the result of conjugation (Manson et al., 2010b), a very effective method of gene transfer that involves cell-to-cell connection, and which represents an important route by which mobile genetic elements (MGEs) such as plasmids and transposons move to a new host (Manson et al., 2010a). Bacteriophage-mediated transfer of antibacterial resistance genes play a critical role in mobilizing chromosomal resistance genes of environmental bacteria and passing them to human and animal pathogens (Muniesa et al., 2013). Also, it has been demonstrated that phage released from a subpopulation of *S. aureus* cells allows the undamaged, prophage-containing population to obtain antibiotic resistance genes from competing, phage-susceptible strains existing in the same environment, a process referred to as “auto-transduction” (Haaber et al., 2016).

1.2.2 Mechanistic basis of antibacterial resistance

Antibacterial resistance in bacteria is mediated through a variety of mechanisms, which can be classified into three fundamental categories including; modifications of the antibiotic molecule, decreased antibiotic accumulation, and alteration of the drug target. In my opinion, these can be further subdivided into a total of nine mechanisms (Figure 1.1).

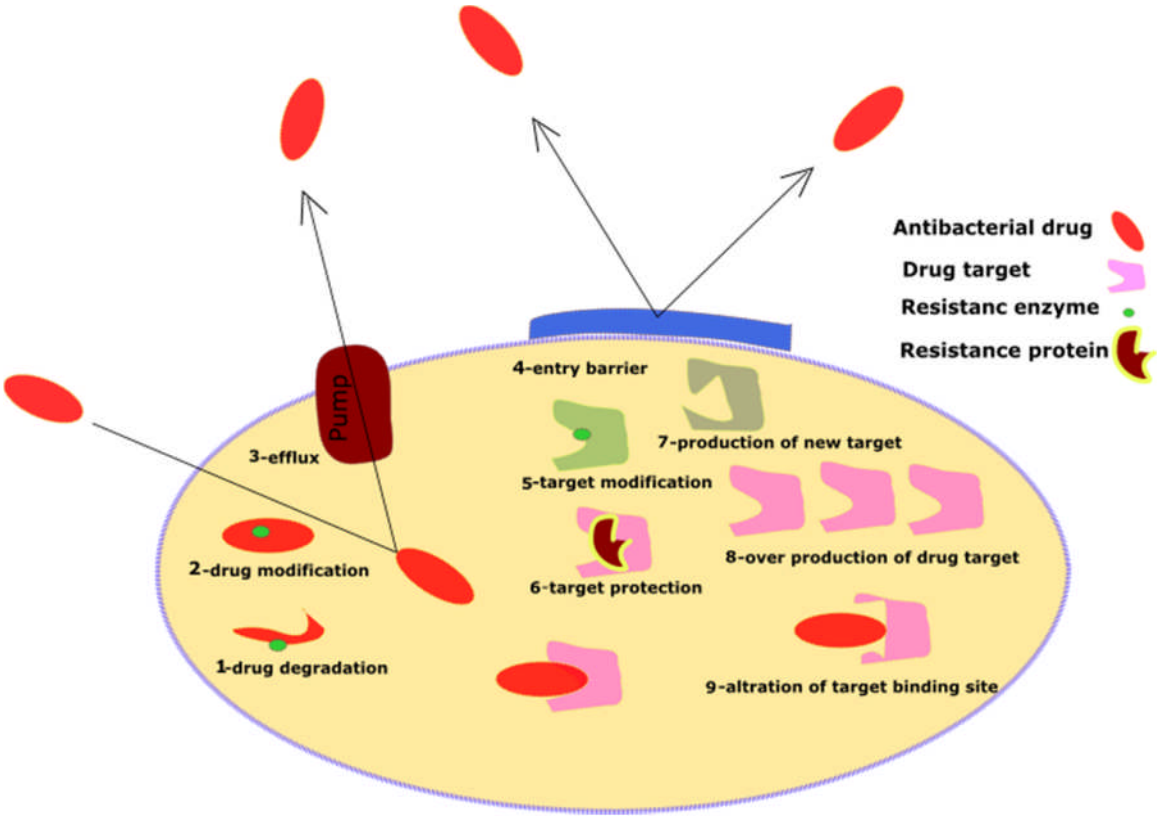


Figure 1.1. Mechanisms of antibacterial resistance. 1-enzymatic degradation of antibiotic, 2-modification of drug molecule, 3- antibiotic efflux, 4- decreased penetration, 5- enzymatic modification of target, 6- target protection, 7-production of completely new target, 8-overproduction of drug target, and 9-alteration of target binding site, adapted from Lewis (2013). See text for more details.

1.2.2.1 Modification of the antibiotic molecule

One of the most effective strategies used by bacteria to deal with the presence of antibiotics is to produce enzymes that hydrolyse the drug or otherwise inactivate it by adding specific chemical moieties to the compound that render it incapable of binding to its target.

The most clinically important example of drug degradation by bacterial enzymes is the hydrolysis of β-lactams by β lactamases produced by both Gram-positive and Gram-negative bacteria (Alekshun and Levy, 2007). These enzymes break the amide bond of the β-lactam ring, thereby rendering the drug ineffective. β-lactamases were first discovered in the early 1940s, one year before penicillin was introduced into clinical use; however, it is evident that they have existed for millions of years (Abraham and Chain, 1988, D'Costa et al., 2011). Infections caused by penicillin-resistant *S. aureus* became clinically relevant after penicillin became widely available and the mechanism of resistance was found to be a plasmid-encoded penicillinase that was readily transmitted between *S. aureus* strains, resulting in rapid dissemination of the resistance trait (Bush, 2013).

One of the best examples of resistance through modification of the drug is presented by the aminoglycoside modifying enzymes (AMEs), which covalently modify the hydroxyl or amino groups of the aminoglycoside antibiotics. Numerous AMEs have been identified to date, and they constitute the major mechanism of

aminoglycoside resistance worldwide (Garneau-Tsodikova and Labby, 2016). These enzymes are usually carried on MGEs, but genes coding for resistance determinants have also been found integrated into the chromosome in particular bacterial species, as seen with some aminoglycoside acetyltransferases in *Providencia stuartii*, *E. faecium* and *S. marcescens* (Ramirez and Tolmasky, 2010).

1.2.2.2 Decreased antibiotic accumulation

Bacteria may resist antibiotics by reducing accumulation of an antibiotic in the vicinity of the target, either through efflux pumps and/or decreased outer membrane permeability. Efflux transporters are membrane proteins involved in the transfer of toxic molecules (including antibiotics) to the bacterial cell exterior. The first efflux system was identified in 1979 and mediated export of tetracycline from the cytoplasm of *E. coli* (Ball et al., 1980). Since then, various classes of efflux transporters have been categorised in both Gram-negative and Gram-positive pathogens. Efflux transporters may be specific for a particular antibiotic (such as the *tet* determinants for tetracycline and *mef* genes for macrolides) or may exhibit broad antibiotic specificity; the latter are typically found in multidrug resistant bacteria (MDR) (Poole, 2005). The majority of antibiotic classes are susceptible to this mechanism of resistance including fluoroquinolones, protein synthesis inhibitors, β-lactams, carbapenems and polymyxins. The genes coding for efflux pumps may be found in MGEs (as in the case of *tet* gene) or on the chromosome (Piddock, 2006).

The accumulation of antibiotics in the bacterial cell may be further decreased by reduced outer membrane permeability (Giedraitiene et al., 2011) (Lima et al., 2013) (Aminov, 2010) (Dever and Dermody, 1991). Bacteria have evolved

mechanisms to avoid the antibiotic reaching its intracellular or periplasmic target by reducing the penetration of the drug into the cell. This intrinsic mechanism is particularly important in Gram-negative bacteria due to the outer membrane, preventing the entry of molecules from the external milieu. Hence, the outer membrane acts as the first-line of protection against the diffusion of the toxic compounds, including several antibacterial agents. In particular, hydrophilic molecules such as tetracyclines, β-lactams, and some fluoroquinolones are compromised by changes in permeability of the outer membrane since they often use water-filled diffusion channels (“porins”) to traverse this barrier (Pages et al., 2008). A good example of the effectiveness of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is inactive against Gram-negative organisms as it is unable to penetrate through the outer membrane (Yarlagadda et al., 2016). Similarly, the innate reduced susceptibility of *Pseudomonas* and *Acinetobacter baumanii* to β-lactams (compared to *Enterobacteriaceae*) can be linked, at least in part, to a decreased number of types and/or reduced expression of porins (Hancock and Brinkman, 2002).

1.2.2.3 Alteration of drug target

Bacteria may evolve antibiotic resistance by avoiding the action of the antibiotic through changes to the drug target. A well-characterized example of point mutations in genetic determinants coding for the target site is the mechanism of FQ resistance. FQs work on DNA replication via the inhibition of two critical enzymes, DNA gyrase and topoisomerase IV. Mutations in the genes encoding these enzymes (*gyr*A-*gyr*B and *par*C-*par*E for DNA gyrase and topoisomerase IV,

respectively) represents the most common route to resistance against these compounds (Redgrave et al., 2014).

Target related resistance mechanism may include production of new target to bypass of the original one. The most relevant example of this mechanism is the acquisition of *mec*A gene, codes for an exogenous PBP (PBP2a) by *S. aureus*, which result in methicillin resistance in this pathogen (Alekshun and Levy, 2007) (Hiramatsu et al., 2001). Methicillin resistance caused by PBP2a will be discussed in chapter 4 of this thesis. Bacteria can also avoid the antibacterial action of drugs by overproducing the antibiotic target to achieve “metabolic bypass”. An applicable example of this strategy is resistance to trimethoprim-sulfamethoxazole (TMP-SMX). Resistance to these antibacterial drugs can result from the production of increased amount of the enzymes, dihydropteroate synthetase (DHPS), and dihydrofolate reductase (DHFR). Overproduction of these drug targets overwhelms the ability of TMP-SMX to interfere with folate production, and enables bacterial survival (Flensburg and Skold, 1987) (Huovinen, 2001). Enzymatic modification of the binding site also occurs; one of the best identified examples of this mechanism is macrolide resistance, which is caused by methylation of the ribosome mediated by an enzyme, erythromycin ribosomal methylase, encoded by the *erm* genes (Pechere, 2001).

Antibacterial resistance may also rise from target protection, which involves continued or repeated interaction between a drug target and a resistance protein. Even though some of the proteins that mediate target protection resistance have been found to be encoded in bacterial chromosome, most of the clinically related proteins involved in this mechanism of resistance are encoded by MGEs. Example of drug inhibited by this mechanism is the antibiotic fusidic acid, which

affected by expression of FusB and FusC proteins (Tomlinson et al., 2016). Resistance to fusidic acid mediated by these proteins will be discussed in more details in chapter three.

The final effect of all these resistance mechanisms is that the affected antibacterial drug is no longer effective for treating infections. As mentioned earlier in this chapter, the emergence of antibacterial resistance against each antibiotic class is inevitable. Therefore, the continued search for new classes of antibacterial drugs will be essential. The following section will provide a brief history of antibacterial drug discovery with a focus on the reasons why the innovation in antibacterial drug development has dried up.

1.3 The challenge of antibacterial drug discovery

1.3.1 History of antibiotic discovery

Figure 1.2 shows the year of discovery and year of initial introduction of the main antibacterial classes in clinical use. The first class of antibacterial agents to be introduced into the market were the sulfa drugs, which were launched in the early 1930s (Jayachandran et al., 2010). The antibacterial activity of sulfa drugs results from the competitive inhibition of the bacterial enzyme dihydropteroate synthase, essential for synthesis of folate (Henry, 1943).

In 1929, Fleming discovered penicillin by chance when he found a *Penicillium notatum* colony clearing a plate streaked with *Staphylococcus aureus* (Fleming, 1929). In the early 1940s, a successful discovery platform of antibiotics was introduced by Selman Waksman. This platform was based on screening soil streptomycetes for antimicrobial activity against a susceptible test organism by

identifying zones of growth inhibition on an overlay plate (Lewis, 2017). The “Waksman platform” was extensively adopted by the pharmaceutical industry and produced the most important classes of antibiotics over the next 20 years (Figure 1.2).

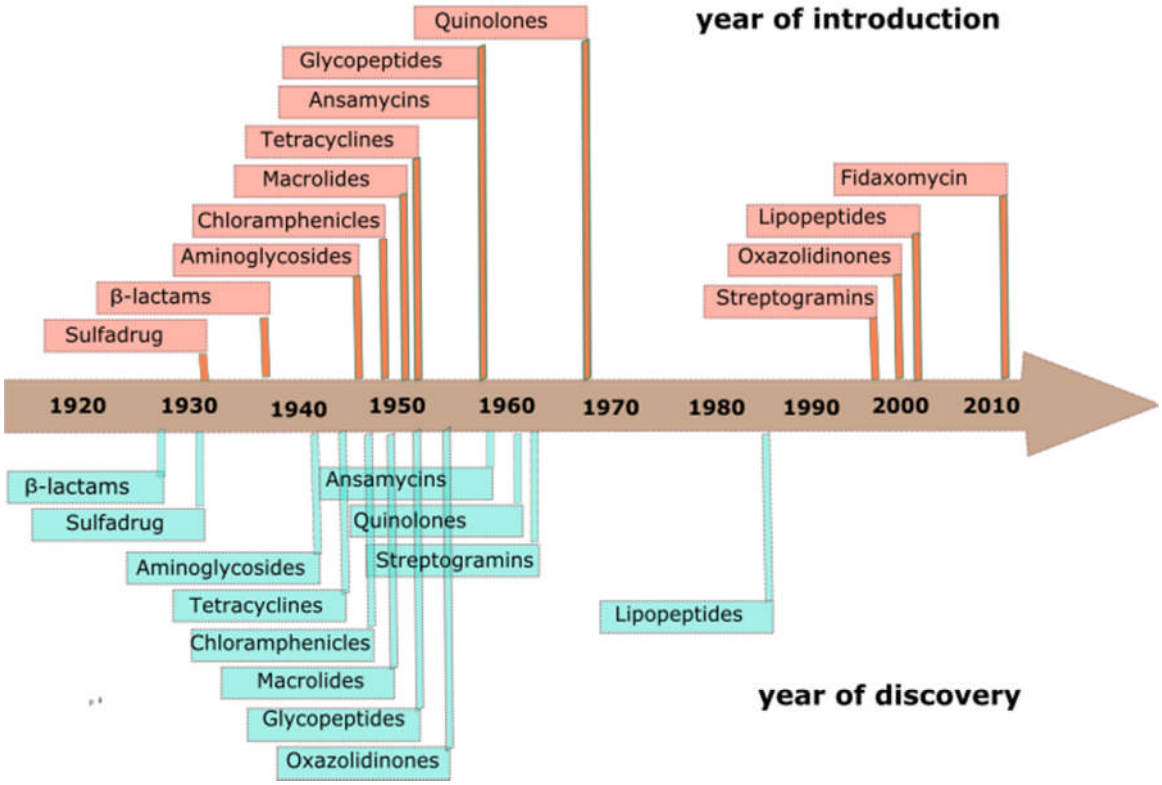


Figure 1.2. The timeline of antibacterial drug discovery and introduction. Adapted from (Lewis, 2017)

However, frequent screening of the soil streptomycetes as well as other actinomycetes (by the same approach) led to this platform has become less

productive due to rediscovery of known compounds (Aminov, 2017). Resistance to introduced antibacterials was emerging, prompting the development of semisynthetic versions of these agents that are active for effective therapy, an approach that was successful for a while (Fischbach and Walsh, 2009). By the 1990s, however, it became clear that resistance was emerging faster than the development of new antibiotics by these means.

The following section gives an overview of the approaches that have been in use for antibacterial drug discovery, and why they have failed over many decades to solve the problem of the lack in antibacterial drugs introduced into the market.

1.3.2 Conventional approaches to the discovery of antibacterial agents

1.3.2.1 Natural Product Screening

This strategy has focused on the screening of fermentation broth of antibiotic producing bacteria by placing filter discs containing fermentation samples onto agar plates inoculated with a bacterial indicator culture. Natural product antibiotics can then be isolated from broths exhibiting a zone of inhibition (Fedorenko et al., 2015). This methodology was the dominant approach for identifying novel lead structures in the early years of antibiotic discovery, from which most natural product antibiotic classes in clinical use have been developed, including the β-lactams, glycopeptides, macrolides, lincosamides, aminoglycosides, streptogramins, tetracyclines, rifamycins and lipopeptides (Wright, 2014). The complex scaffolds produced by this method have also proved excellent platforms for re-engineering and re-modelling to create new generations

of semi-synthetic antibiotics (Gootz, 1990). Lately, drug discovery has used alternative approaches for mining these resources due to the frequent rediscovery of known compounds, which leads to a significantly increased cost of discovery of each novel natural product (Singh et al., 2011). The discrimination of known scaffolds from unknown, a process called dereplication, is a considerable challenge even with the availability of advanced chemical dereplication methods (Wright, 2014).

A more recent approach to find new antibiotics from natural products includes the use of recombinant DNA technology to isolate biosynthetic gene clusters directing the production of antibiotics; several gene clusters encoding metabolic pathways involved in antibiotic production have been identified by cloning genes from streptomycetes (Singh et al., 2011). Although cloning of these genes may achieved either by metagenomic “capture of clusters” or by synthesis, heterologous expression of these clusters continuous to present a huge challenge. Another approach to the discovery of novel natural product antibiotics involves screening previously uncultured bacteria using an “iChip”. This device contains hundreds of small cavities used to capture a single microbe in each cavity after a diluted sample of soil being poured on this device. Subsequently, the device covered with membranes and returned into the soil sample, these membranes comprise pores that allow chemical nutrients to flow, and prevent movement of any bacteria. This enables the single organism in each of the cavities in the iChip to consume all the natural nutrients for growth and prevent contamination with other bacteria that are present in the surrounding environment. These bacteria then transferred to be grown under lab conditions to isolate antibiotics that they produce. This approach has recently resulted in the discovery of a new antibiotic termed teixobactin (Ling et al., 2015).

1.3.2.2 Synthetic antibacterial agents

Synthetic chemistry has provided a second strand of antibacterial discovery, and involves the production of antibacterial compounds from chemicals that are not found in nature. The first group of synthetic antibacterial drugs that have been in use since their introduction in 1930 are the sulfa drugs (Walsh, 2003); sulfanilamide (para-aminobenzenesulfonamide) was identified as an active compound of this group, which was modified over the years to generate less toxic and broader spectrum antimicrobial compounds (Smith and Powell, 2000). Fluoroquinolones, such as ciprofloxacin, are another group of synthetic antibiotics; this class inhibit bacterial DNA replication and repair (Maxwell, 1997). The most recent synthetic antibacterial class introduced to the market is the oxazolidinones, exemplified by linezolid, introduced in 2000 (Walsh, 2003). While chemical/synthetic scaffolds are dominant in the development of new leads for non-antibacterial biological targets, completely synthetic chemical collections have not provided a large number of starting points for antibacterial drug development. The reason may be that most chemical library components are extensions of the medicinal chemistry programs for other disease targets (Wright, 2014, Singh et al., 2011). Consequently, these compounds are often not suitable for bacterial targets due to the complex obstacles to penetration and retention of compounds that bacteria have evolved. Therefore, this method has been unsuccessful in development of new antibacterial drugs for tackling the problem of antibacterial resistance (Wright, 2014).

1.3.2.3 The genomic revolution and its disappointment

Starting in the 1990s, the field of antibiotic discovery adopted a genomic-based approach in the search for new antibiotics. This approach was facilitated by the advances in molecular biology, including the sequencing of whole genomes of multiple bacterial pathogens (Schnappinger, 2015), and utilising this genome sequence data for the identification of bacterial targets present in all clinically relevant pathogens but lacking homologues in mammalian cells (Livermore, 2011). This approach also offered the potential to discover new narrow spectrum antibacterials by targeting genes present in one or few pathogens, and thereby provide species-specific treatments that do not adversely affect the bacterial flora (Chan et al., 2002).

In this approach, a high-throughput biochemical assay is used to screen identified (usually) proteins targets against existing compound libraries for candidates that bind to these targets that should in principle be capable of inhibiting bacterial growth (Livermore, 2011). Despite early enthusiasm about genomics and its use for antibiotic discovery, no antibacterial drugs have reached the market through this approach (Donkor, 2013). Indeed, among 67 high throughput screens conducted by the pharmaceutical company GlaxoSmithKline between 1995 and 2002, only five yielded lead compounds (Payne et al., 2007). The compound libraries are designed to obey Lipinski rules (Lipinski et al., 2001), which is not always applicable for antibacterial agents. Furthermore, many antibiotics (approved or in late stage clinical trials) comprise functional groups that are “undesirable” in standard drug-development programmes (Blaskovich et al., 2017). Therefore, this HTS approach has only been effective in identifying inhibitors for targets both in and outside of human cells. For bacteria, however, even when potent enzyme inhibitors were identified, they lacked antibacterial activity due to a failure to penetrate prokaryotic cells (Blaskovich et al., 2017).

In addition to the aforementioned obstacles to antibacterial drug discovery which is specific for each approach, there are other problems that apply to antibiotic drug discovery regardless of the approaches that are used to identify them.

1.4 Reasons underlying the failure of antibacterial drug discovery

A series of factors have contributed to the failure to bring new antibacterial agents to the market. Most significant, is the challenge of drug delivery into bacterial cells. The outer membrane is a barrier for amphipathic compounds - which basically all antibacterial therapeutics are - since they need to be soluble and capable of crossing the cytoplasmic membrane (Brown and Wright, 2016). Multidrug efflux transporters extrude any compounds that escape through the outer membrane, and distinguish chemically unrelated molecules based essentially on polarity, favouring amphipathics. The inner membrane limits penetration of hydrophilic substances, providing a barrier that restricts the entry of antibiotics into bacterial cells (Lomovskaya and Lewis, 1992) (Li and Nikaido, 2009). And even when some compounds reach the intracellular milieu, toxicity resulting from the need to deliver high concentrations of a compound represents another important barrier that diminishes the possibility of developing good leads (Silver, 2011).

targeting a single bacterial protein is another factor that directly contributes to the failure of antibacterial drug discovery programmes, which leads to problems in respect of rapid development of bacterial resistance (Fedorenko et al., 2015).

Even when a single antibacterial target is essential, well conserved across bacterial species, and lacks homology in mammalian host, discovery of new antibacterial agents targeting single targets still fail. The reason is that they are subject to single-step high level resistance selection (Silver, 2011). Multiple molecular targets or targets encoded by multiple genes are necessary to restrict the rapid development of resistance (Silver, 2011), as bacteria are unlikely to develop high level resistance against these antibiotics via single genetic changes (Silver and Bostian, 1990). To reduce development of resistant bacteria during the course of treatment, and in order for the treatment to be successful, the probability of resistance development should stay below/in 10^9 (a number of bacterial cells readily reached within an infected patient). This necessity for low resistance frequency has hindered the introduction of antibacterial agents acting against single targets. For instance, advanced leads with good efficacy in animal models have been developed against the bacterial enzyme deformylase, but failed owing to frequency with which resistance develops (Chen et al., 2004). Another example is the compound commonly referred to as “GSK ‘052” (short for GSK2251052), developed as broad spectrum inhibitor of a single enzyme (leucyl tRNA synthetase). It has been evaluated in a phase II clinical trial, in which resistance against this compound rapidly developed in three of fourteen (21%) patients during therapy (Hernandez et al., 2013) (O'Dwyer et al., 2015).

Empiric observations of growing data on existing antibiotics suggest that antibiotics working on more than one biological targets (protein, RNA, DNA) have a much lower tendency for target-based resistance than those interacting with a single enzyme target (East and Silver, 2013). The β-lactams, fluoroquinolones, and ribosomal inhibitors such as streptomycin or linezolid are examples of currently used antibiotic classes that target multiple different enzymes in a given

species (Silver, 2007). β-lactams attack penicillin binding proteins (PBPs) (Beise et al., 1988), fluoroquinolones inhibit the catalytic subunit of both DNA gyrase (GyrA) and topoisomerase IV (ParC) (Chen et al., 1996), and ribosomal inhibitors bind to rRNA which is encoded by multiple genes. The successful application of targeting dual or multiple targets can be exemplified by these antibacterial classes, and support the hypothesis that multiple targets are required to reduce the rate of antibacterial drug resistance (Silver, 2011).

In addition to these scientific difficulties, there are also more fundamental obstacles associated with the business of antibiotic discovery. In contrast to treatments for chronic illness, antibiotic treatment is normally short (typically for a period of few days) and resistance to any antibiotic will ultimately develop, limiting the drug's useful lifespan. Therefore, antibacterial agents have poor returns on investment which has led to Big Pharma losing interest in, and ultimately leaving, the field of antibacterial drug discovery (Kealey et al., 2017). This is highlighted by the fact that as of March 2015 the development pipeline contained only 28 antibacterial drug candidates compared to 504 cancer drug candidates in Phase II/III clinical trials (Blaskovich et al., 2017).

1.5 Alternatives to antibiotics

In view of the challenges of conventional antibacterial drug discovery and development that have led to an insufficient pipeline of new drugs, it would be practical to explore the potential of non-conventional strategies (Payne et al., 2007, Tommasi et al., 2015). Several alternative approaches are being explored for their potential to treat and prevent bacterial infection, of which ten should be

given special significance according to Czaplewski et al. (Czaplewski et al., 2016) (Table 1). Top of the list is the use of antibodies to bind bacteria or their virulence factors, either to inactivate them or present them to the immune system. As will be explained below in this chapter, although antibodies possess excellent potential as antibacterial therapeutics, they have limitations that hinder their practical application in this field. Thus, the focus of the work presented in this thesis was on exploring the potential of antibody mimetics – which have improved properties over antibodies – as antibacterial agents.

Table 1.1. Ten alternatives to antibiotics that should be given serious consideration (Czaplewski et al., 2016)

Strategy	Mechanism	Probable use	reference
Antibodies	Antibodies that work by inactivating a pathogen, its virulence factors, or its toxins	Prevent Gram-positive and Gram-negative infection ; probably adjunct use	(Hauser et al., 2016)
Probiotics	Live micro-organisms employed to compete bacterial pathogens when administered in sufficient amounts	Prevent or treat Clostridium difficile-related diarrhoea or diarrhea caused by antibiotics	(Fijan, 2016)
Lysins	Enzymes produced by bacteriophages and used in cell wall destruction of a target bacterium	Inhibition of Gram-positive infection	(Daniel et al., 2010)
Wild-type bacteriophages	Viruses that infect and kill bacteria	Directed to Gram-positive and Gram-negative infection	(Bragg et al., 2014)
Engineered bacteriophages	Genetically engineered bacteriophages with new characteristics to infect and kill bacteria	Treat Gram-positive and Gram-negative infection	(Pires et al., 2016)
Immune stimulation	Innate proteins or bacterial extracts that enhance the immune system	Prevent or provide synergistic therapy for infection caused by both Gram-positive and Gram-negative	(Del-Rio-Navarro et al., 2012)
Vaccines	Disabled bacteria or bacterial proteins that boost the immune system	Prevent infection, Gram-positive more than Gram-negative	(Bronze and Dale, 2010)
Antimicrobial peptides	Small peptides that exhibit direct antibacterial action	Treatments or adjuvants for Gram -positive and Gram-negative infection	(Marshall and Arenas, 2003)
Host defense peptides and innate defense peptide	Peptides that led to an increased expression of anti -inflammatory chemokines and cytokines , and reduced expression of pro-inflammatory cytokines	Adjunct for infection caused by Gram-positive and Gram-negative bacteria	(Hancock et al., 2016)
Antibiofilm peptides	Peptides that specifically inhibit formation of bacterial biofilm	Adjunct for Gram-positive and Gram-negative infections	(Pletzer and Hancock, 2016)

1.5.1 Antibodies in antibacterial chemotherapy

1.5.1.1 Structure and function of antibodies

Antibodies are a key part of the mammalian immune response and are responsible for binding to foreign bodies, marking them for degradation by components of the immune system such as the phagocytes. Antibodies are clustered into five classes depending on the sequence of their heavy chain constant regions: IgM, IgD, IgG, IgE and IgA. Among them, IgG is the most commonly used for research and in therapeutic applications. IgGs are Y-shaped molecules consisting of two long heavy chains connected to two shorter light chains by disulphide bonds (Figure 1.3) (Ruigrok et al., 2011).These chains are made up of several units known as immunoglobulin folds, composed of several anti-parallel β-sheets (Bork et al., 1994). Antibodies are constructed of two distinct functional units: the fragment of antigen binding (Fab) and the constant region (Fc). The Fab includes the variable region, which contains three hypervariable complementarity determining regions that form the antigen binding site of the antibody and determine antigen specificity (Ruigrok et al., 2011). Antibodies mediate immune effector functions through the Fc fragment, which is able to activate complement-dependent cytotoxicity (Weiner et al., 2010).

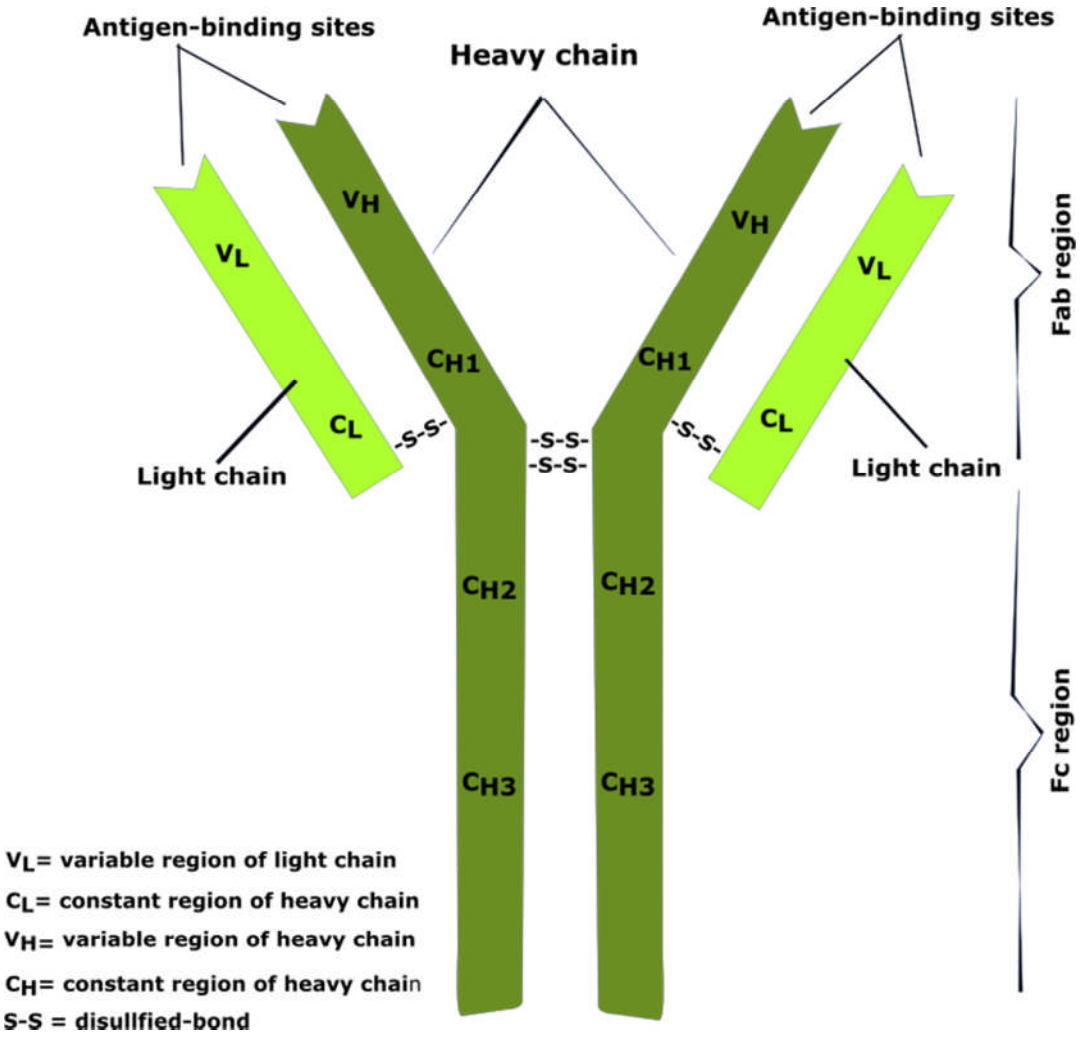


Figure 1.3. Structure of an IgG molecule. Adapted from (Schroeder and Cavacini, 2010)

1.5.1.2 Antibody-based therapeutics

Since 1975, when Kohler and Milstein established a process to produce monoclonal antibodies (mAbs) (KÖHler and Milstein, 1975),these molecules have been considered by many as near perfect molecules for imaging and therapy, comparable to the magic bullets envisioned by Paul Ehrlich at the beginning of the 20th century (Bebbington and Yarranton, 2008). Antibodies are capable of

binding to a wide range of molecules with high specificity and affinity and now have an established track-record as therapeutic agents (Wang, 1999).

The 1990s witnessed the cloning of genes encoding mAbs of interest in eukaryotic expression vectors (Winter and Milstein, 1991); as a result it has been possible to obtain recombinant versions of any mAb in a reproducible manner (Chames and Baty, 2000). This was the first step towards the modification of antibodies, which made it conceivable to enhance recombinant antibodies and ushered in the age of antibody engineering (Hoogenboom and Chames, 2000). Recent years have seen substantial advances in mAbs technology and production including phage-displayed antibody libraries (Nixon et al., 2014) (Lloyd et al., 2009), hybridoma technology (Green, 2014), and the isolation of immunoglobulin directly from human B cells after infection (DiGiandomenico et al., 2012). An example of the success of antibody-based therapeutics is their use in the treatment of cancer. Over the past decade, the efficacy of antibodies in treating patients with cancer has been demonstrated (Sliwkowski and Mellman, 2013). Many of these antibodies are specifically directed against antigens produced by the tumour itself. Antibodies combined with radiotherapy or chemotherapeutic drugs have also successfully been used in treatment of hematological malignancies (Weiner et al., 2010). Unconjugated antibodies targeting growth factor receptors, such as epidermal growth factor receptor (EGFR) are generally used for the treatment of non-leukaemic cancers (Weiner et al., 2010).

Anti-cancer mAbs are in many ways similar to what would be required of an ideal antibacterial mAb: in both fields, mAbs are required to specifically identify and

eradicate pathogenic cells that are reproducing/evading the immune response in severely ill, or immunocompromised patients.

Engineering of mAbs to be effectively used in antibacterial chemotherapy is therefore an attractive proposition (Oleksiewicz et al., 2012).

1.5.1.3 Antibodies to treat bacterial infection

The concept of passive immunization is not new; antibodies were used, in the form of serum therapy, to prevent or treat bacterial infection before the antibiotic era (Dolman, 1936). However, toxicity issues and expensive production processes associated with these materials led to their replacement by broad spectrum antibiotics. After half a century of highly productive antibiotic development, it has now become clear that antibiotics are unlikely to offer the ultimate solution in the battle against bacterial infections. The success and huge increase in mAbs based therapy in recent years have made mAbs a viable option to prevent infectious diseases, and intensive efforts have been made towards novel vaccines addressing antibiotic resistant bacterial infections, particularly against *S. aureus*. The antibacterial mAbs paradigm concentrates mostly on generating antivirulence compounds that disarm pathogenic bacteria by neutralizing their virulence factors (Bebbington and Yarranton, 2008). However, the pathogenesis of *S. aureus*, for instance, is mediated by an enormous number of surface proteins, carbohydrate molecules, and secreted factors that are used for suppression of complement action, inhibition of antibody function, destruction of host cells, and exhibition of toxic effects (Thammavongsa et al., 2015). Possessing multiple virulence factors enables the pathogen to invade multiple sites inside the human body. A number of these *S. aureus* virulence proteins have been targeted by monovalent vaccine and immunization approaches, and

none of them have yet proceeded to approval for clinical (Sause et al., 2016). The reason may be that these compounds require combination therapy to cover multiple, redundant or strain-variable virulence factors. Also they may not be useful in all types of disease that are caused by the same pathogen. Furthermore, organism-specific antivirulence compounds necessitate rapid identification of a bacterial pathogen, and even if they were successful in treating bacterial infection, bacteria may persist and cause harm after therapy (Dickey et al., 2017).

In the antibacterial area, currently the only mAbs in clinical use are antitoxin mAbs produced against *Clostridium botulinum* and *Bacillus anthracis* (Table 1.1). However, there are a number of antibacterial mAbs in development (Table 1.2).

Table 1.2. Examples of clinically approved antibacterial antibodies

	Subtype or chemistry	Molecular target	Furthest developmental stage	Refs
Clostridium botulinum				
BabyBIG	Human, mostly IgG, plasma-derived immune globulin	BoNT serotypes A and B	FDA approved	(Arnon et al., 2006)
BAT	Equine, Fab and F(ab′)2; plasma-derived immunoglobulin	BoNT serotypes A–G	FDA approved (NCT00360737)	-
Bacillus anthracis				
Raxibacumab	Human, mAb IgG1	Protective antigen of anthrax toxin	FDA approved	(Migone et al., 2009)
Obiltoxaximab	Human, mAb IgG1	Protective antigen of anthrax toxin	FDA approved	(Greig, 2016),(Yamamoto et al., 2016)

Table 1.3. Examples of antibacterial antibodies against *S. aureus that are in development*

	Subtype or chemistry	Molecular targets	Furthest developmental stage	Refs
Staphylococcus aureus				
Compound name				
MEDI4893	Human, mAb IgG1	α-Toxin	Phase II (ongoing)	(Oganesyan et al., 2014)
AR-301	Human, mAb IgG1	α-Toxin	Phase II (ongoing; NCT01589185	-
ASN-100 (a combination of ASN-1 and ASN-2)	Human, mAb IgG1	α-Toxin, PVL, LukED, LukGH and γ-haemolysin	Phase II (ongoing)	(Rouha et al., 2015), (Badarau et al., 2016)

1.5.1.4 Limitation of antibodies as therapeutics

In spite of their successes, monoclonal antibodies face a variety of limitations that restrict their applicability. Several of these limitations relate to the size of the antibody molecule, which, in the case of the frequently used IgG isotype, is in the range of 150 kDa (Chames et al., 2009). The size of this molecule prevents penetration into cells. Furthermore, the structure of antibodies is complex; they contain two different chains (heavy and light) comprised of six different domains. Assembly of the antibody molecule and much of its stability depend on the accurate formation of disulfide bonds between these chains and on post-translational glycosylation of the constant region (Rouet et al., 2014). Together, these requirements make the heterologous production of human antibodies by bacteria difficult (Birch and Racher, 2006) (Rouet et al., 2012). To overcome the size and stability limitations of monoclonal antibodies, a large body of work has focused on designing small non-antibody scaffolds “antibody mimetics” for therapy and imaging applications (Skerra, 2007) (Lofblom et al., 2011).

1.5.2 Antibody mimetics

Antibody mimetics are small proteins which imitate the binding activity and specificity of full length antibodies, whilst being smaller in size and exhibiting higher stability (Banta et al., 2013). These binding proteins are derived from small and stable none-immunoglobulin scaffolds that can be embued with target-specific binding functions using combinatorial protein design techniques (Banta et al., 2013). Libraries of binding protein are screened for a specific binder using several approaches, with the most common method being phage display, which

involves displaying the protein of interest fused with one of the viral coat proteins of bacteriophage (Galan et al., 2016). They also have the added advantage of comparatively easy expression and purification using bacterial cells. Consequently, antibody mimetics are of substantial interest for drug discovery (Parizek et al., 2012). Typically, they are protein molecules that employ the immunoglobulin fold. However, over the past few years a range of mimetics based upon diverse protein scaffolds that enable protein-protein interactions have been developed (Banta et al., 2013). Non-antibody scaffolds may generally fall into two structural classes, namely domain-sized scaffolds with molecular size of 6–20 kDa, and constrained peptides which are 2–4 kDa and peptide-associated scaffolds. Table 1.4 shows some examples of these scaffolds.

Table 1.4. Examples of non-antibody scaffolds (Banta et al., 2013)

domain-sized scaffolds	**peptide-associated scaffolds**
Affibodies, Affilins, Anticalins, Atrimers, DARPins, FN3 scaffolds (e.g. Adnectins and Centyrins), Fynomers, Kunitz domain, Pronectins, Scannins and OBodies**.**	Avimers, bicyclic peptides and Cys-knots.

A great number of candidates derived from these scaffolds are presently under academic, preclinical and clinical development and have collectively shown substantial potential in terms of affinity, target inhibition and stability (Vazquez-Lombardi et al., 2015) (Binz et al., 2005). More recently a new artificial antibody protein, originally named “Adhiron”, was developed (Tiede et al., 2014); these proteins are now known as Affimers (Tiede et al., 2017), and this term will be used hereafter. As will be discussed in the following section, Affimers meet all the requisite criteria of an artificial binding protein scaffold, and they are developed at the University of Leeds and established as a technique that has been successfully used for many applications, it was therefore a good opportunity to investigate their potential as antibacterial agents.

1.5.2.1 Affimers

Affimers are a novel class of antibody mimetic based on a scaffold derived from a cysteine protease inhibitor in plants called phytocystatin. This protein is a member of the cystatin family, all of which are characterized by a highly conserved fold, containing a central α-helix surrounded by four anti-parallel β-sheets (Ochieng and Chaudhuri, 2010). Phytocystatin is an important protein in plants and has been shown to play roles in plant defence against proteases released by pathogens as well as environmental stress such as drought (Martinez and Diaz, 2008). Additionally, it plays a role in the regulation of proteases during seed maturation and is involved in programmed cell death (Chan et al., 2014). Its small size, stability and natural lack of either cysteines or

glycosylation sites make it ideal for the development of a protein library capable of binding targets of interest (Tiede et al., 2014). Affimers have been shown to bind several different targets specifically, such as a chemically biotinylated yeast small ubiquitin-like modifier (SUMO) protein with low nanomolar affinity (Tiede et al., 2014). More recently, Affimers have been successfully used in a wide range of applications including (among others) studying intracellular signalling pathways, modulating ion channel function, super-resolution microscopy and single particle tracking (Tiede et al., 2017).

In order to construct Affimers, the N-terminus of the scaffold was truncated and its two inhibitory loops reformed with a random sequence of nine amino acid residues. This insertion creates two variable regions in the loops placed between the β-sheets (Figure 1.3). The design of the scaffold and library offers a highly stable scaffold with extended flexible binding loops that adapt to allow appropriate molecular interactions with protein pockets, protein surfaces, peptides and small molecules (Tiede et al., 2014). Affimers exhibit a far greater degree of thermostability compared to full length antibodies, with a melting temperatures of 101°C, and they express well in *E. coli* (Tiede et al., 2014).

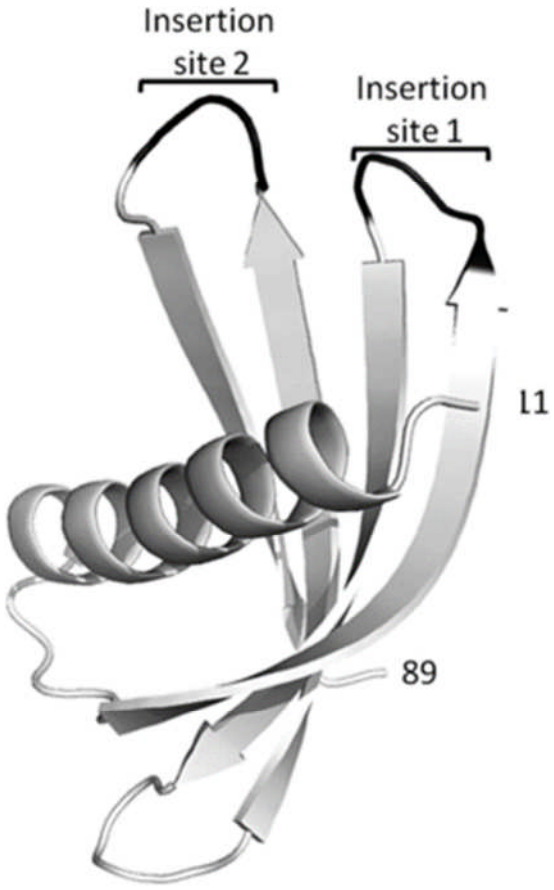


Figure 1.4. The X-ray crystal structure of an Affimer: The structure is at a resolution of 1.75 Å and illustrates a truncated Affimer, from residue 11 – 89. The regions of the randomised loops are coloured in black (Tiede et al., 2014).

1.6 Objectives

Due to the increasing emergence of multi-drug resistant bacteria and the lack of novel antibiotics in development, this study aimed to investigate the potential of a new approach for antibacterial drug discovery. This approach involves the use of novel artificial binding proteins, Affimers, for selection of molecules with inhibitory effect on bacterial target proteins.

In the first instance, this work examined whether Affimers are capable of inhibiting antibacterial resistance mechanisms, with the intention to investigate the use of these proteins to augment existing antibiotics. Successful Affimers will then be used as tools for overcoming antibacterial resistance. Subsequently, this study, explored the potential of these Affimers as antibacterial agents in their own right, to target and inhibit essential bacterial proteins.

To achieve these goals, two main aspects needed to be investigated; the first one was to examine the possibility of generating single Affimers to recognise, bind, and inhibit multiple target proteins. This was of interest because antibacterial resistance is often mediated by closely related families of proteins, and also, variation of drug targets is an important factor for reducing rapid development of resistance against an antibacterial agent.

The other important issue that is to be considered in this work was the concept of how to deliver these molecules into bacterial cells, which was addressed by targeting well-validated, essential target proteins that are accessible from outside the cell. *S. aureus* was used as a model organism in this study, as a considerable expertise for genetic manipulations of this organism are available in our lab, and the goals were: (1) to select dual-targeted Affimers for inhibiting FusB-type proteins (FusB and FusC), thereby abrogate fusidic acid (FA) resistance that mediated by these proteins. (2) to generate dual-targeted antibacterial Affimers by inhibiting essential membrane proteins (PBP2 and PBP2a).

The first step towards achieving these goals was to establish an appropriate phage display method to enable the selection of single Affimers for more than one target protein. The second step in the processes was to evaluate the ability of selected Affimers to inhibit their target protein. In tandem with microbiological studies using bacteriology and DNA manipulation, biochemical methods with purified protein were used to characterise selected Affimers. Results from this study report for the first time proof of the concept that single Affimers can be selected to bind dual target proteins. Furthermore, Affimers were shown not only to be capable of blocking antibacterial resistance proteins, but also exhibiting antibacterial activity by inhibiting essential bacterial target proteins.

2. Materials and Methods

2.1 Materials

2.1.1 Bacterial strains and plasmids

Table 2.1 lists bacterial strains used and constructed in this study. Plasmid vectors used in this study are listed in Table 2.2. For routine culture of *E. coli* strains, Luria-Bertrani Broth (LBB) and agar (LBA) (Sigma-Aldrich, UK) were used. *S. aureus* strains were grown in Mueller-Hinton broth (MHB) and agar (MHA) (Oxoid, UK), unless otherwise stated. Bacterial strains were stored as glycerol stocks (800 µl of saturated culture and 200 µl of 80% (v/v) glycerol) at -80 ˚C.

2.1.2 Antibiotics and chemicals

All antibiotics and chemicals used in microbiological and biochemical studies were from (Sigma-Aldrich), and all chemicals used in phage display and phage ELISA were from Thermo Scientific, unless otherwise stated. IPTG (Isopropyl β-D-thiogalactopyranoside) was purchased from (Calbiochem, UK).

Table 2.1. Bacterial Strains

Strain	Description	Source
S. aureus SH1000	Derivative of *S.aureus* 8325-4, used as a source of *pbp2* gene	(Horsburgh et al., 2002)
S. aureus COL	Methicillin resistant *S. aureus*, used as a source of *pbp2a* gene	(Gill et al., 2005)
S. aureus RN4220	Restriction-deficient cloning host	(Kreiswirth et al., 1983)
S. aureus CYL12349	RN4220 with an engineered *attB* site, containing (pYL112 Δ19) for integrase expression	(Lei et al., 2012)
E. coli ER2738	Allows translational read-through stop codon (TAG) to create an Affimer-truncated-pIII fusion protein Genotype: F′*proA*$^{+}$*B*$^{+}$ *lacIq* Δ(*lacZ*)*M15 zzf*::Tn10(TetR)/*fhuA2 glnV* Δ(*lac-proAB*) *thi-1* Δ(*hsdS-mcrB*)5	(Tiede et al., 2014)
E. coli DH5α	Used for propagation of recombinant plasmids Genotype: *fhuA2 lac*(del)U169 *phoA glnV44* Φ80' *lacZ*(del)M15 *gyrA96 recA1 relA1 endA1 thi*-1 *hsdR17*	Novagen
E. coli BL21 (λDE3) Gold	For protein expression from T7 promoter	Novagen

	Genotype: F-*ompT gal dcm lon hsdSB*(rB-mB-) λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*])	
E. coli Rosetta 2 (DE3)	BL21 derivatives designed to enhance the expression of proteins that contain codons rarely used in *E. coli* Genotype: F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen

Table 2.1. Continued. Bacterial strains

S. aureus RN4220 (pRAB11: *pbp2*)	*S. aureus* RN4220 bearing pRAB11:*pbp2* Used for expression of PBP2 in *S. aureus*	This study
S. aureus RN4220 (*fusC)*	*S. aureus* RN4220 with *fusC* integrated at the engineered L54a *att*B under control of *cap*1A promoter	This study
S. aureus RN4220 (*fusB*)	*S. aureus* RN4220 with *fusB* integrated at the engineered L54a *att*B under control of *cap*1A promoter	This study
S. aureus RN4220-pRAB11	*S. aureus* RN4220 carrying plasmid pRAB11	This study
S. aureus RN4220 (*fusC*) : pRAB11-fusB/C Affimers 1-4	*S. aureus* RN4220 (*fusC*) with fusB/C binders 1-4 under control of Pxyl/tet promoter on pRAB11	This study
S. aureus RN4220 (*fusB*) : pRAB11-fusB/C Affimers	*S. aureus* RN4220 (*fusB*) with fusB/C Affimers 1-4	This study

1-4	under control of Pxyl/tet promoter on pRAB11	
S. aureus RN4220 (pRAB11: PBP2/PBP2a Affimer)	*S. aureus* RN4220 with PBP2/PBP2a Affimer under control of Pxyl/tet promoter on pRAB11	This study

Table 2.2. Plasmid and phagmid vectors used in this study

Plasmid	Description	Source
pET28a	Expression vector C-terminal (6xHis) Kanamycin selection	Novagen
pET11a	Expression vector Carbencillin selection	Novagen
pRAB11	Tightly controlled tetracycline-dependent gene expression in *S. aureus* Carbencillin selection in *E. coli* Chloramphenicol selection in *S. aureus*	(Helle et al., 2011)
pLL102	A single-copy integration vector, integrates at specific site in the chromosome of *S. aureus* Spectinomycin selection in *E.coli* Tetracycline selection in *S. aureus*	(Lei et al., 2012)

pBSTG1 (phagmid)	M13 phagmid developed from pHEN1phagmid vector Used as sources of DNA coding regions of selected Affimers Carbencillin selection	(Tiede et al., 2014)

2.2 Molecular biology techniques

2.2.1 Purification of plasmid DNA

The E.N.Z.A plasmid DNA Miniprep kit (OMEGA, USA) was used to extract and purify plasmid DNA according to the manufacturer's instructions. Purified DNA was subsequently stored at -20˚C. When purifying plasmids from *S. aureus*, 100 µg/ ml of lysostaphin was added to the cell resuspension mixture and incubated at 37˚C for 30 minutes to lyse the staphylococcal cell wall before proceeding with the rest of the protocol.

2.2.2 Polymerase chain reaction

Synthesised oligonucleotide primers were from Eurofins MWG Operon (Ebersberg, Germany). PCR was performed in T100™ Thermal Cycler (Bio Rad, UK). Q5 High-fidelity DNA polymerase (New England Biolabs, Hertfordshire, UK) was used according to manufacturer's guidelines and cycling conditions were optimised as appropriate. Nucleotides were from Promega (Madison, WI, USA).

2.2.3 Colony PCR

Single colonies were suspended in 20 µl of dH2O and 2 µl of this suspension used as a template for each colony PCR reaction. Reactions consisted of 12 µl Go Taq Green Master Mix (Promega), 1 µl forward and reverse specific primers (100 pmol/µl stock concentration). Cycling conditions were as follows: 98 ˚C for 15 minutes 35 cycles of 98˚C for 30 seconds, 50˚C for 30 seconds and 1 minute/kb at 70˚C. Samles were subsequently analysed by agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis and gel extraction

Gels were used at concentration of 0.8 % (w/v) agarose in TAE buffer containing SYBR® Safe gel stain (Invitrogen). The MinElute® gel extraction kit from QIAGEN (West Sussex, UK) was used to purify DNA fragments according to the manufacturer's instructions.

2.2.5 Restriction digest and ligation of DNA

Restriction enzymes and buffers were obtained from New England Biolabs (Hertforshire, UK). Digestion of PCR products and plasmid vectors was performed in a total reaction volume of 50 µl. Each reaction comprised 1 µg DNA, 5 µl reaction buffer and 1 µl of each restriction enzyme (20,000 U/ml), and was incubated at 37°C for 4 hours. Digestion products were gel purified using a QIAGEN gel extraction Kit (QIAGEN). Purified DNA fragments were ligated using T4 DNA Quick Ligase according to the manufacturer's instructions.

2.2.6 Transformation of *E. coli* strains

Competent *E. coli* strains were prepared as previously described (Chung et al., 1989). Ligated DNA (5-10 ng) was added to 100 µl aliquots of competent cells on ice. The transformation mixture was held on ice for 30 minutes, heat-shocked at 42°C for 45 seconds, returned to ice for 2 minutes. Transformations were then transferred to a 25 ml universal containing 900 µl of SOC medium, and incubated for 1 hour at 37°C with vigorous aeration. Transformed cells were plated on LBA under appropriate antibiotic selection, and plates incubated overnight at 37°C. Individual transformants were screened for the presence of insert by colony PCR (2.2.3).

2.2.7 Transformation of *S. aureus* strains

Recipient cells of *S. aureus* strains were made competent according to a method described by Kreamer and Iandolo (Kraemer and Iandolo, 1990). For electrotransformation, cells were thawed at room temperature and placed on ice. An aliquot (50 µl) of the cells was then centrifuged at 10,000 × *g* for 1 minute and resuspended in 50 µl of 10% glycerol (v/v) and 500 mM sucrose (filter sterilized). The cell suspension was then mixed with 0.2-0.5 µg of plasmid DNA in a 1 mm electroporation cuvette (Geneflow, Elmhurst, UK), and electroporated with a single pulse at 21 kV/cm, 100 Ω, and 25 µF. Immediately after the pulse, 1 ml of TSB containing 500 mM sucrose (filter sterilized) was added to the cuvette. The resulting suspension was transferred to a universal and incubated at 37°C with shaking for 1 h before plating onto TSB with appropriate antibiotics for selection. Plates were incubated at 37°C overnight.

2.2.8 Construction of strains and over expression plasmids

All primers used in this work to construct the strains and plasmids are described in Table 2.3. To investigate the ability of FusB/FusC binding Affimers to restore the antibacterial action of FA in the presence of FusB and FusC determinants, a strain of *S. aureus* RN4220 carrying *fusB* and another strain carrying *fusC* on the chromosome were constructed. To construct these strains, DNA corresponding to the open reading frame for each protein were PCR amplified using primer pairs Cap1a FusB-102-F/Cap1a FusB-102-R and Cap1a FusC-102-F/Cap1a FusC-102-R, respectively (Table 2.3), and the purified PCR product was cloned into the integrative vector pLL102. Transformants were selected in *E. coli,* confirmed DNA sequencing and introduced into *S.aureus* CYL12349 by electroporation after which they became integrated in the chromosome at the engineered *attB* site (*attB* 2). Integration of cloned DNA into this site was confirmed by PCR as previously defined by (Lei et al., 2012). Then these constructs were transduced to *S.aureus* RN4220 by phage transduction as previously described (Novick, 1991), to generate RN4220 *fusB*$^{+}$ and RN4220 *fusC*$^{+}$ strains.

For creation of plasmid constructs from which FusB/FusC Affimers were expressed in *S. aureus*, DNA regions coding for these proteins were PCR amplified using primers RAB-AFFI-S-F and RAB-AFFI-S-R (forward primer was designed to include ribosome binding site). Amplified PCR products were purified, ligated into the plasmid pRAB11, and the correct constructs electroporated into RN4220 *fusB*$^{+}$ and RN4220 *fusC*$^{+}$ strains.

To construct the *S. aureus* strain for controlled expression of PBP2, DNA encoding this protein was PCR amplified using primers PBP2-RAB-F and PBP2-

RAB-R (Table 2.3). Purified PCR product was cloned into the plasmid pRAB11, verified by DNA sequencing and introduced to S*. aureus* RN4220 by electroporation.

Plasmids for overexpression of Affimers in *E. coli* were constructed as follows: the DNA coding sequences of selected Affimers were PCR amplified using primers AFFI-pET-F and AFFI-PET-R (Table 2.3), and the purified PCR products were ligated into pET11a, confirmed by colony PCR and DNA sequencing.

Table 2.3. Oligonucleotide primers used in this study. Sequences complementary to the pLL102 and pRAB11 plasmids used for Gibson assembly are lower case, restriction sites are underlined, and expression elements (promoters and ribosome binding sites) are indicated by bold letters.

Name	Nucleotide sequence	Restriction enzyme	Description
Cap1a FusB-102-F	gaattcgagctcggtacccggggatcctct**TTGCAA**AATATACAGGGGATTATA**TATAAT**GG AAAACAAGAAAGGAAAAT**AGGAGG**TTTATATGAAAACAATGATTTATCCT		For introduction of *fusB* into plasmid vector pLL102 under control of the *cap1A* promoter
Cap1a FusB-102-R	ctaaagaagttgtaggtaataaaaaagcttTTAATCTAGTTTATCAATAAAAT		
Cap1a FusC-102-F	gaattcgagctcggtacccggggatcctctAGAGT**TTGCAA**AATATACAGGGGATTATA**TAT AAT**GGAAAACAAGAAAGGAAAAT**AGGAGG**TTTATATGAATAAAATAGAAGTGT ATAAG		For introduction of *fusc* into plasmid vector pLL102 under control of the *cap1A* promoter
Cap1a FusC-102-R	ctaaagaagttgtaggtaataaaaaagctt CTATTTTATTTTAACAATAAATTCGTAAA		
RAB-AFFI-S-F	GCAACGGTACCTA**AGGAGG**ATGATGAAAAAGATTTGGTTGGCTC	KpnI	For introduction of selected PBP2/PBP2a Affimers into pRAB11 plasmid vector
RAB-AFFI-S-R	GCAACGAGCTCCTAGTGGTGATGATGGTGATG	SacI	
PBP2-RAB-F	tcattgatagagtatgatggtaccgttaac**AGGAGG**ATGACGGAAAACAAAGGATCTTCTCAGCC		For introduction of *PBP2* into

PBP2-RAB-F	acgacggccagtgaattcgagctcagatctTTAGTGTGTACTACGATTTGTAGTGTT	pRAB11 plasmid vector

Table 2.3. Continued. Oligonucleotide primers used in this study.

AFFI-RAB-F	GCAACGGTACCTA**AGGAGG**ATGGCTACCGGTGTTCGTG	KpnI	For introduction of selected FusB/FusC Affimers into pRAB11 plasmid vector
AFFI-RAB-R	GCAACGAGCTCCTAGTGGTGATGATGGTGATG	SacI	
AFFI-pET-F	GCTAGCATGGCTACCGGTGTTCGTG	NheI	For introduction of selected Affimers into pET11 plasmid vector
AFFI-PET-R	CGCCGGCGCTAGTGGTGATGATGGTGATG	NotI	
pET-PBP2-F	GCATTACGGGATCCATGTTACAAGATCCGATTCCTGC	BamHI	For introduction of *pbp2* into pET28a plasmid vector
pET-PBP2-R	CGTAATGCCTCGAGGTGTGTACTACGATTTGTAGTGTT	XhoI	
pET-PBP2a-F	GCATTACGGGATCCATGTATGCTTCAAAAGATAAAGAAATTAATAATAC	BamHI	For introduction of *pbp2a* into pET28a plasmid vector
pET-PBP2a-R	CGTAATGCCTCGAGATCTATATCGTATTTTTTATTACCGTTCTCATATAG	XhoI	

2.3 Over-expression and purification of recombinant proteins

2.3.1 Expression and production of target proteins

FusB type proteins (FusB and FusC) were expressed and purified exactly as previously described (Cox et al., 2012). PBP2 and PBP2a were produced and purified following the methods defined in previously published work by (Lovering et al., 2007) and (Kim et al., 2012), respectively.

2.3.2 Expression and purification of target-binding Affimers

The pET11 plasmids expressing selected Affimers were constructed as described above (Section 2.2.8), and expression of these Affimers was performed in *E. coli* BL21 (λDE3) cells. Expression levels of these Affimers were analysed in small-scale cultures. For each Affimer, 7 ml of LBB supplemented with 1% glucose was inoculated with a single freshly transformed colony of BL21 (λDE3) strain. Cells were then grown at 37°C with vigorous shaking until an OD_{600} of about 0.6, whereupon cultures were induced with IPTG at a final concentration of 0.5 mM and allowed to grow for 6 hours at 30°C. Aliquots (1 ml) of cultures were pelleted at 10,000 x *g* for 10 minutes, and cell pellets resuspended in 200 µl of lysis buffer (20 mM NaH2Po4, 500 mM NaCl, 20mM imidazole). The resultant suspensions were centrifuged at 30,000 x *g* for 15 minutes to separate soluble and insoluble fractions. The supernatant was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Affimers were expressed in large scale cultures and the resulting proteins were purified as previously described (Tiede et al., 2014), Affimers were eluted in phosphate buffered solution containing 500 mM NaCL, 300mM imidazole and 10% glycerol and then buffer exchanged using the S75 gel filtration column (GE Healthcare).

2.4 Phage display

2.4.1 Biotinylation of target proteins

Target proteins were labelled with EZ-Link® NHS-SS-Biotin. A solution of 5 mg/ml NHS-SS-Biotin in dimethyl sulfoxide (DMSO) was prepared immediately before use. NHS-SS-Biotin solution was added to the target proteins to achieve 2–4 moles of biotin per mole of target protein in a total volume of 100 µl Phosphate Buffered Saline (PBS- pH 7.4). The reaction mixture was incubated at room temperature for 1 hour and then desalted using Zeba Spin Desalting Columns, 7K MWCO (Thermo Scientific) according to the manufacturer's guidelines. Subsequently, an equal amount (100 µl) of 80% glycerol was added to biotinylated protein samples and aliquots stored at -20°C.

2.4.2 Assessing biotinylation by Enzyme Linked Immunosorbent Assay (ELISA)

The extent of biotinylation was determined using streptavidin conjugated to horseradish peroxidase (HRP). Biotinylated target protein (1-1.5 µg) in a total volume of 50 µl PBS per well was incubated overnight at 4°C in Nunc-Immuno™

MaxiSorp™ strips (Thermo Scientific). Wells were then washed three times with 300 µl of PBST (PBS + 0.1% Tween-20) per well on a plate washer (TECAN HydroFlex), and blocked with 250 µl of 10x casein based blocking buffer (Casein, Sigma). Blocked wells were incubated at 37°C for 3 hours prior to washing 3 times with 300 µl PBST per well. Biotinylated proteins were detected by incubating each well with 50 µl of 1:1000 dilution of High Sensitivity Streptavidin-HRP for 1 hour at room temperature. Following washing, biotin labelled proteins were visualised with 3,3`,5,5`-tetramethylbenzidine (TMB) (Seramun) and measured at 610 nm.

2.4.3 Affinity selection of phage-displayed target-binding Affimers

Essentially, affinity selection of target-binding Affimers was conducted as described (Tiede et al., 2014). Figure 2.1 shows schematic illustration of phage display technique. Screening for phage displayed Affimers that bound to multiple targets were conducted using two approaches.

Method 1. Individual phage displayed binders selected for one protein were rapidly tested for their ability to bind second target protein by phage ELISA (Section 2.4.5).

Method 2. Phage pool collected from biopaning the whole library against one target protein was biopanned on the other target. In this procedures, enriched phage selected from the first panning round against one target was used as an input phage to be biopanned on the second target. Phage display course involved total three rounds of panning and conducted as previously described (Tiede et al., 2014).

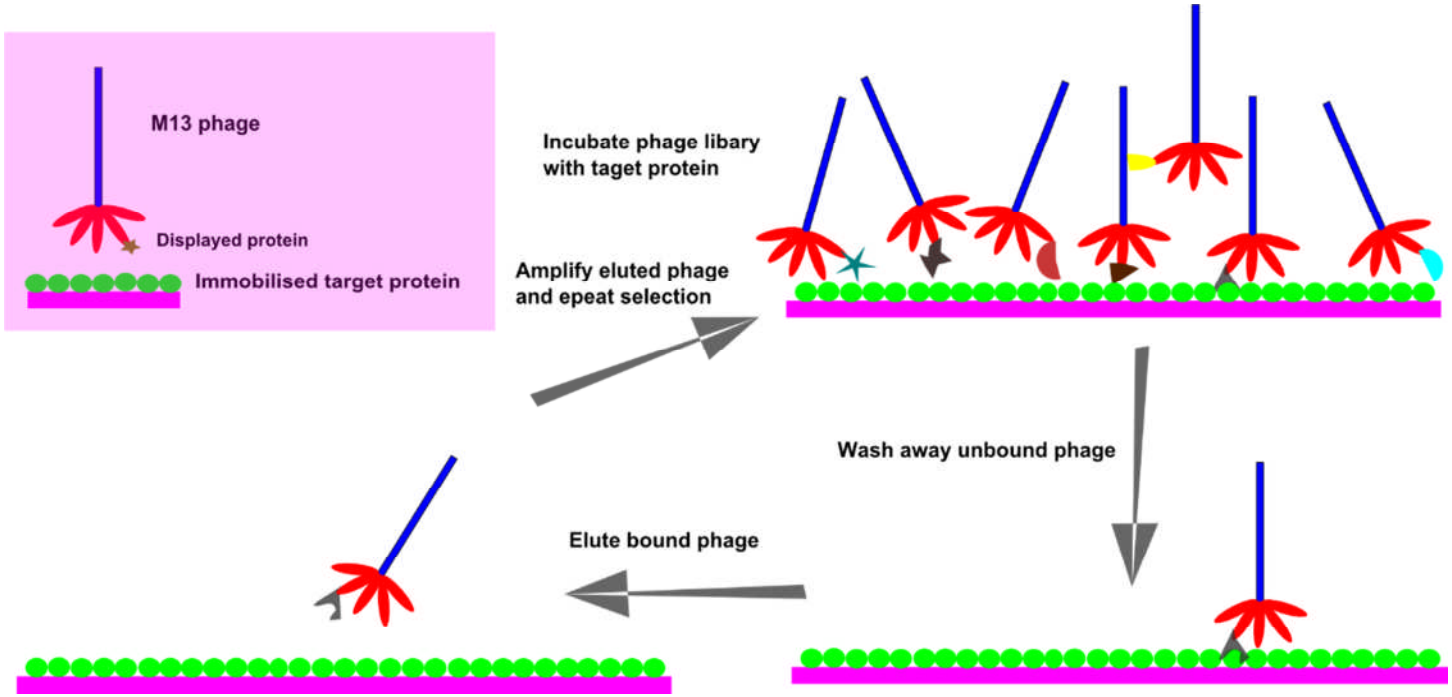


Figure 2.1. Schematic presentation of biopanning. Phage display library is incubated with target protein immobilized on solid support. Specific library phage is bound to the target and unbound phages are washed out. The specific phages are eluted and amplified in bacteria. After several panning rounds, specific phages are analysed (Bazan et al., 2012).

2.4.4 Phage titration

To determine the phage output (for both target protein and negative control) from each round, a 10-fold dilution series of the eluted phages was used to infect RE2738 cells grown to OD600 0.5 at 37 °C. Phage infection was allowed to proceed for 30 min at 37 °C. At least three 10 µL aliquots for each phage dilution were plated on LB agar plates and incubated at 37 °C overnight. The titre of phage was calculated from the dilution with the highest number of countable colonies.

2.4.5 ELISA detection of phage binding

2.4.5.1 Preparation of Phage (individual binders)

Following a full course of binding enrichment, a single colony of each clone (*E. coli* ER2738 cells infected with individual phage displayed Affimer) was used to inoculate 200 µl of 2TY media in a 96-well V-bottom deep well plate (Greiner Bio-One). Cultures were incubated overnight at 37°C, 1050 rpm in an incubating microplate shaker (Heidolph Incubator 1000 and Titramax 1000). To amplify the phage, 25 µl per well of the overnight cultures was transferred to 200 µl 2TY media into fresh plates, and incubated for 1 hour at 37°C, 1050 rpm. M13K07 helper phage (titre ca. 10^{14}/ml) was diluted 1/1000 in 2TY and 10 µl per well was added to the freshly grown cultures. Phage infected cultures were then incubated at room temperature, 450 rpm for 30 minutes, in an incubating microplate shaker (VWR Incubating Microplate Shaker), followed by adding kanamycin to a final concentration of 25 µl/ml. Cultures were incubated overnight at room

temperature, 750 rpm. Phage containing supernatant was obtained by centrifuging the phage-infected culture plates at 3,500 x *g* for 10 minutes.

2.4.5.2 Preparation of Streptavidin-coated 96-well plates

Streptavidin (Molecular Probes®) was obtained from Life Technologies in lyophilized form in 10 mM phosphate buffered saline, pH 7.4. Streptavidin was reconstituted at 5 mg/ml in deionized water then stored in small volumes at -20°C. These aliquots were diluted to 5 µg /ml in PBS and 50 µl per well were transferred to a F96 Maxisorp Nunc-Immuno Plates (Thermo Scientific) and incubated for a minimum of 4 hours at room temperature or overnight at 4°C.

2.4.5.3 Phage ELISA

Streptavidin-coated 96-well plates, prepared as described in Section 2.4.3.2, were blocked with 200 µl per well of 2x Blocking Buffer and incubated overnight at 37°C, washed with 300 µl per well of PBST on a plate washer (TECAN Hydro Speed). Coating solution (50 µl per well) was prepared by diluting each target protein to a concentration of 2 µg/ml in 2x blocking buffer, and 50 µl per well of the solution was incubated for 1 hour at room temperature on the vibrating platform shaker. As a negative control, 50 µl/well of 2x blocking buffer was added to a separate row in the same plate. Plates were emptied, washed with 300 µl/well of PBST, and plotted to dry on stacked paper towels. To test against both target and negative control, 40 µl of each Affimer-displaying phage supernatant was added to each target coated and control well of the plate, and incubated for 1 hour at room temperature on a vibrating platform shaker. After washing the plate with 300 µl per well of PBST, aliquots of 50 µl diluted Anti-Fd-Bacteriophage-HRP (Seramun Diagnostica GmbH) (1:1000) was added to each

well, and incubated for 1 hour at room temperature. The unbound phage was washed as described above, followed by the addition of 50 µl per well of TMB (SeramunBlau® fast TMB/substrate solution, Seramun). The reaction was allowed to develop for 3-4 minutes prior to measuring the absorbance at 620 nm using a microplate reader Multiskan Ascent (Thermo Scientific).

2.5 Protein-protein interaction studies

2.5.1 ELISA analysis with purified protein

Target proteins (5-10 ng) were absorbed onto Immuno 96 Microwell™ Nunc MaxiSorp ™ plate wells overnight at 4°C. Plates then were blocked by adding 100 µl per well of 3 x blocking buffer at 37°C for 4 hours without shaking. Biotinylated target-binding Affimers (5-10 µg) per well in phosphate-buffered saline with Tween 20 (PBST) containing 2 µl blocking buffer were incubated in wells for 1 h with shaking. Plates were washed 3 x in PBST, and streptavidin conjugated to HRP (Invitrogen) diluted 1: 1000 in 50 ml PBST was added for 1 h. After washing, Affimer binding to targets was detected with 50 µl TMB (Seramun) and the absorbance measured at 610 nm.

2.5.2 Analytical size exclusion chromatography

All experiments were performed at 4°C. Purified FusB or FusC (3mg), were incubated with FusB/C-binding Affimer (10 mg) in a final volume of 2 ml, for one hour. Reaction mixtures were injected into a S75 (16/60) pre-backed column (GE Healthcare), and eluted in running buffer (50 mM NAH2PO4, 250 mM NaCl, 10% glycerol and 1mM DDT [pH7.4]). PBP2 and PBP2a at concentrations of 2 mg/ml were bound to their cognate ligand (10 mg), in a total reaction volume of 2

ml, for 1 hour. Samples were run through HiLoad 26/600 Superdex 200 pg pre-backed column (GE Healthcare), and eluted in the same buffer except containing 1 mM DDT.

2.5.3 Isothermal titration calorimetry

ITC experiments were carried out using the isothermal titration calorimeter Microcal 200 (MicroCal, USA), with a cell volume of 200 µl. The optimal mixing speed was 750 rpm. Each protein and its cognate ligand was titrated at 25°C in degassed 50 mM NAH2PO4 buffer (pH 7.4) comprising 300 mM NaCl and 10% glycerol. Target proteins were used at concentrations of 40-70 µM with ligands (FusB, FusC, PBP2 and PBP2a) at 400-700 µM. Volumes of 0.5 µl and 19 x 2 µl injections of ligands were added to target proteins at intervals of 2 minutes. The data point from the first injection was discarded before data analysis, data was analysed using Origin (OriginLab).

2.5.4 Crystallisation trials

Crystal screens JCSG Core (I, II, III, IV) from Qiagen were evaluated for their ability to provide conditions appropriate for crystallisation. Conditions were tested in sitting drops (0.4 µl drops) using the vapour diffusion reagent (60 µl reservoir volume). 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 inspected after one day and then observed at regular intervals for up to two months.

2.6 Investigating the activity of target-binding Affimers in *S. aureus*

2.6.1 Re-sensitization of *S. aureus* to fusidic acid by endogenous expression of Fus B/C binding-Affimers

2.6.1.1 Broth microdilution

Constructed fusidic acid resistant strains of *S. aureus* (one expressing FusB and the other one expressing FusC) were electroporated with plasmid constructs encoding FusB/FusC Affimers and unrelated Affimer (SUMO Affimer) (Tiede et al., 2014), as a negative control. Cultures of these strains and final inoculums were prepared according to the guidelines previously defined (Wiegand et al., 2008). A single colony of each strain was picked from a fresh agar plate and used to inoculate Mueller-Hinton Broth (MHB) supplemented with 10 µg/ml chloramphenicol. Cultures were grown at 37°C with aeration until an OD_{625} corresponding to 1 x 10^8 cfu ml^{-1} was reached. Cells then were induced with anhydrotetracycline (AT) at final concentration of 25 ng/ml for 30 minutes, to enable expression of Affimers. The cultures were then adjusted to McFarland standard 0.5 and diluted to 1:100 in MHB to reach the final desired inoculum of $5x10^5$ cfu ml^{-1} when distributed to the wells of the microtiter plate, and induced with AT at final concentrations of 25, 50, 75, 100, 125, 150 ng/ml. Cultures were induced in the presence and absence of fusidic acid (FA) at final concentrations of 0.06, 0.125, 0.250, 0.50, 1, and 2 mg/ml. Bacteria treated with FA alone and bacteria with the addition of no drug were included as controls. The antibiotics mupirocin and erythromycin at their MIC against tested strain (0.125 mg/ml) were

also used as control. The plates were covered, sealed and incubated at 37°C with shaking for 18 hours. The effect of Affimers on the bacterial growth was recorded as the lowest concentrations of AT at which no bacterial growth was visible in the presence of FA compared with control.

2.6.1.2 Agar dilution

The bacterial inoculum was prepared and preinduced as described above (2.6.1.1). Preinduced cultures were then diluted to 1:10 to give a final cell density of 10^4 cfu per spot when delivered on to MHA plates using a 21-pin replicator (Wiegand et al., 2008). FA at final concentrations of two fold dilutions down from 2 mg/ml was added to agar plates containing 75 ng/ml AT. Cultures were grown at 37°C for 18 hours.

2.6.1.3 Delivery of FusB/FusC-binding Affimers into *S. aureus* using NanoCargo™-PRO

Selected FusB/FusC dual targeted Affimers were entered in a trial to deliver them (as purified protein) into constructed FA resistant strain of *S. aureus* (RN4220 *fusC*$^+$). The nanoparticles, NanoCargo™-PRO, was purchased from Tecrea Ltd, (UK) and used to formulate these Affimers. The first step in this process was to investigate the effect of NanoCargo™-PRO on bacterial growth, and in order to do so, the MIC of this agent against tested strain was determined. Serial, two fold dilutions of the NanoCargo™-PRO were performed in a total volumes of 100 µl PBS. Cultures of RN4220 *fusC*$^+$ strain were prepared as described above (2.6.1.1), and bacterial inocula of $5x10^5$ cfu/ml were incubated with

NanoCargo™-PRO at final concentrations of 0.06, 0.125, 0.250, 0,50, and 1µg/ml in a 96 well plate. Cultures then were grown at 37°C with aeration for 18 hours, and the MIC of this agent was determined as the lowest concentration that inhibited visible growth.

Formulation mixtures containing different concentrations of nanocin (0.6, 1.25, 2.5, 5 µg/ml) and 5 mg/ml of each Affimer in 30 µl PBS were incubated at room temperature for 30 min. Bacterial cells ($5x10^5$ cfu/ml) of *S. aureus* RN4220 *fusC*$^+$ were incubated with 20 µl of each mixture in a total culture volume of 200 µl in 96 well plate, cells were grown at 37°C for 18 hours. This was performed in the presence and absence of FA at 0.06, 0.125, 0.250, 0.50, 1, and 2 µg/ml.

2.6.2 Determination of the antibacterial effect of PBP2/PBP2a-binding Affimer against *S.aureus*

2.6.2.1 Production of PBP2/PBP2a Affimer from *S. aureus* RN4220 and *S. aureus* USA300 strains

Fresh plates of S*. aureus* RN4220 and the methicillin resistant *S. aureus* USA300 strains containing PBP2/PBP2a-binding Affimer into plasmid pRAB11 were prepared as discribed above (2.6.1.2), and 1 µl aliquots of around 10^4 cfu per spot were delivered to MHA plates containing AT at final concentration of 25, 50, 75, 100 and 150 ng/ml.

2.6.2.2 Testing the effect of purified PBP2/PBP2a-binding Affimer on the growth of *S. aureus* and *S. epidermidis*

The minimum inhibitory concentration (MIC) of Affimer was determined using the broth microdilution technique according to the guidelines previously defined (Wiegand et al., 2008). Bacterial inoculum of 1 x 10^5 colony forming units (CFU) were used to determine the effect of PBP2/PBP2a Affimer on bacterial growth. The Affimer was tested against *S. aureus* SH1000 and a panel of six isolates including five methicillin resistant *S. aureus* (MRSA), one susceptible strain *S. aureus* (MSSA), and species specificity was tested using a methicillin resistant *Staphylococcus epidermidis* strain (Table 2.4).

Table 2.4. *S. aureus* and *S. epidermidis* isolates tested against PBP2/PBP2a Affimer

Strain	Resistance phenotype	Source
S.aureus		
USA300	MRSA	NARSA
MRSA252 ATCC BAA-1720	MRSA	ATCC
Mu50 ATCC 700699	MRSA	ATCC
UAMS-1 ATCC 49230	MRSA	ATCC
N315 NRS70	MRSA	NARSA
MSSA476 ACTT BAA-1721	MSSA	ATCC
S. epidermidis		
RP62A ATCC	MRSE	ATCC
35984		

MRSA = Methicillin resistant *S. aureus*. MRSE = Methicillin susceptible S*. aureus*

2.6.2.3 Testing specificity of dual Affimer for PBP2

To test specificity of selected Affimer for PBP2, the effect of over expression of target protein (PBP2) on the MIC of this Affimer was tested. A recombinant strain of *S. aureus* that enables regulated expression of PBP2 was constructed (Section 2.2.8). MIC of Affimer against this strain, in the presence and absence of inducer (AT) was determined using broth microdilution method as described in Section 2.6.1.1.

2.6.2.4 Investigating combined effect of Affimer and oxacillin

The synergistic effect (Berenbaum, 1989) between PBP2/PBP2a binding Affimer and oxacillin was assessed by checkerboard assay (Orhan et al., 2005). Serial two-fold dilutions of the antibiotic and Affimer to 2x MIC were prepared immediately prior to testing. A 96 well microdilution plate was used and total of 50 µl of Mueller-Hinton broth was distributed into each well. The Affimer was serially diluted along the ordinate, while the antibiotic was diluted along the abscissa. Cultures of *S. aureus* USA300 were adjusted to a 0.5 McFarland turbidity standard in Mueller-Hinton broth (MHB). This was further diluted to achieve a bacterial inoculum of 5×10^5 CFU/ml in each well. Plates were incubated at 37°C for 18 hours with aeration. The resulting checkerboard comprises each combination of two agents with wells that contain the highest concentration of each agent at opposite places. The MIC was identified as the lowest concentration of compound that inhibits visual growth. The fractional inhibitory concentrations (FICs) were calculated as follows: MIC of Affimer in combination/MIC of Affimer alone + MIC of antibiotic in combination/ MIC of

antibiotic alone. The combination is considered synergistic when the FIC is less than or equal to 0.5, indifferent when the FIC is between 0.5 and 2, and antagonistic when the FIC is more than 4 (Orhan et al., 2005).

The same procedures was performed to determine the effect of this combination on growth of methicillin resistant *S. epidermidis* strain (RP62A). Since PBP2/PBP2a Affimer does not inhibit growth of this organism, for calculation of FIC, MIC of Affimer was set to 1500 µg/ml, the highest concentration tested which did not have inhibitory effect on bacterial growth of this pathogen.

2.7 *In vivo Galleria mellonella* killing assay

Wax moth larvae (*G. mellonella*) were from Livefood UJ Ltd (Rooks Bridge, somerest, UK) and were kept on wood chips in the dark at 14°C for no longer than two weeks. Bacterial infection of *G. mellonella* was achieved as described by (Wand et al., 2011), with compound treatment of *G. mellonella* performed according a method described in (Peleg et al., 2009). The concentration of the dual Affimer injected into *G. mellonella* was based on the solubility limit of this protein. All work with *G. mellonella* was performed by Charlotte Hind and Mark Sutton (Public Health England, Salisbury, UK).

3. Inhibition of FusB-type fusidic acid resistance in *S. aureus* with Affimers

3.1 Abstract

This chapter investigated the potential of Affimers to inhibit antibiotic resistance. A well-understood example of antibiotic resistance was chosen; FusB-type fusidic acid resistance in *S. aureus*. This resistance is mediated by FusB-type protein family (FusB or FusC). These proteins bind to elongation factor G (EF-G), the target protein of FA, and promote the dissociation of ribosome•EF-G•GDP complex that is held on the ribosome by FA, thereby allowing protein synthesis to resume. To block this resistance mechanism and thereby resensitise *S. aureus* to the antibacterial action of FA, dual targeted Affimers were generated that bind to and inhibit the action of these resistance proteins. Selected Affimers were able to restore the antibacterial effect of FA when expressed in a recombinant FA-resistant strain of *S. aureus*. Nanocin™-PRO (a nanopolymer) was used to prompt entry of these Affimers (as purified protein) into bacterial cells, however, this appears to be hindered by the intrinsic sensitivity of *S. aureus* to this agent. The ability of selected Affimers to bind both FusB and FusC *in vitro*, with high affinity and a 1:1 stoichiometry, was established using size exclusion chromatography and isothermal titration calorimetry. Although the FusC•Affimer complex could be crystallized, none of the tested crystallization conditions were able to generate crystals suitable for X-ray diffraction.

3.2 Introduction

This chapter investigated whether Affimers can be selected to inhibit resistance proteins and thereby provide useful potentiators of antibiotics against which resistance exists. A suitable system was chosen to be used in this study. This system involved an extensively studied mechanism of antibiotic resistance, the FusB-type fusidic acid resistance (mediated by FusB or FusC proteins) (Tomlinson et al., 2016), in a clinically relevant organism, *S. aureus*.

3.2.1 The concept of pairing an inhibitor of resistance protein with an antibiotic (antibiotic adjuvants)

Adjuvants are compounds that enhance the activity of existing drugs and can eliminate, and even directly inhibit resistance (Bernal et al., 2013, Kalan and Wright, 2011a). Adjuvants are administrated together with antibiotics and therefore are combination drugs. In contrast to antibiotic combinations, antibiotic adjuvants exhibit little or no antimicrobial activity alone (Gill et al., 2015, Worthington and Melander, 2013). Instead, when delivered with drugs, they boost antibiotic activity under specific conditions.

The most successful and clinically used adjuvants to date are the inhibitors of β – lactamases. These enzymes hydrolyse penicillins, cephalosporins, and carbapenems, collectively the most widely used antibiotics in the clinic. β-lactamases are divided into two major classes; (1) Ser β -Lactamases which use the active site Ser in hydrolysis of antibiotic; and (2) metallo β-Lactamases that use active-site Zn^{2+} atoms to activate a water molecule that is positioned for

hydrolysis of the antibiotic (Bush and Jacoby, 2010). β-lactamase inhibitors potentiate the action of the antibiotic by inhibiting the function of these enzymes and thereby restoring the activity of the β-lactam antibiotic against β-lactamase producing pathogens. The first β-lactamase inhibitor/antibiotic drug combination successfully approved for clinical use was clavulanic acid combined with the antibiotic amoxicillin, a combination marketed as Augmentin® (Brown et al., 1976). Clavulanic acid has potent activity against one type of Ser- β-lactamases and has been in clinical use for more than 30 years; however, the efficacy of this compound has been eroded in recent years due to the spread of the inhibitor resistant β-lactamases (Bradford, 2001). More recently, a new class of β-lactamase inhibitor, the diazabicyclooctanes (DABCOs), was introduced into clinical practice. The combination of DABCO avibactam with the cephalosporin ceftazidime (Avycaz) was approved for clinical use by the FDA in 2015 (Wright, 2016).

Inhibitors for aminoglycoside-modifying enzymes and erythromycin ribosomal methylases have also been identified (Feder et al., 2008) (Vong et al., 2012), but none of them has been considered adequately persuasive for further development as antibiotic adjuvants.

In this study, the potential of dual targeted Affimers as potentiators of antibiotic fucidic acid will be explored.

3.2.2 FusB-type fusidic acid resistance in *S.aureus*

FA was introduced into clinical practice in 1962 (Godtfredsen et al., 1962). Since then it has been effectively used as a topical and oral agent to treat various strains of *Staphylococcus aureus* including methicillin-resistant *S. aureus* (MRSA), the most commonly identified antibiotic-resistant pathogen in many countries (Ippolito et al., 2010) (Ferry et al., 2010, Howden and Grayson, 2006). However, FA resistance has dramatically increased rendering this antibiotic ineffective (Cassir et al., 2014).

FA resistance in *S. aureus* is usually the result of acquisition of determinants that have been designated *fusB* and *fusC* (O'Neill et al., 2007, O'Neill and Chopra, 2006). These determinants encode FusB-type proteins (FusB and FusC), both 25 kDa proteins with 42% amino acid sequence identity. The structure of both proteins is very similar (RMSD of 1.4 Å) comprising two-domains with an unusual zinc-binding fold in the C-terminal domain (Figure 3.1. B, C) (Cox et al., 2012),(Guo et al., 2012). FusB and FusC bind EF-G, the target of FA, and protect it from the antibacterial action of the antibiotic (Cox et al., 2012).

EF-G is a 65 kDa translational GTPase, cosisting of five domains (Figure 3.1.A), with an essential role in protein synthesis. Two steps of protein synthesis are catalysed by this protein; first, translocation of tRNAs and mRNA with respect to the ribosomal 30S subunit to make a new mRNA codon available for decoding, and second, splitting the ribosomal post-termination complex. Both of these steps require hydrolysis of GTP by EF-G to provide the energy source, and in both cases FA stops the release of EF-G from the ribosome after GTP hydrolysis, thereby disrupting protein synthysis (Bodley et al., 1969, Hirokawa et al., 2002)

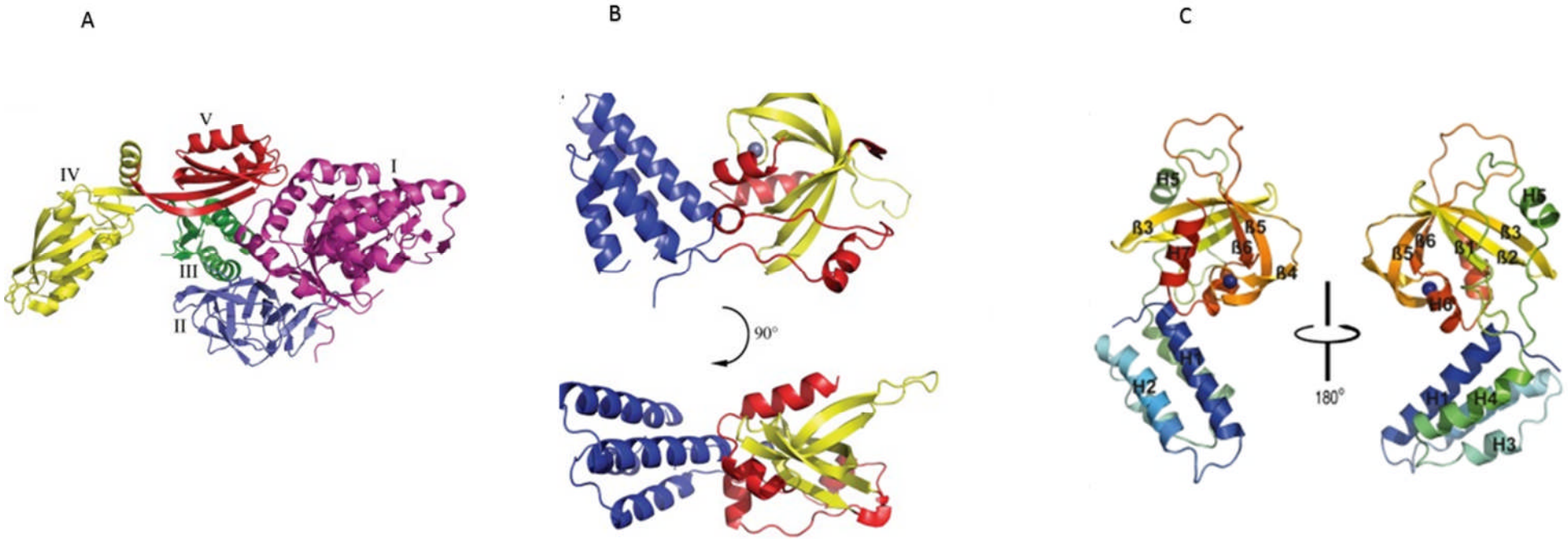


Figure 3.1. (A) Structure of EF-G from *S. aureus*, the five domains are shown in different colours and indicated by roman numerals; (B) FusB structure showing its two domains indicated by (blue) and (red ,yellow) respectively (Guo et al., 2012). (C) structure of FusC, revealing similar structure to FusB, the α-helices and β-strands constructing the protein are labeled (Cox et al., 2012). the zinc ion is shown as a purple sphere for both FusB and FusC.

3.2.3 Mechanisms of FusB/FusC mediated FA resistance

As mentioned earlier, resistance proteins FusB and FusC bind EF-G on the ribosome and enable the release of GDP•EF-G•ribosome complex that otherwise would not be possible in the presence of FA (Cox et al., 2012) (Figure 3.2). The precise mechanism of interaction between the resistance protein FusB and EF-G that leads to dissociation of post-translocation complex is relatively well-understood (Tomlinson et al., 2016). The resistant protein does not compete with FA or with the ribosome for EF-G; the drug and FusB-type proteins bind to their joint target (EF-G) at distinct binding sites, and conformational changes occur in EF-G upon binding to FusB is the basis for resistance to FA. When they come in contact, FusB and EF-G interact with each other through two regions involving the C- and N-terminal domains of the resistance protein to domains 4 and 5 of EF-G, respectively (Tomlinson et al., 2016). Dissociation of EF-G from the ribosome after translocation requires disruption of the contact between domain 4 and the 30S subunit of the ribosome. When FA binds EF-G between domains 2 and 3 it prevents transmission of conformational changes that produced by GTP hydrolysis to domain 4, thereby prevents its release from the ribosome. Binding of FusB to EF-G results in altered conformational flexibility of domain 3 and consequently accelerate the release of the complex from restraint (Tomlinson et al., 2016).

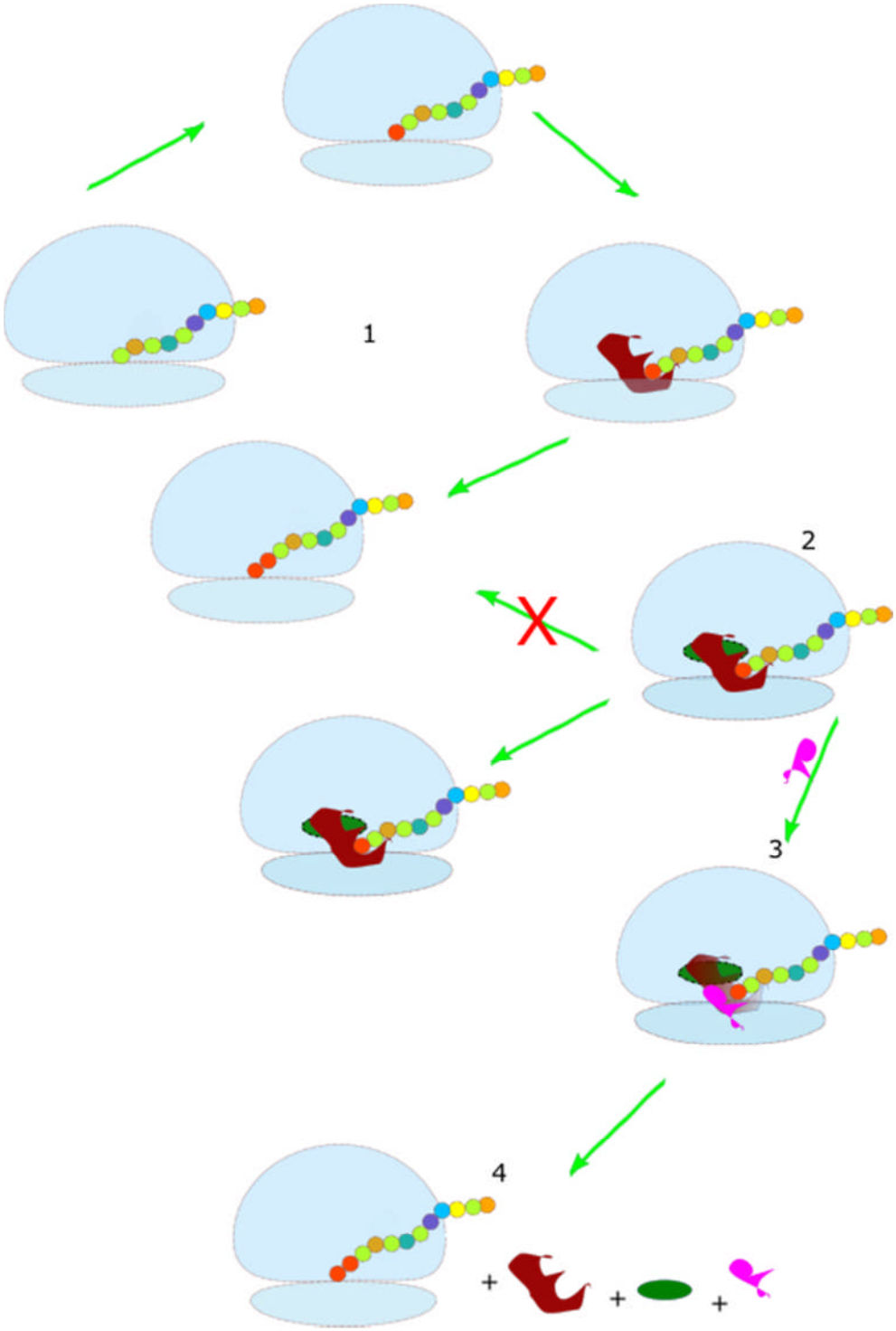


Figure 3.2. FusB mediated FA resistance. (1) EF-G (brown) binds to the ribosome

(light blue) to mediate translocation of amino acids to P and E sites, and then detaches to enable the A site to accommodate next amino acid, (2) FA (green) binds EF-G on the ribosome and prevent its release, (3) binding of FusB (pink) to EF-G promotes the dissociation of stalled complexes and resume protein synthesis. Adapted from (Tomlinson et al., 2016)

In this chapter, a phage display approach was employed to select Affimers that bind to both FusB and FusC and are capable of inhibiting FusB-type FA resistance in *S. aureus*. This approach involved alternative biopanning of Affimer phage library (Tiede et al., 2014, Tiede et al., 2017) on the two target proteins to isolate dual targeted Affimers. (Tiede et al., 2014).

3.3 Aims

The aim of the work presented in this chapter was to generate and characterise dual Affimers for FusB and FusC, with the intention of blocking FA resistance. Binding of isolated Affimers to both target proteins was confirmed by phage ELISA and ELISA with purified protein assays. Microbiological study to test desired function of isolated Affimers was performed by expression of selected Affimers from inside bacterial cells of a FA resistant *S. aureus* strain. Biochemical characterization of interaction between a representative Affimer and both FusB and FusC was also carried out using isothermal titration calorimetry (ITC) and gel filtration chromatography techniques.

3.4 Results

3.4.1 Screening of an Affimer library for target-binding proteins

FusB type proteins (FusB and FusC) were produced and purified from E. coli as previously described (Cox et al., 2012) and approximately 20 mg of highly purified protein was obtained per 1 litre of IPTG-induced cells. Figure 3.3 shows elution profile and SDS-PAGE for purified FusB and FusC. The identity of purified proteins was confirmed by mass spectrometry analysis.

Target-binding Affimers were isolated by screening the whole library (1.3×10^{10}) against both proteins FusB and FusC; each target protein in a separate phage display experiment. After being pre-panned three times to eliminate nonspecific Affimers, the Affimer library was added to immobilized biotinylated FusB or FusC and biobanned for three rounds as described (Tiede et al., 2014), to select target specific Affime

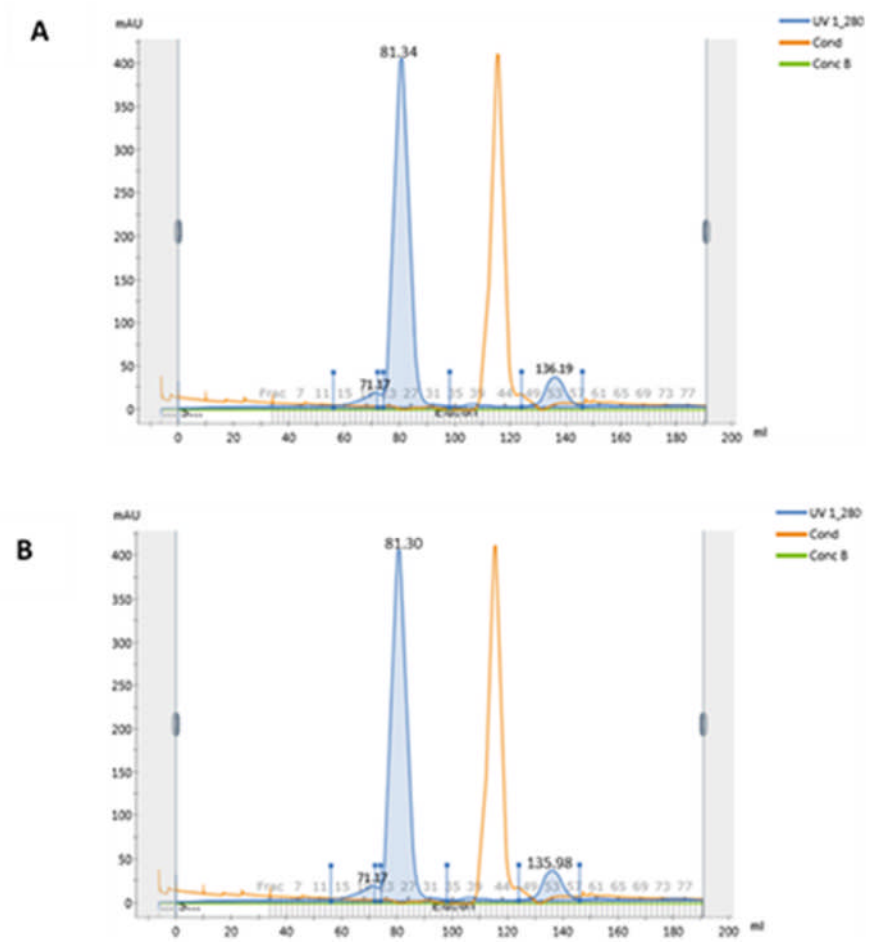


Figure 3.3. Gel filtration elution profile of FusB (A) and FusC (B). (C), SDS-PAGE of eluted FusB; lane 1, FusC; lane 2, (M; protein marker).

3.4.1.1 Progression of phage enrichment

The recovery of amplified phage with increasing rounds of selection was evaluated, to monitor the enrichment of a phage pool specific for the target protein. A functional selection is characterised by an increase in the ratio of target binding phage pool to non-specific phage throughout the course of selection/amplification process. Phage selected from each round of biopanning against both target protein and negative control (blocked wells) was titered as described in section 2.4.4. In the last round of panning for FusB and FusC, the relative enhancement of target specific binding versus unspecific binding was approximately 10^7 fold (Table 3-1).

Table 3.1. Phage display details for FusC and FusB. Output and enhancement calculation for the last round of selection

Target	Output (cfu) against target	Output (cfu) against blocked wells	Enhancement fold
FusC	2×10^{10}	1×10^{3}	1×10^{7}
FusB	3×10^{10}	2×10^{3}	1×10^{7}

3.4.1.2 Confirmation of selected Affimers by phage ELISA

After the last panning round, 48 individual clones for each target protein were grown in 200 µl of 2TY media. Phage then were amplified and tested by phage ELISA assay to confirm binding to corresponding target. For FusC, 37 clones exhibited binding by phage ELISA (Figure 3.4). FusB biopanning with Affimer library resulted in 35 Affimers that were confirmed to bind FusB by phage ELISA (Figure 3.5).

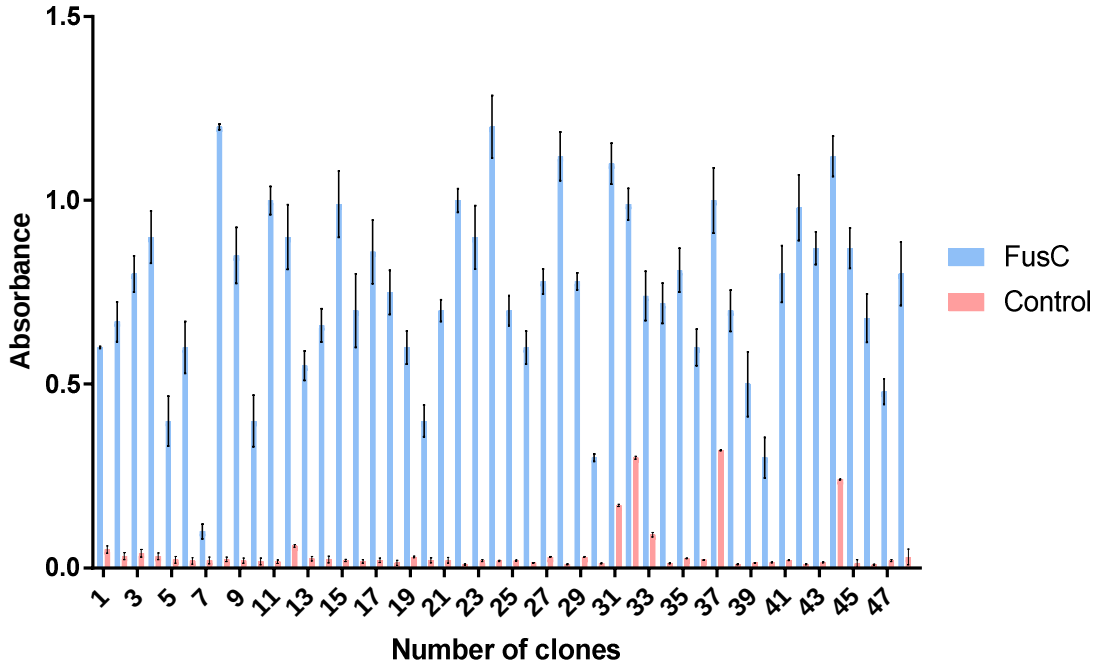


Figure 3.4. Binding of selected clones to FusC by phage ELISA. A total of 37 Affimers are specifically bound to FusC, as indicated by a high signal of absorbance compared to the negative control.

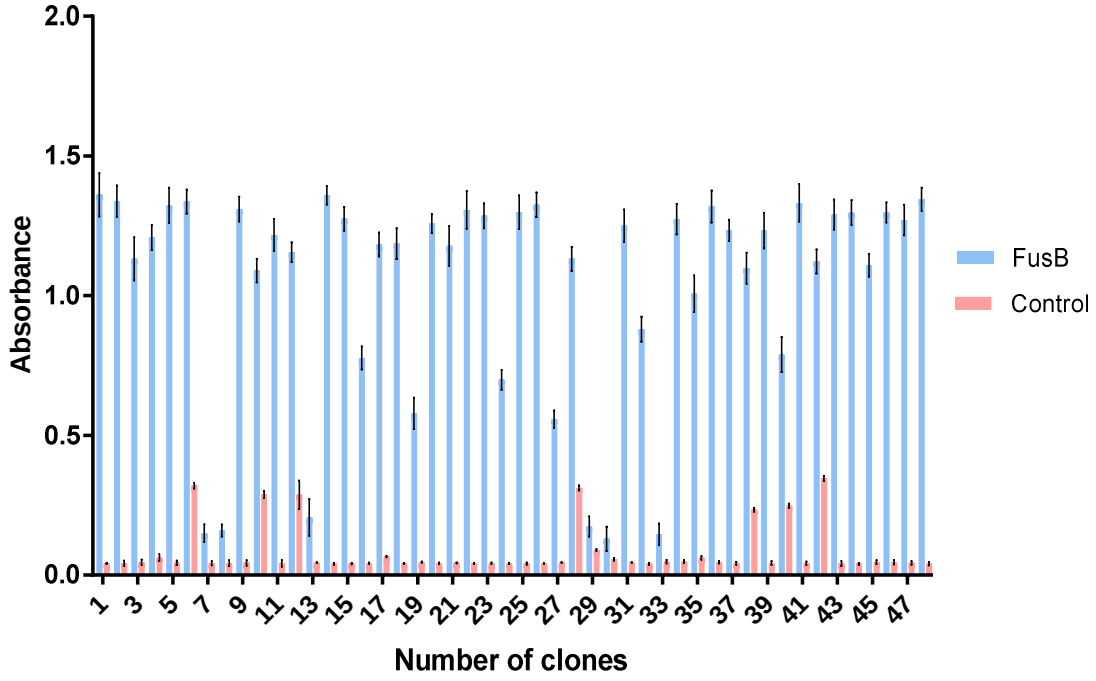


Figure 3.5. Binding of selected clones to FusB by phage ELISA. A total of 35 Affimers are specifically bound to FusB, as indicated by a high signal of absorbance compared to the negative control.

3.4.1.3 DNA sequencing of selected Affimers

DNA sequence analysis of clones selected for FusC (37 clones) established that these clones comprised 20 different Affimers (Table 3.2), whilst of 35 sequenced FusB-binding Affimers, 11 Affimers were distinct (Table 3.3).

Table 3.2. Amino acid sequence of variable loops of FusC Affimers obtained by phage display (20 Affimers)

Representative clone	Sequence		Frequency of appearance (number of clones out of 37)
	Loop 1	Loop 2	
4	ARWYKDYEW	SLKDMFPFK	6
5	HEMNLGAPG	HPHFRKMPW	4
6	REGRVYIYS	GKWAPDFVV	4
2	PTHATLRNG	WRMTRKMFT	3
8	YMPIWKLPP	PHHIHKDKH	2
3	PKYIFKRSV	GPKWDLWIN	2
11	RKFIFRHPH	YYDISYPRA	2
14	KKYIFIAPN	MMYSNMNNR	2
15	IRKYVFKGP	GDIYLWNLV	1
16	PKFVFKMPA	MHLRGYKFI	1
21	IVPGMPVLW	RKAPFKERK	1
25	ETDGKHMWA	NYTLNPIFK	1
27	VMIHAEYHY	NGLHPGPFM	1
30	IKGVKHNLY	NMDIHSKPK	1
34	YSIYTEEGF	MHGFFITGA	1
35	KTHYGKYYT	WILIGNGDY	1
38	ATVMIYFHH	RDKSYPIDA	1
41	KHAVIARDM	WYTGEKYIL	1
46	REGRVYIYS	GKWAPDFVV	1
48	PHMQQYILP	LKWHHGTIV	1

Table 3.3. Amino acid sequence of variable loops of FusB Affimers obtained by phage display (11 Affimers)

Representative clone	Sequence		Frequency of appearance (number of clones out of 35)
	Loop 1	**Loop 2**	
1	KAYAERRGW	GKTPWHGRA	5
3	WHYAVEFTA	GYKMWYVHA	5
5	GNRMNLQKY	APGKVGKPP	4
9	TPRKHAEKG	GPLMEARTA	4
21	QIYKDTIVR	PFKSMWEPQ	4
16	AELKFAHTS	MAGVMRTHQ	3
19	AMFKPQVSP	YSIAVERGA	3
31	AGVKKWMHG	YAGRFLHFG	3
47	WHTYKGFPG	MWKIGHTFA	2
35	EIQQMYEWG	YGQLWVKTI	1
45	AGFQMAKYP	VQKKAPHHI	1

3.4.3 Isolation of Affimers with cross-reactivity to FusB and FusC

To test whether selected Affimers (20 FusC-binding Affimers and 11 FusB Affimers) were capable of binding to the other FusB-type target protein, these Affimers were cross-screened against the other target using phage ELISA. Only one Affimer exhibited the desired function, however, this Affimer had no inhibitory effect on target proteins.

A modified phage display approach was then successfully applied to generate further Affimers able to bind to both FusB and FusC. This approach is detailed in section (2.4.3), and involved using a FusC enriched phage pool (selected from the first round of panning Affimers library on FusC) as input phage to be panned against FusB. After three rounds of biopanning, this process resulted in 31 Affimers that exhibited specific binding to both FusB and FusC by phage ELISA (Figure 3.6). The DNA sequences of these Affimers revealed that they comprised 4 distinct species (Table 3.4).

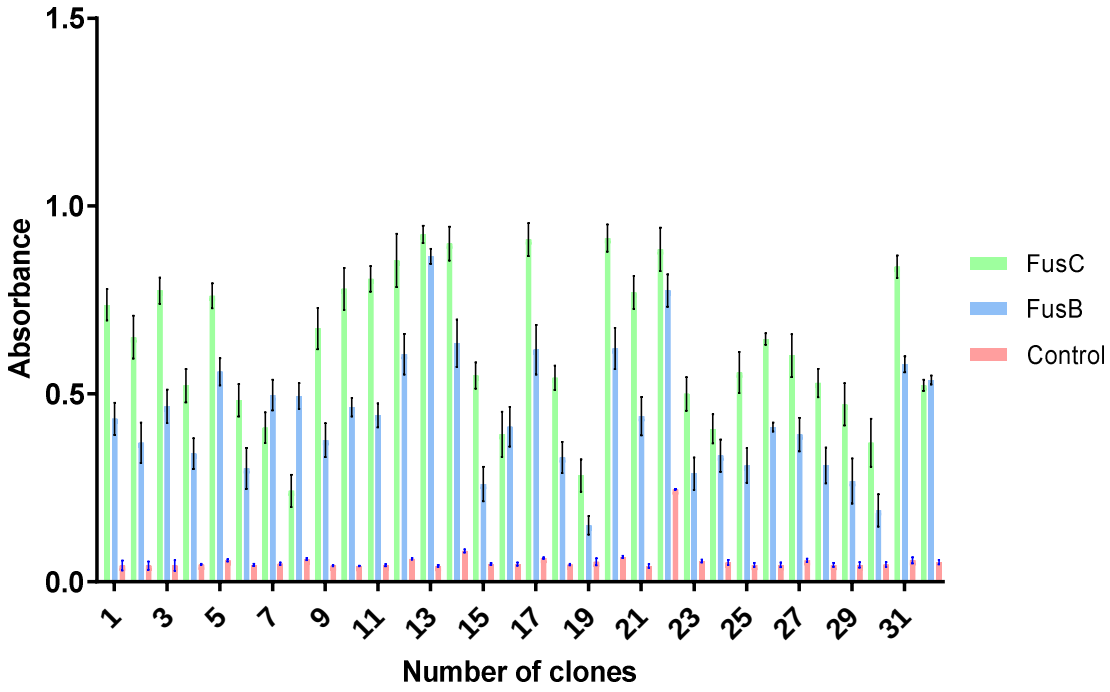


Figure 3.6. Individual FusB/FusC Affimers isolated from FusB biobanning with FusC enriched phage. 32 Affimer tested for their cross-reactivity to FusB (blue) and FusC (green) using phage ELISA. The presence of cross-reactivity was clearly shown by high signal of absorbance measured for target proteins compared to control wells.

Table 3.4. Amino acid sequence of Affimers exhibiting binding to both FusC and FusB

Representative clone	Sequence		Frequency of appearance (number of clones out of 31)
	Loop 1	Loop 2	
1	FVEWAEYDS	QWSGGIYRL	10
2	HMSLPFITD	FAWGASEIF	6
3	PTHQTLRNG	WRMTRHMFE	7
4	PKYIFRDNV	LAKNKKQYV	8

3.4.4 Construction and evaluation of FA resistant strains of *S. aureus*

To evaluate the activity of selected dual targeted Affimers, and to determine whether these proteins are capable of inhibiting FA resistance mediated by FusB and FusC, expression of selected Affimers in FA resistant strains of *S. aureus* was performed. In this experiment, two stable FA resistance strains of *S. aureus* (RN4220 *fusB*$^{+}$ and RN4220 *fusC*$^{+}$) that constitutively produce resistant proteins were generated as described in section 2.2.8; one strain expressing FusB, and the other strain, FusC.

To determine the ability of these strains to continuously produce FusB and FusC, and thereby resist the antibacterial action of antibiotic FA, the MIC of FA against constructed strains and the wild type strain (*S. aureus* RN4220) was determined. The results showed that FA MIC of both recombinant strains was 16 fold higher than that of the parent strain (2 µg /ml versus 0.125 µg /ml).

3.4.5 Expression of FusB/FusC binding Affimers in FA resistant *S. aureus* (RN4220 *fusB*$^{+}$ and RN4220 *fusC*$^{+}$)

To test whether restoration of FA antibacterial action against *S. aureus* can be achieved via intracellular expression of an Affimer, DNA sequences encoding FusB/FusC Affimers were cloned in the staphylococcal expression plasmid pRAB11 (Section 2.2.8), RN4220 *fusC*$^{+}$ and RN4220 *fusB*$^{+}$ strains were then transformed with resulting plasmids. Cultures of these strains harboring plasmids containing FusB/FusC Affimers, empty plasmid and plasmid containing an unrelated Affimer (SUMO Affimer) (Tiede et al., 2014), as

negative controls, were grown to early log phase and preinduced with anhydrotetracycline (AT) to induce expression of these Affimers. All induced cultures reached approximately the same level of growth after 30 mintues of induction at 37°C. After being preinduced, cells were grown for 18 hours on MHA containing inducer at a final concentration of 75 ng/ml, in the presence or absence of FA.

The results showed that expression of each of the four Affimers was able to restore the antibacterial action of FA against both strains (RN4220 *fusB*$^{+}$ and RN4220 *fusC*$^{+}$), and there was no effect of these Affimers on the MIC of antibiotics used as control (mupirocin and erythromycin). Figure 3.7 shows expression of these Affimers from a resistant strain that constitutively express FusC from the chromosome (RN4220 *fusC*$^{+}$). Comparable results were obtained for a recombinant FA resistant strain that continuously expressed FusB (data not shown).

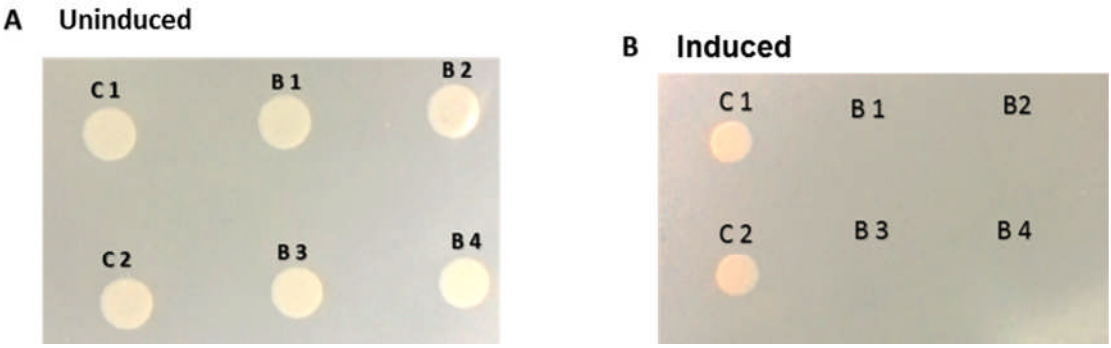


Figure 3.7. The effect of FusB/FusC Affimers in FA resistant *S.aureus*. In the presence of 0.125 µg/ml FA (A), combination of 0.125 µg/ml FA and 75 ng/ml AT was

added (B). C1; (control 1), FA resistant *S. aureus*, C 2; (control 2), FA resistant *S. aureus* harboring unrelated Affimer (SUMO Affimer) on plasmid pRAB11, and B1-B4; four FusB/FusC Affimers.

3.4.6 Delivery of FusB/FusC Affimers into *S. aureus*

Having shown that FusB/FusC Affimers were able to abrogate FusB-type resistance and restore the antibacterial effect of FA when expressed in FA-resistant *S. aureus* strains, I sought to determine whether entry of these molecules (as purified proteins) into cells could be achieved. In an attempt to overcome the cytoplasmic membrane barrier and promote entry of these molecules into bacterial cells, Nanocin™-PRO ,a cationic nanopolymer that has been developed for delivery of macromolecules into cells (Ridden and Good, 2016) was used as described in section 2.6.1.3. Results from this experiment demonstrated that Nanocin™-PRO itself has an inhibitory effect on growth of bacterial cells, even when small amounts of the agent were added to the cells; the MIC of this agent was 0.250 µg /ml, with some growth inhibition noticed at 0.125 µg /ml. Figure 3.8 shows a representative cell entry trial for one Affimer into a FA resistant strain of *S. aureus* (RN4220 *fusC*$^{+}$), where a formulation mixture containing 100 µg of Affimer was mixed with Nanocin™-PRO at different concentrations (to monitor the inhibition of bacteria growth that caused by addition of this agent) was added to bacterial cells. These results shown that Nanocin™-PRO had no effect on bacterial growth only when it was used at 0.06 µg/ml, which is probably insufficient for delivery of Affimer molecules enough for inhibiting target proteins.

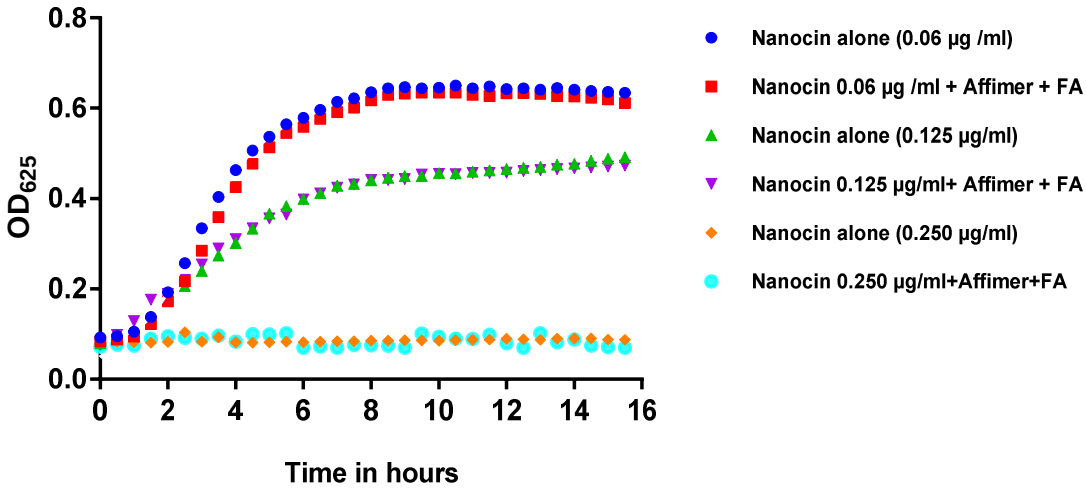


Figure 3.8. Delivery of FusB/FusC Affimers into FA resistant *S.aureus* using Nanocin™-PRO. After addition of formulation mixture, cells were grown at 37 ºC for 16 hours, and the optical density at 625 nm was monitored every 30 minutes. This trial was performed to deliver 100 µg protein along with 0.250 µg/ml FA combined with different concentrations of entry prompting agent. Nanocin alone was used as control.

3.4.7 Further characterization of FusB/FusC Affimers

3.4.7.1 Detection of FusB and FusC by ELISA with purified Affimers

To further confirm binding of dual targeted Affimer to their target proteins, binding of purified FusB/FusC Affimers to FusB and FusC was tested by ELISA, where microtitre wells were coated with purified FusB, FusC and negative control protein (for determining specificity of these Affimer for their target proteins). The biotinylated Affimers and a non-related Affimer (SUMO Affimer as a negative control), were added, and bound protein was detected by streptavidin conjugated to HRP and visualized by TMB. The results shown that FusB/FusC Affimers specifically bound FusB and FusC; control (non-related Affimer) did not bind these proteins, and these Affimers did not detect negative control protein, suggesting that dual targeted Affimers were specific for their target proteins (Figure 3.9).

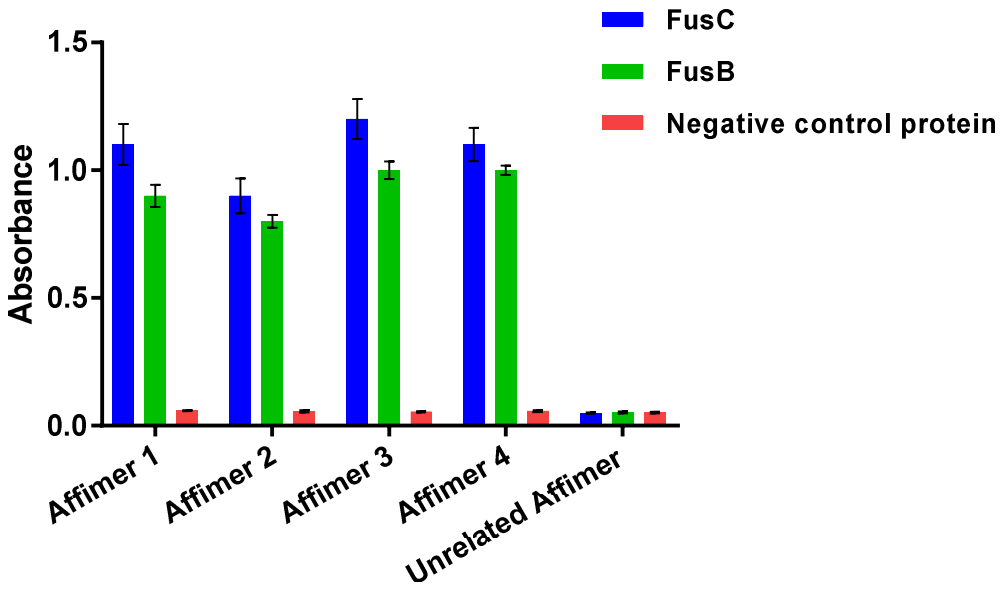


Figure 3.9. ELISA assay to confirm binding of purified FusB/FusC Affimers to their target protein. Biotinylated Affimers were used to detect FusC (blue), FusB (green), and control protein (red). TMB product visualised at 560 nm.

3.4.7.2 Analytical gel filtration chromatography

To determine the ability of selected dual Affimers to bind their target proteins in solution and identify quantities of molecules involved in binding, interaction between these Affimers and target proteins was analysed by gel filtration chromatography. FusB/FusC Affimer (10 mg) was incubated with FusC or FusB (3 mg), a molar ratio of 5:1 (to allow excess amount of Affimer) at 4°C for about 2 hours to allow complete binding of the Affimer to its target protein. The sample then was passed through gel filtration column and the results indicated that FusB/FusC Affimer bound to FusC with 1:1 stoichiometry. The chromatogram has two major peaks, the first peak consists of FusC•Affimer complex as it has higher molecular weight than the second peak which corresponds to unbound FusB/FusC Affimer (Figure 3.10). Results of FusB/FusC Affimer binding to FusB were consistent with the results shown for FusC.

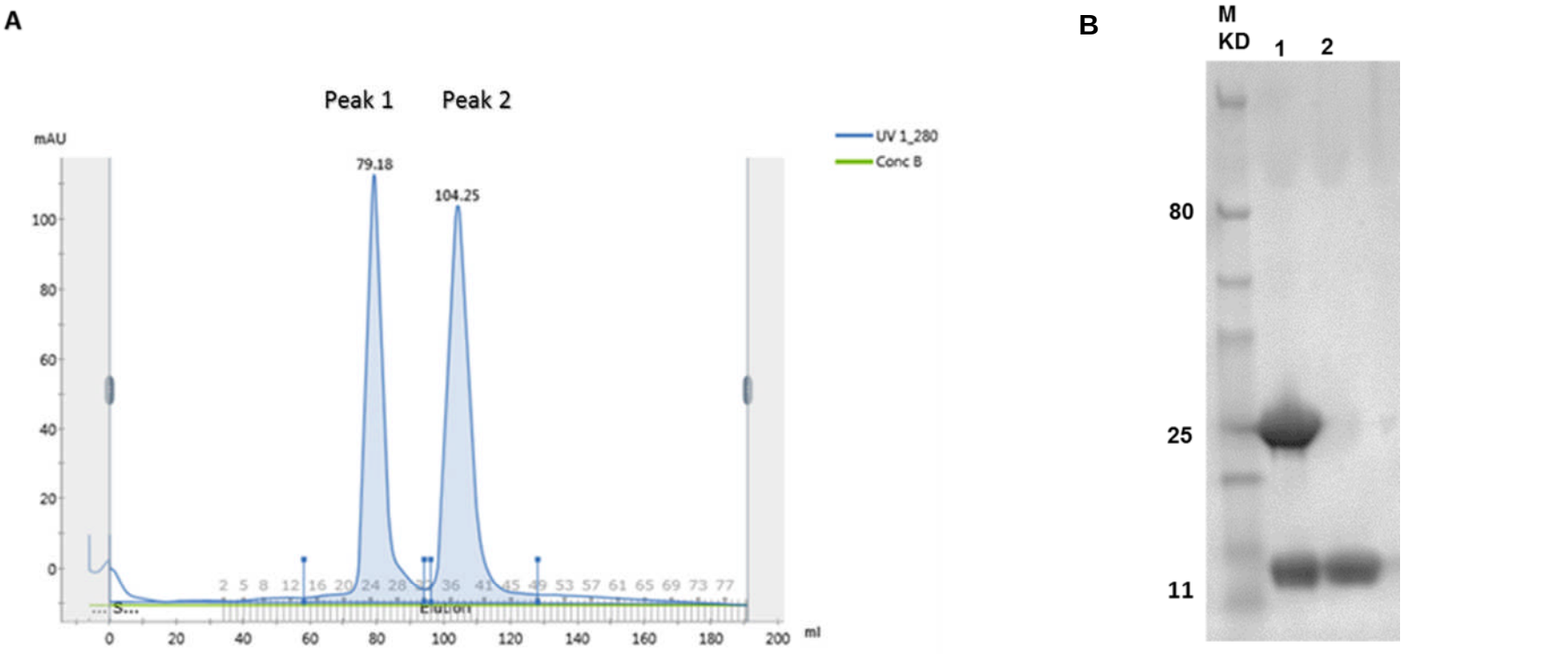


Figure 3.10. Analytical gel filtration chromatography analysis of the interaction between FusC and FusB/FusC Affimer. (A) The chromatogram of FusB/FusC Affimer•FusB; peak 1 represents the eluted complex and peak 2 excess Affimer. **(B)** SDS-PAGE of the fractions; lane 1; marker, lane 2; eluted complex, and lane 3; represent excess Affimer. Lane 2 and lane 3 correspond to (peak 1) and (peak 2) respectively.

3.4.7.3 Isothermal titration calorimetry (ITC)

ITC experiments were conducted to gain further insight into the interaction between the FusB/FusC Affimer and its target proteins (FusB and FusC).

Figure 3.11 shows base-line corrected heat peaks and integrated peaks of a representative titration of FusB/FusC Affimer with FusB and FusC, respectively, fitted with a single-site model, and the mean of triplicate determination of thermodynamic data are given in Table 3.5. A triplicate titration of FusC with FusB/FusC Affimer revealed that the protein bound with high affinity (Kd = 33.3 ± 3 nM, ΔG = - 42.67 ± 1 kJ mol^{-1}), unfavourable entropy (TΔS = - 5.78 ± 1 kJ mol^{-1}) compensated by an increase in enthalpy (ΔH = - 48.45 ± 2 kJ mol^{-1}), compared with FusB, which bound the Affimer with slightly lower affinity (Kd = 83.5 ± 3 nM, ΔG = - 40.55 ± 1 kJ mol^{-1}) and favourable enthalpy and entropy (ΔH = - 26.35 ± 3 kJ mol-1 TΔS = 14.2 ± 2 kJ mol-1). The ITC data established that both complexes have a 1:1 stoichiometry.

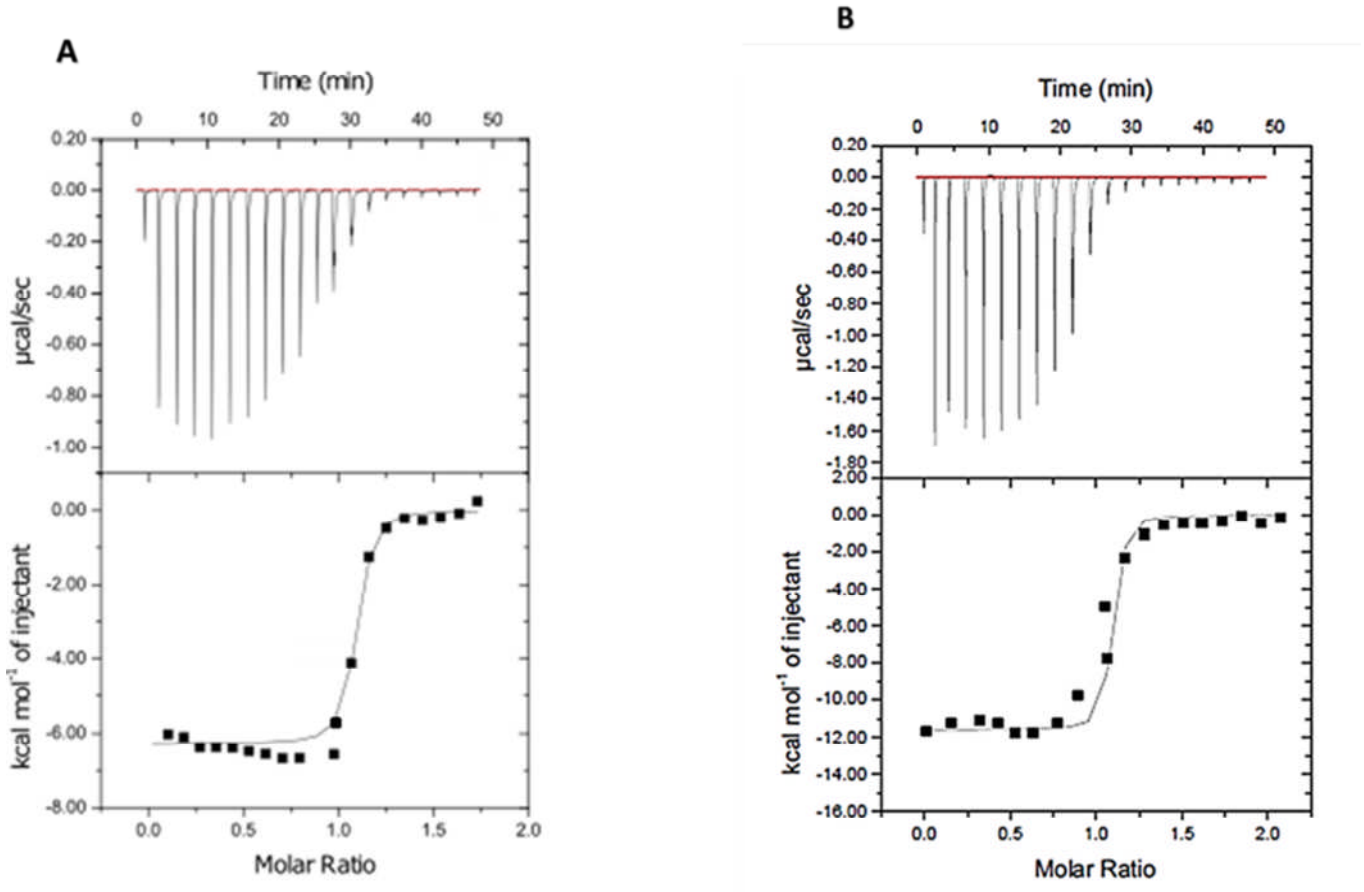


Figure 3.11. ITC analysis of FusB/FusC binding Affimer with FusB and FusC. ITC data of representative Affimer titrated with FusB (A) and FusC (B)

Table 3.5. Thermodynamic profile of FusB/FusC Affimer binding FusB and FusC

Interaction	Kd/nM	ΔH/kJ mol^{-1}	N	-TΔS/kJ mol-1	ΔG/kJ mol-1
FusB•Affimer	83.3 ± 5	- 26.35 ± 3	1.06 ± 0.03	-14.2 ± 2	- 40.55 ± 1
FusC•Affimer	33.3 ± 3	- 48.45 ± 2	1.05 ± 0.02	5.78 ± 1	- 42.67 ± 1

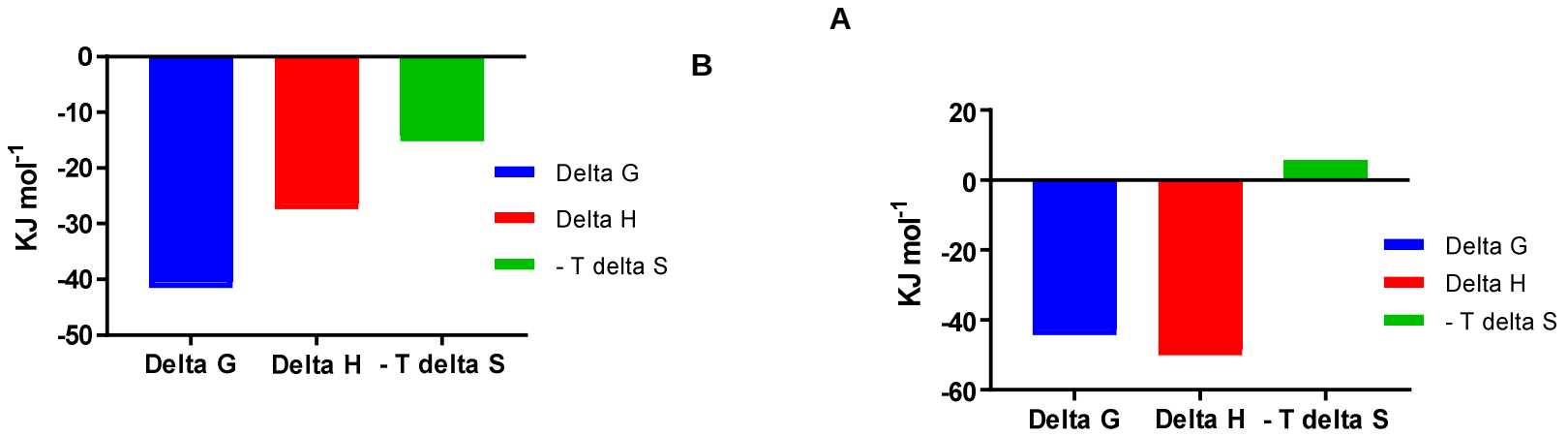
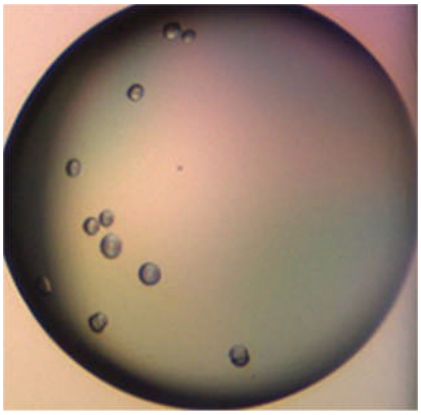
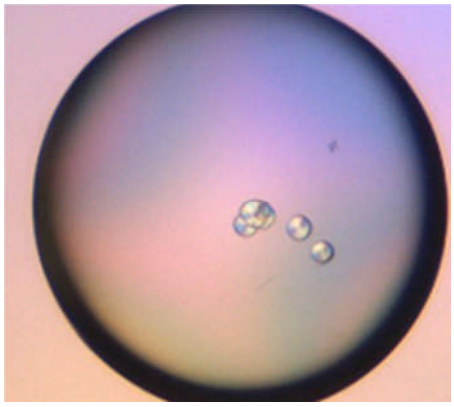


Figure 3.12. The binding signature (free energy, binding enthalpy, and entropy factor) plotted for the binding of FusB/FusC Affimer to FusB (A) and FusC

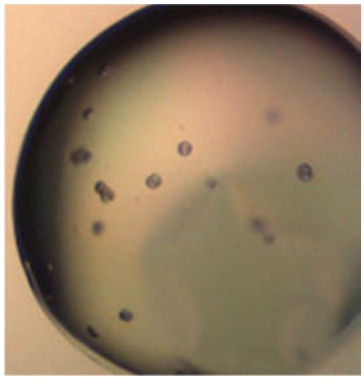
3.4.7.4 Crystallization trials of the FusC•FusB/FusC Affimer complex

In an attempt to perform structural studies of the dual targeted Affimer in complex with its target protein, concentrated protein complex was used to set up four commercially available crystallisation screens as described in Section 2.5.4. Crystallisation conditions, including protein concentration and ratio of crystallisation buffer to protein in each drop were varied for each screen. Phase separation (Figure 3.13. A) and spherulites (Figure 3.13. B) were produced under several conditions. The conditions that gave phase separation and spherulites were optimised (one variable at a time) by decreasing the concentration of precipitant in the well, lowering the temperature, increasing well volume and by vortexing these crystals and using them as seeds for new screen. However optimisation of these conditions resulted only in small crystals (Figure 3.13.C) that were too small and not suitable for x-ray diffraction, optimization of conditions that gave these crystals did not result in any crystals.





C



A B

Figure 3.13. Crystallization trials of FusC•FusC/FusB Affimer complex. Phase separation formed in (30% w/v PEG 6K precipitant, 0.1 M N,n-Bis (2-Hydroxyethyl) glycine (BICINE) 8.5 PH) (A), spherulites produced from a well containing 40% v/v PEG 400 precipitant, 0.1 M MES 6PH (B) , and small crystals formed in LiCI (salt), 0.1 M N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES) 7 PH, 10% w/v PEG 6K precipitant (C).

3.5 Discussion

Using phage display, five Affimers exhibiting cross-reactivity to both FusB and FusC were selected. Standard phage display of the Affimer library as described by Tiede (Tiede et al., 2014) resulted in many Affimers for each target protein; however, only one of the selected Affimers exhibited cross-reactivity to both protein targets. Conventional methods of phage-display by panning libraries on immobilized purified protein, followed by extensive washing steps in detergent-supplemented buffers, results in selection of binders that are highly specific for their targets (Derda et al., 2011). The results presented here strongly suggest that this method is poorly effective for selecting binders for dual targets due to the loss of these binders during the course of biopanning against one target. As the goal of this study was to select dual targeted Affimers, a modified strategy for phage display was successfully established to generate Affimers with the desired function, involving alternative biopanning of FusB and FusC with the Affimer phage library.

Since binding of a protein to its target does not equal inhibition of this target, selected Affimers were initially tested for their ability to bind and inhibit protein target inside bacterial cells. This study demonstrated that intracellular expression of four selected FusB/FusC Affimers in a FA resistant strains of *S. aureus* blocked FusB-type FA resistance in this organism; that is, they were able to resensitize these strains to the antibacterial action of FA. Upon

induction, the growth of FA resistant strains of *S.aureus* harboring Affimers was only inhibited in response to the addition of FA, with no detectable inhibition of growth in the absence of antibiotic. By contrast, expression of an unrelated Affimer did not have any effect on growth in either the presence or absence of FA. Thus, the growth defect seen in response to expression of FusB/FusC Affimers, in the presence of FA, is consistent with inhibition of resistance proteins (FusB and FusC) that renders this antibiotic active once again.

Although the idea of inhibiting antibacterial resistance has been previously established and some antibiotic adjuvants are already in clinical use (Kalan and Wright, 2011b), to date, artificial binding proteins have not been used as antibiotic potentiators. To our knowledge, the only phage displayed biological agent that has been investigated for inhibiting an antibiotic resistance protein is the β-lactamase inhibitory protein (BLIP), where derivatives of (BLIP) with high affinity for β-lactamase were obtained (Huang et al., 2000).

This work demonstrated that Affimers can mediate inhibition of FusB-type FA resistance. Even though delivery of these molecules into bacterial cells has not been achieved, this study may highlight the usefulness of these Affimers as surrogate ligands for detection of small molecule inhibitors. A previous study has successfully used phage displayed peptides in a rapid binding assay to identify small molecule inhibitors for enzyme function (Hyde-DeRuyscher et al., 2000).

Biochemical characterization studies with purified protein were performed to gain further insight into the interaction of selected Affimers with FusB and FusC. My work has shown, for the first time that one Affimer can be selected

to bind two proteins from the same family, with comparable affinity and stoichiometry. The comparable affinities of this Affimer for FusB and FusC strongly suggests that conserved residues are involved in complexation. The thermodynamic profiles obtained from ITC experiments revealed that the interactions between the dual targeted Affimer and both target proteins are enthalpically driven (Figure 3.12); an enthalpically driven interaction suggests that binding of this Affimer to its target proteins is governed by the formation of hydrogen bonds (Holdgate and Ward, 2005). Specific interactions are expected to produce more negative enthalpy, and are less favorable entropically (Reynolds and Holloway, 2011).

Data obtained from ITC showed that FusB and FusC bind their dual Affimer with comparable affinities to their affinities for EF-G. These proteins bind EF-G with a Kd value of 63.0 ± 5 nM and 25 ± 2 nM, respectively (Cox et al., 2012), whilst, their affinities for the Affimer were 83 ± 3 nM and 33 ± 3 nM, respectively. This, together with the percentage of amino acid sequence identity between FusB and FusC (42%), and the location of identical residues predominantly in regions critical for their interactions with EF-G (Tomlinson et al., 2016), suggests that this Affimer is most likely to compete directly with EF-G for binding to the resistance proteins, rather than allosterically mediating inhibition of resistance protein binding to EF-G.

Although crystallisation trials did not produce crystals suitable for X-ray diffraction, structure studies could, in future, be performed using cryo-EM technique by fusing Affimers to a homo-oligomeric scaffold to generate suitable protein size to allow a cryo-EM analysis for the complex (Coscia et al., 2016).

This study highlights the potential of Affimers in targeting antibacterial resistance to thereby achieve inhibition of the resistance mechanisms that they mediate.

4. Affimer mediated inhibition of PBP2 and PBP2a in *Staphylococcus aureus*

4.1 Abstract

Having shown Affimers can be isolated that inhibit antibiotic resistance to potentiate the action of antibiotics against which resistance exists (Chapter 3), this chapter investigated the potential of these proteins as antibacterial agents in their own right. Affimers were used as new tool to revisit “old” antibacterial targets in *S. aureus*; the penicillin binding proteins (PBPs), which are the bacterial target of the β-lactams. PBPs are membrane-associated proteins that have an essential role in the biogenesis of bacterial peptidoglycan (PG), a major component of the bacterial cell wall, and inhibition of these proteins leads to bacterial cell lysis and death. *S. aureus* has four penicillin binding proteins (PBP1-PBP4), of which only PBP2 is essential. However, methicillin resistant *S. aureus* (MRSA) strains have acquired an additional PBP (PBP2a), which mediates resistance to β-lactams. Work presented in this chapter reports, for the first time, selection of an Affimer that binds and inhibits the action of both PBP2 and PBP2a in *S. aureus*. This Affimer was able to inhibit methicillin sensitive (MSSA) as well as methicillin resistant *S. aureus* when it was endogenously expressed in, or when exogenously added to bacterial cells. Isothermal titration calorimetry

(ITC) and gel filtration chromatography techniques established that selected Affimer interacts with its target proteins with high affinity and 1:1 stoichiometry.

4.2 Introduction

Chapter three reported isolation and characterisation of dual targeted Affimers for inhibiting antibiotic resistance mediated by intracellular resistant proteins. The inhibitory activity of isolated Affimers was demonstrated through their expression inside bacterial cells. Since delivery of Affimers into bacterial cells has not been achieved, it was therefore of interest to investigate whether Affimers (inhibitors) may be selected for extracellular, essential target proteins in the same organism. As a Gram-positive bacterium, *S. aureus*, is protected by a single cytoplasmic membrane layer followed by a thick, rigid cell wall called peptidoglycan (PG) (Silver, 2016). Peptidoglycan is an irregular, mesh-like macromolecule that located outside the cytoplasmic membrane, and in Gram positive bacteria presents the outer layer of the cell envelope. This central element of the bacterial cell envelope is crucial for preserving cellular integrity by maintaining the structure of cell wall and protecting it from the effect of osmotic pressure. Formation of new cell walls is critical prior to progression to division process, and failure of synthesis of functional walls leads to a serious inhibition of cell division and growth (Teo and Roper, 2015). Biosynthesis of PG involves several stages, both in and outside the cytoplasm, with the last steps taking place on the cell

membrane mediated by the penicillin binding proteins (PBPs), the bacterial target of β-lactam antibiotics (Leski and Tomasz, 2005). The accessibility and previous validation of PBPs as antibacterial targets make them an excellent target for Affimers.

4.2.1 Peptidoglycan biosynthesis

The biosynthesis of peptidoglycan involves three major stages. The first stage takes place in the cytoplasm where a series of enzymes mediate synthesis of uridine diphosphate-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide). The first step in this process includes conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-N-acetylmuramic acid (UDP-MurNAc) by the MurA and MurB enzymes. Next, is the formation of the final cytoplasmic peptidoglycan precursor, UDP-MurNAc-pentapeptide by addition of the pentapeptide stem to the MurNAc through successive action of the enzymes MurC-MurF (Broughton et al., 2016). The composition of the stem peptide varies between bacterial species, but is normally l-Ala–γ-d-Glu–diaminopimelate–d-Ala–d-Ala in Gram-negative bacteria and l-Ala–γ-d-Glu–l-Lys–d-Ala–d-Ala in Gram-positive bacteria such as staphylococci (Giesbrecht et al., 1998). The second stage is initiated by transferring the phosphorylated MurNAc-pentapeptide precursor to the membrane-embedded acceptor, undecaprenyl-phosphate, resulting in undecaprenyl-pyrophosphoryl-MurNAc (lipid I), a step catalysed by MurY. Lipid I is then glycosaminylated by MurG and receives a GlcNAc from UDP-GlcNAc to yield undecaprenyl-

pyrophosphoryl-MurNAc-pentapeptide-GLcNAc (lipid II), with the release of UDP (Bouhss et al., 2008, Broughton et al., 2016). Lipid II is then translocated to the outer surface of the cytoplasmic membrane, where the last stage of peptidoglycan synthesis takes place. This stage is catalysed by penicillin binding proteins (PBPs), and can be further divided in to two main reactions. Transglycosylation, in which synthesis of the glycan chain occurs, is a step catalysed by a glycosyltransferase (GT) that assembles lipid II (N-acetylglocosamin and N-acetylmuramic acid-pentapeptide core) (Wright, 2007). Transpeptidase (TP) catalyses the subsequent step, the transpeptidation reaction, cross-linking these carbohydrate polymers to each other by formation of peptide ponds between polymer strands providing the wall with more rigidity (Figure 4.1) (van Heijenoort, 2001). In the case of *S. aureus*, the position 4 d-Ala on one peptidoglycan strand is linked to pentaglycine extension attached to the position 3 L-Lys of the peptide stem of another (Giesbrecht et al., 1998).

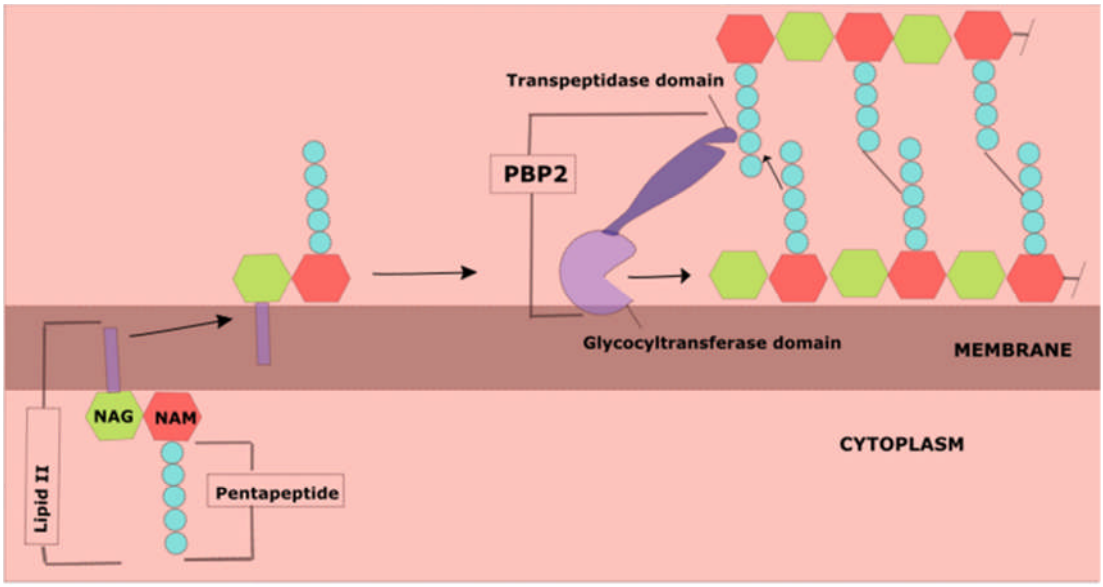


Figure 4.1. PBP2 contribution in peptidoglycan biosynthesis. TP is located outside of the cell, where it can find its substrate (the pentapeptide chains). GT is interacted with the membrane where lipid II is found. Adapted from (Wright, 2007)

4.2.2 Classification and overview of PBPs

Bacteria have a distinct number of PBPs, and the availability of whole genome sequencing has made it possible to determine the number of PBPs in each species. PBPs have been grouped into two main classes, the high molecular mass (HMM) PBPs and the low molecular mass (LMM) PBPs. HMM PBPs are essential for cell survival and represent the major target for β-lactams; they are multifunctional proteins responsible for both transpeptidation and transglycosylation. These proteins consist of two domains joined by a linker positioned on the outer surface of the cytoplasmic

membrane where peptidoglycan synthesis takes place, they also have a cytoplasmic tail and transmembrane anchor (Sauvage et al., 2008). HMW PBPs are further subdivided to two groups (A and B), depending on the structure and function of their N-terminal domain. The C-terminal domain, penicillin-binding (PB) domain, of both class A and class B is responsible for their transpeptidase activity where the adjacent glycan chains are cross-linked. The N-terminal domain has two different functions; in class A, it has glycosyltransferase activity, catalysing the formation of glycan chains, while in class B it plays a cooperative role in cell morphogenesis. Class C PBPs are non-essential under laboratory conditions and therefore represent minor targets of β-lactam antibiotics. Drugs that target peptidoglycan exhibit low toxicity due to the lack of this component in mammalian cells (Zervosen et al., 2012). In *S. aureus*, four PBPs are present (PBP1-PBP4), though only PBP2 is essential for viability and is capable to act as transglycosylase in addition to providing transpeptidase activity (Figure 4.2,A).

Methicillin resistant *S. aureus* (MRSA), has acquired an additional PBP, termed PBP2a (Figure 2.4,B). This protein becomes essential in the absence of PBP2, and mediates methicillin resistance in this organism (Peacock and Paterson, 2015). The following section will provide an overview on PBP2a mediated resistance in *S. aureus*.

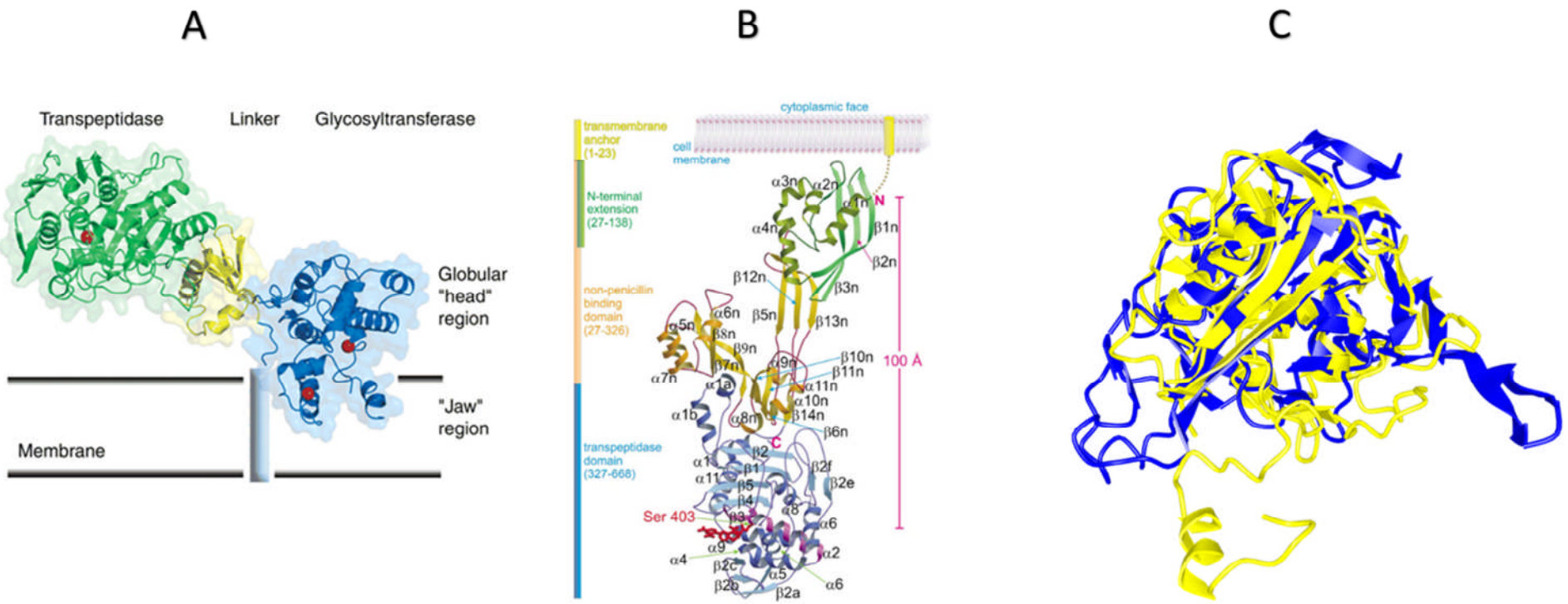


Figure 4.2. Penicillin binding protein 2 (PBP2) with its two domains TP and GT connected by a linker (A), (Lovering et al., 2007). PBP2a structure (B) (Lim and Strynadka, 2002). The C-terminal TP domain is present on the outside of the cell, the N-terminal domain interacts with the membrane. Structures of TP domains of both proteins, amino acid residues 327-668, (PBP2, yellow and PBP2a, blue. PDB codes 2OLU and 1MWS, respectively) are superimposed, where they show similar structure (C).

4.2.3 Methicillin resistance in *Staphylococcus aureus*

Resistance of *S. aureus* to methicillin is distinct from resistance to penicillin, which is mediated by a (usually plasmid-borne) β-lactamase (Dyke et al., 1966). Methicillin resistance in this organism is mainly mediated by expression of a foreign PBP, referred to as PBP2a or PBP2′, this protein is of reduced affinity for β-lactams compared to the original PBP2 (Stapleton and Taylor, 2002). PBP2a is encoded by a highly conserved gene (*mecA* gene) which is located on “foreign” DNA region (named *mec* element) of the chromosome of resistant strains but lacking in susceptible strains (Ubukata et al., 1989) (Stewart and Rosenblum, 1980)**.** This gene has been suggested to initiate from *Staphylococcus sciuri* (Wu et al., 2001); however, its mechanism of acquisition from this organism is not known.

PBP2a catalyses the transpeptidation step of PG biosynthesis in the absence of transpeptidase domain of the native PBP2 in a cooperative manner with transglycosylase activity of this protein. Therefore, this protein performs an essential function for the organism (Pinho et al., 2001).

In this chapter, a dual targeted Affimer was generated to bind and inhibit the action of both PBP2 and PBP2a in *S. aureus*. Binding of this Affimer to PBP2 and PBP2a was confirmed by phage ELISA and ELISA with purified protein. Both endogenous expression of selected Affimer and its exogenous addition as purified protein showed inhibition of growth of methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA).

The PBP2/PBP2a Affimer was subsequently shown to bind both target proteins (PBP2 and PBP2a) with high affinity and 1:1 stoichiometry by gel filtration chromatography and ITC.

4.3 Aims

The main aim of this chapter was to further investigate the potential of Affimers as antibacterial agents. In this respect, work described here aimed to select phage displayed dual targeted Affimer against well validated antibacterial targets (PBPs) that are extracellularly accessible in the model pathogen, *S. aureus*. An antistaphylococcal dual targeted Affimer was isolated and characterised by microbiological and biochemical techniques.

4.4 Results

4.4.1 Screening of an Affimer library for PBP2 Affimers

Target protein (PBP2) was expressed and purified as described (Lovering et al., 2007). Purified protein identity was confirmed by mass spectrometry. The Affimer library was biopanned against immobilized PBP2, and three panning rounds were performed as previously described (Tiede et al., 2014). The enrichment of the amplified phage pool selected against target protein (PBP2) versus the phage population selected against blocked wells (Negative control) was evaluated as described in Section 2.4.4. In the last round of panning for PBP2, the relative enhancement of target specific binding versus unspecific binding was approximately 10^7 fold.

After the last panning round (pan three), 96 independent phage clones were randomly picked, grown in 200 µl of LB and tested for binding to PBP2 by phage ELISA. Each phage clone was also tested for binding to system component in the absence of target protein, as negative control. A ninety two selected clones exhibited good binding to target protein with no or little binding to blocked wells (Figure 4.3 A, B).

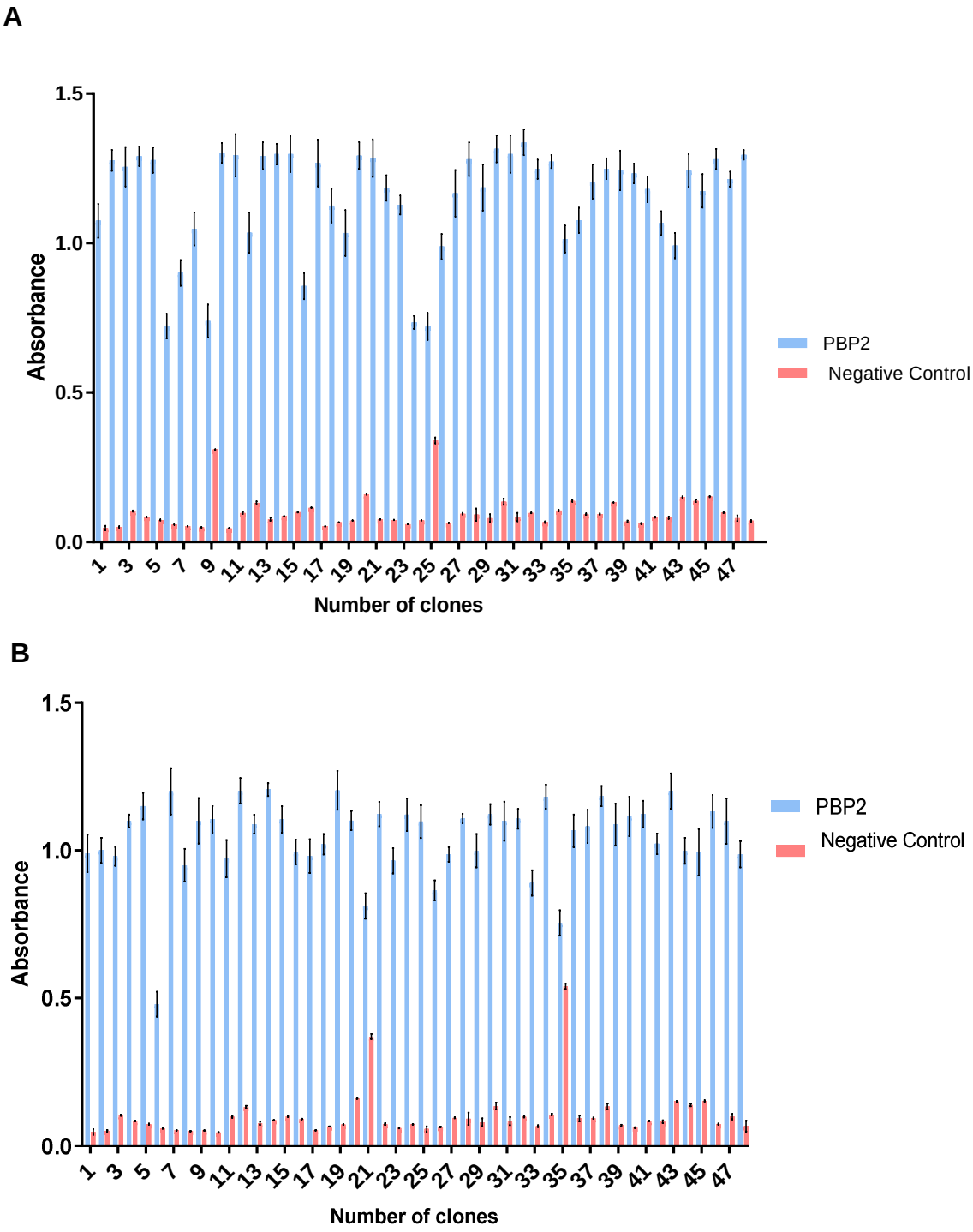


Figure 4.3. (A,B) Phage ELISA results for Affimers identified against PBP2. In total, 96 wells were coated with PBP2, the phage was added and the absorbance for each well was measured after the addition of TMB. Wells containing target protein are shown in blue and control wells are shown in red. Positive Affimers are indicated by high signal of absorbance measured for target protein compared to control wells.

4.4.1.1 DNA sequencing of selected Affimers

The DNA coding sequences of 92 selected PBP2 binding Affimers were determined, and 15 distinct Affimers were identified. Table 4.1 shows the amino acid sequences of the random regions of each of these Affimers. The results show that the variable loops of PBP2 binding Affimers do not share any consensus sequence, implying that the isolated PBP2-targeted phage clones bind their target protein at different epitopes.

Table 4.1. Amino acid sequences of the variable loops of PBP2 binding Affimers.

Representative clone	sequence		Frequency of appearance
	Loop 1	Loop 2	
1-A	FMSHAVWFL	TMPQQNSAR	10
2-A	QGPMLSQKD	KPAEHRRGP	9
39-A	FVHDRQSTI	EERSTNTSQ	8
16-A	MTYYSGDHR	MIGGTNQYV	7
25-A	SINMMQHPE	VYVPGWAAK	7
48-A	YFEGDNHAI	PNQMQGTLI	7
32-B	WHRKDIELD	AMNNGKEER	7
8-A	FQNLLTSHR	EDYYMIQHR	6
12-B	QTLHEILFN	HGTMYTIGG	6
10-A	KQHSFIAQD	ANMAILLEK	5
11-A	GGTMFGTKI	EDQNGPSIA	5
13-A	GEEHRNSMW	KERGGFAHE	4
14-A	HFVSWKEDN	WQKASDHAV	4
23-B	YGQDRPIMH	ITMNQIKHH	4
15-A	YNHERGSFM	GWIQLEEVA	3

4.4.2 Selection of PBP2/PBP2a binding Affimers

After selection of PBP2 Affimers, the next step was to test these Affimers for their ability to bind the other target protein (PBP2a). This protein was purified as described (Kim et al., 2012), and purified protein identity was confirmed by mass spectrometry. Phage clones displaying distinct PBP2 Affimers (Table 4.1) were isolated and tested for cross-binding to PBP2a by phage ELISA, though none of them was found to be capable of binding this target protein.

Screening of Affimer library against PBP2a was conducted, and PBP2 was then biopanned with a phage pool recovered from the first pan of PBP2a biopanning with this library. Two rounds of selection/amplification were carried out, and by the second round 10^5 fold amplification of target specific phage clones compared with phage clones amplified against blocked wells was achieved. After the last pan, 32 phage clones were isolated and tested for cross-reactivity to both target proteins (PBP2 and PBP2a) by phage ELISA. Results shown that all the 32 Affimers were cross reactive towards PBP2 and PBP2a, with no or little binding with control wells (Figure 4.4).

DNA sequencing of PBP2/PBP2a binding Affimers revealed three distinct Affimers. The amino acid sequence of the variable loops of these Affimers are shown in Table 4.2.

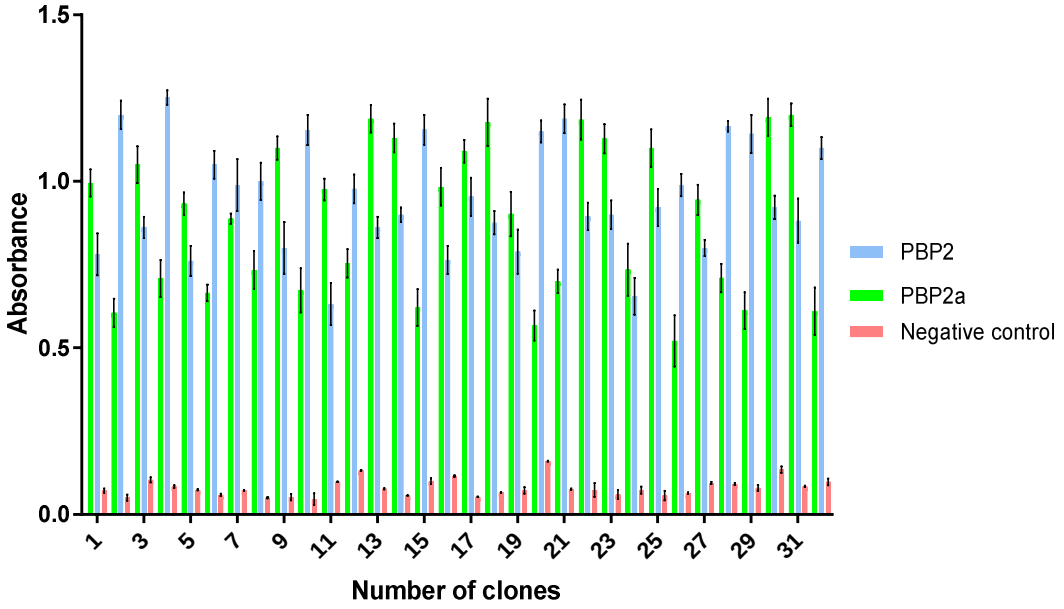


Figure 4.4. Testing of selected phage clones for crossbinding to target proteins (PBP2 and PBP2a) by phage ELISA. Phage obtained after second panning round of PBP2 with PBP2a enriched phage pool were incubated with immobilized PBP2, PBP2a and blocking buffer. Positive phage clones were visualized using an anti-phage antibody. Thirty two Affimers were selected that bind both target proteins. The presence of cross-reactivity was clearly shown by high signal of absorbance measured for target proteins compared to control wells.

Table 4.2. Amino acid sequences of the variable loops of PBP2/PBP2a binding Affimers.

	Sequence		
Representative clone	**Loop 1**	**Loop 2**	**Frequency of appearance**
1	WFMDEVANI	NPAFKLIQT	16
2	TGLMIHFVR	SWLIEYYQL	10
3	SHEVIAREQ	RYIVEPKVA	6

4.4.3 Testing the antibacterial activity of a dual PBP2/PBP2a Affimer

4.4.3.1 Intracellular expression of PBP2/PBP2a Affimers in *Staphylococcus aureus*

To determine whether selected Affimers bind at sites that are crucial for the function of the target proteins, and to provide an initial evaluation regarding the antibacterial properties of these Affimers, the antibacterial activity of these proteins expressed inside cells was investigated. Both methicillin sensitive *S. aureus* (MSSA) and methicillin resistance *S. aureus* (MRSA) strains were used as hosts for regulated expression of selected Affimers. The DNA sequences that encode for the PBP2/PBP2a binding Affimers, and for an unrelated Affimer (FusB Affimer), with a signal peptide (DsbA), were cloned into the pRAB11 plasmid vector in *E. coli*. These constructs were verified by PCR and DNA sequencing and were then introduced by electroporation into *S. aureus* SH1000 (MSSA) and USA300 (MRSA) for expression.

To evaluate the ability of PBP2/PBP2a binding Affimers to inhibit these proteins inside bacterial cells, regulated expression of these Affimers was performed. The constructed strains of *S. aureus* harbouring these Affimers and a control Affimer (Non-PBPs-binding) were grown at 37°C in MHB supplemented with anhydrotetracycline (AT), to act as an inducer of expression. Induced expression of Affimer 1 led to complete inhibition of growth of the bacterial cells (both MRSA and MSSA) at concentration of 150 ng/ml AT, whilst no detectable effect on the growth of both *S. aureus* strains

containing either of the other two Affimers or the control Affimer was observed.

4.4.3.2 Bacterial growth inhibition at various PBP2/PBP2a Affimer concentrations

After effectively inhibititing growth of *S. aureus* when it was expressed from inside the cells, I sought to determine whether exogenous addition of the purified dual targeted Affimer was able to inhibit bacterial growth. This Affimer was purified as described in (Tiede et al., 2014), and added to bacterial cells to determine its antibacterial effect as purified protein on the growth of a panel of six methicillin resistant *S. aureus* (MRSA) strains comprised of USA300, COL, MRSA252, Mu50, UAMS-1 and N315, and two MSSA strains (*S. aureus* SH1000 and MSSA476). Bacterial cells were incubated at 37°C with various concentrations of purified Affimer, and growth was followed by measuring optical density (OD) at 625 nm at 30 minutes intervals. This Affimer inhibited growth of all the *S. aureus* strains in a dose dependent fashion, causing complete growth suppression at 62 µM; Figure 4.5 shows a representative growth curve for inhibition of *S. aureus* USA300 (MRSA) by the Affimer. Species specificity of this Affimer for *S. aureus* was tested by determining its effect on growth of methicillin resistant *Staphylococcus epidermidis* strain (RP62A). Growth of this strain was not inhibited by this Affimer even when it was added to cells at two-fold higher than its MIC against *S. aureus* (Figure 4.6).

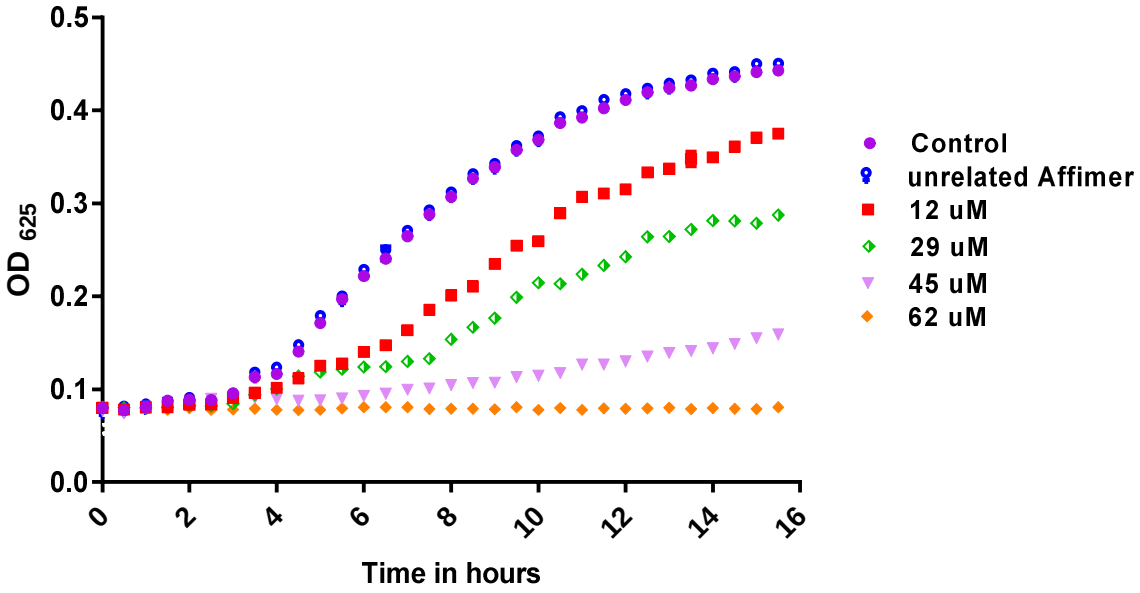


Figure 4.5. Growth of *S. aureus* USA300 cells incubated with different concentrations of PBP2/PBP2a Affimer. Same results were demonstrated for all tested strains of *S. aureus*.

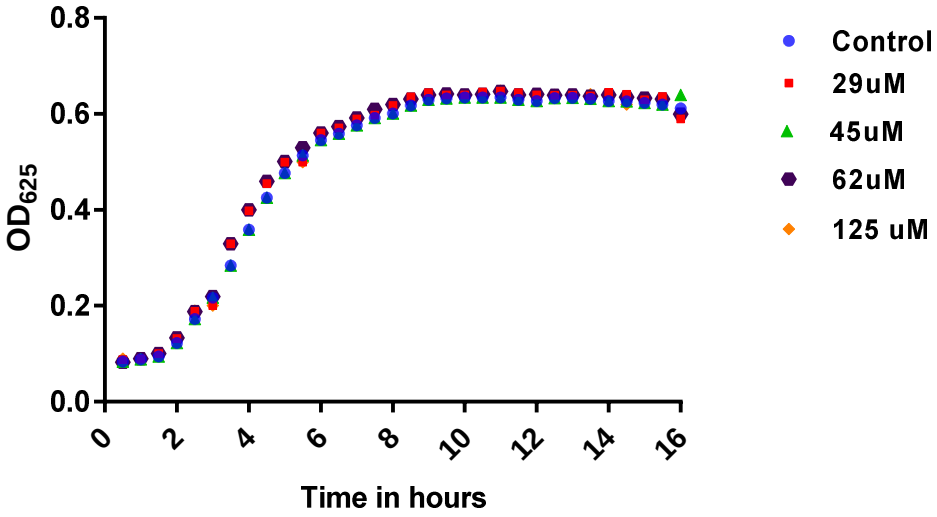


Figure 4.6. Growth of *Staphylococcus epidermidis strain (RP62A)* cells incubated with different concentrations of PBP2/PBP2a Affimer.

4.4.3.3 Determining Affimer specificity for PBP2

To determine the specificity of the dual targeted Affimer for PBP2, I investigated whether overexpression of target protein (PBP2) results in rescue from the inhibitory effect of the Affimer. A strain of *S. aureus* for regulated expression of PBP2 was constructed as described in Section 2.2.8. DNA coding region for PBP2 was cloned into plasmid pRAB11, and correct construct electroporated into *S. aureus* to enable regulated expression of target protein up on addition of inducer (AT). This strain was used for determining the MIC of exogenously added PBP2/PBP2a Affimer in the presence and absence of AT. Induction of target protein with 50 ng/ml AT increased the MIC of Affimer against this recombinant strain two fold compared to parental strain, and there was no detectable effect seen on the growth of induced recombinant strain in the absence of Affimer. Increasing Affimer MIC upon induction of PBP2 expression confirmed that growth inhibition was caused by inhibition of this protein target.

4.4.3.4 Investigating the effect of combining PBP2/PBP2a Affimer with oxacillin on growth of *S. aureus* USA300

Having shown that the dual targeted Affimer is able to inhibit growth of both methicillin sensitive (MSSA) and methicillin resistant (MRSA) *S. aureus*, I sought to determine whether this Affimer is able to synergise the antibacterial effect of oxacillin against the resistant strain, *S. aureus* USA300.

A checkerboard microdilution assay was used to test the effect of pairing this Affimer with the antibiotic oxacillin on growth of *S. aureus* USA300 as described in Section 2.6.2.4. To identify potential synergy, the fractional inhibitory concentration (FIC) is determined by comparing the MIC of each agent alone with the MIC of that agent when paired with other drug. Combination of these two compounds resulted in an FIC of 0.86, which represents a two-fold decrease in the MIC of oxacillin against tested strain.

4.4.4 Further characterization of PBP2/PBP2a Affimer

4.4.4.1 ELISA with purified protein

To further confirm binding, ELISA was employed to test specific binding of the dual Affimer to PBP2 and PBP2a. Biotinylated Affimer was incubated with immobilised PBP2 and PBP2a on a 96 well microtiter plate, and binding of Affimer to target protein detected by streptavidin conjugated to HRP, visualized by TMB. Figure 4.7 shows that this Affimer specifically binds its target proteins, with apparently slightly higher affinity for PBP2a.

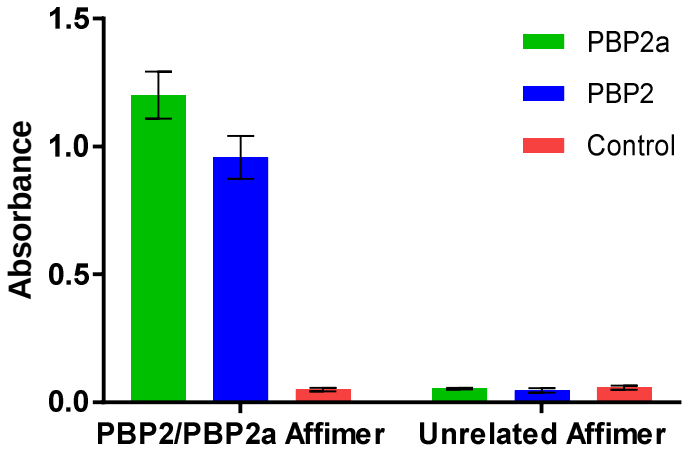


Figure 4.7. ELISA with purified dual targeted Affimer. Biotinylated PBP2/PBP2a Affimer was used to detect PBP2a (green), PBP2 (blue) and control protein (red) by ELISA.

4.4.4.2 Analytical size exclusion chromatography

To further confirm binding of this Affimer to its target proteins and to purify formed protein complexes for structure studies, size exclusion chromatography was used to analyse target proteins complexes with PBP2/PBP2a Affimer in solution. A mixture containing PBP2a or PBP2 with the dual Affimer was loaded on to size exclusion column, and both target proteins formed a 1:1 complex with this Affimer. Figure 4.8 shows PBP2a•Affimer and excess Affimer eluted at correct expected size of about 85 KD for complex and 12 KD for Affimer alone.

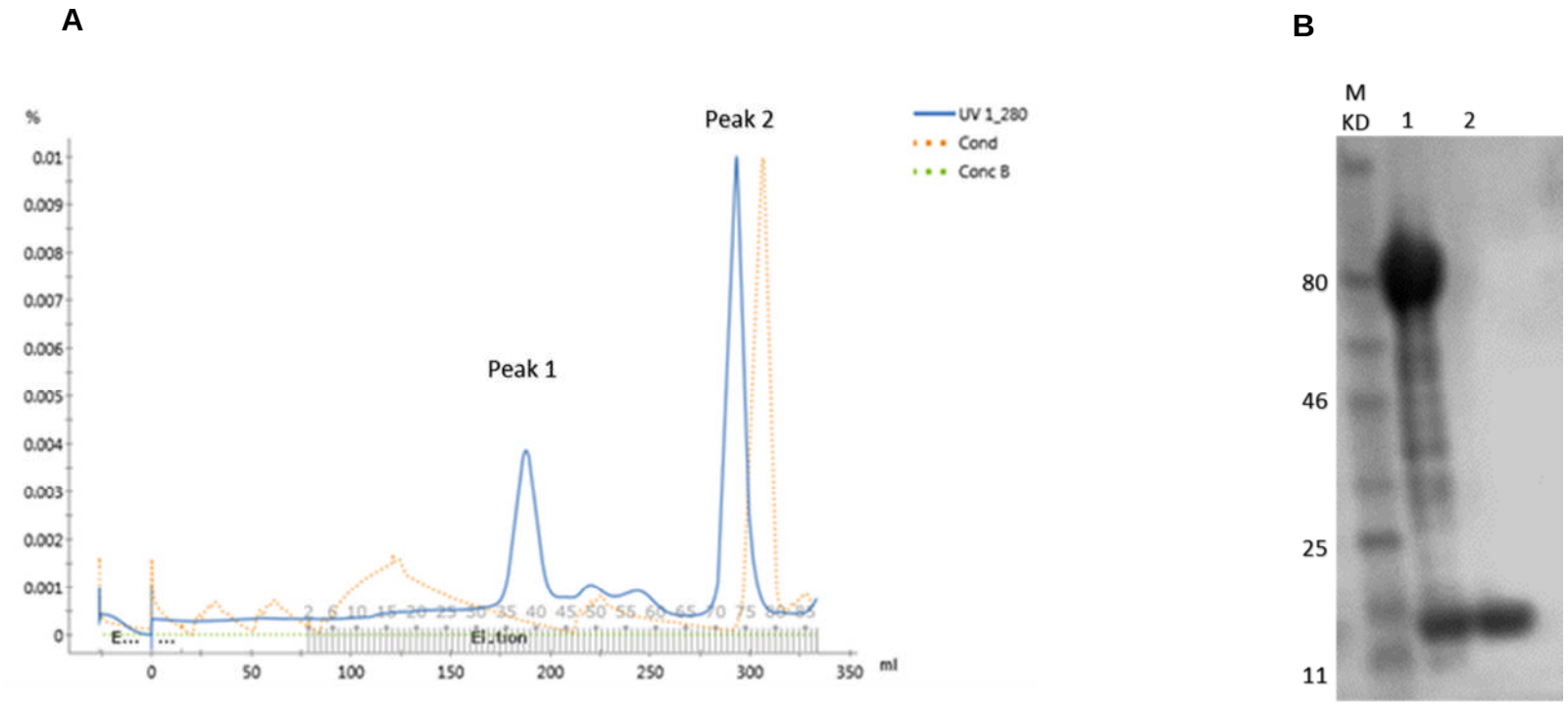


Figure 4.8. Gel filtration chromatography for PBP2a•Affimer complex formation (A). The first peak corresponds to the protein complex and the second peak represents excess dual Affimer. **(B) SDS-PAGE of the fractions**; lane 1; marker, lane 2; eluted complex, and lane 3; represent excess Affimer. Lane 2 and lane 3 correspond to (peak 1) and (peak 2) respectively.

4.4.4.3 Determining binding affinity and thermodynamic parameters of binding interactions

The affinity of PBP2/PBP2a binding Affimer for its target proteins (PBP2/PBP2a), along with the thermodynamic profile of the binding interactions, were determined by ITC. This Affimer bound both target proteins (PBP2a and PBP2) with 1:1 stoichiometry, with dissociation constants in the nanomolar range (kd values of 730 ± 5 and 950 ± 5 nM respectively) (Figure 4.9 A,B). Figure 4.10 shows thermodynamic parameters of binding interactions obtained by ITC, analysis of these parameters shows both favourable enthalpic (- 4.8 ± 0.8, - 2.95 ± 0.6) and entropic contribution (30.17 ± 1, 31 ± 1), for PBP2a•Affimer and PBP2•Affimer complexes respectively.

These comparable thermodynamic profiles for both interactions suggest that the same amino acid residues and mechanism of interaction are potentially involved in the association of Affimer and its two target proteins. The favourable enthalpy contribution is due to the formation of hydrogen bonds upon binding, while the favourable entropic contribution indicates the involvement of hydrophobic interactions upon complex formation (Chaires, 2008)

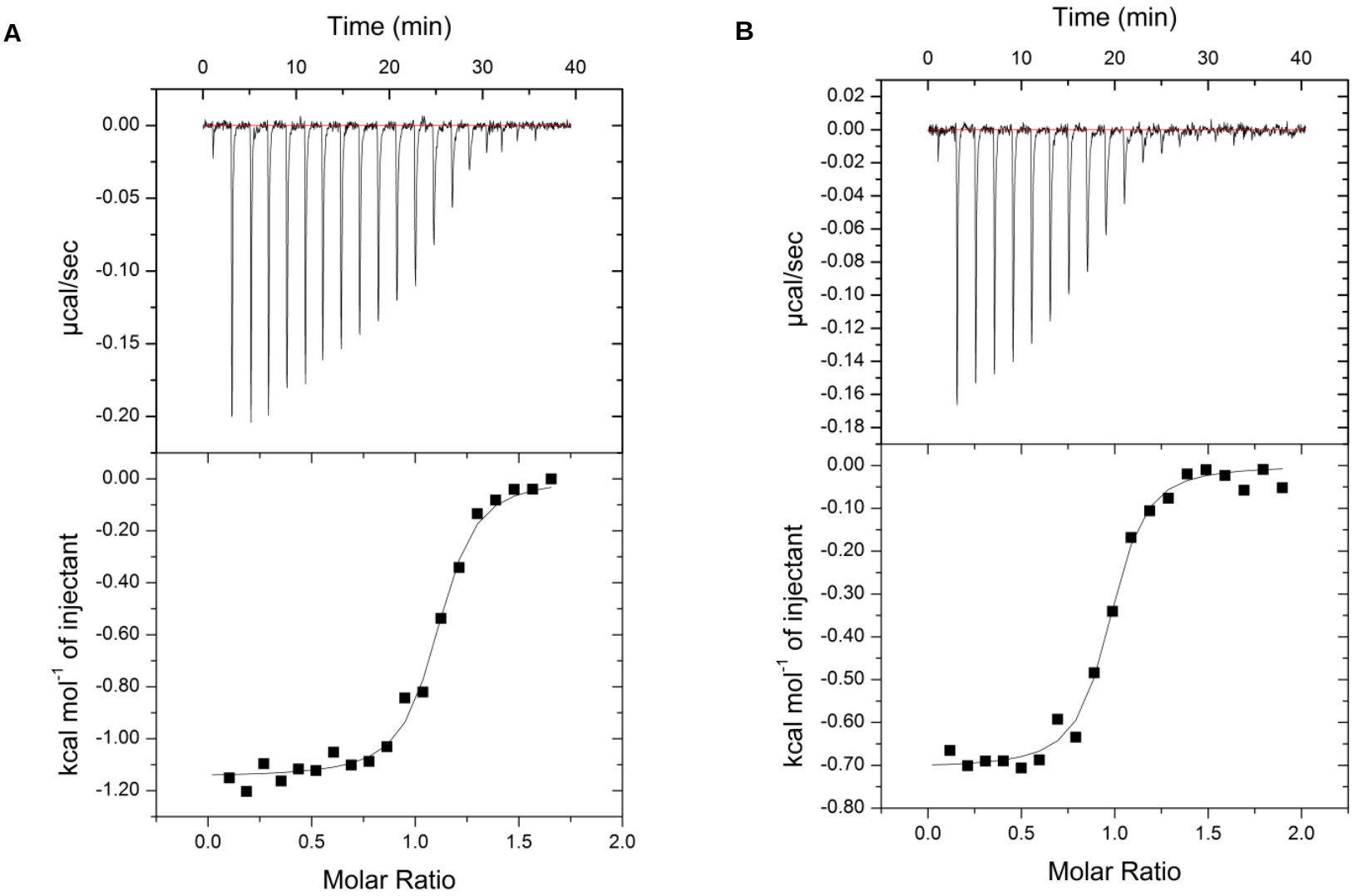
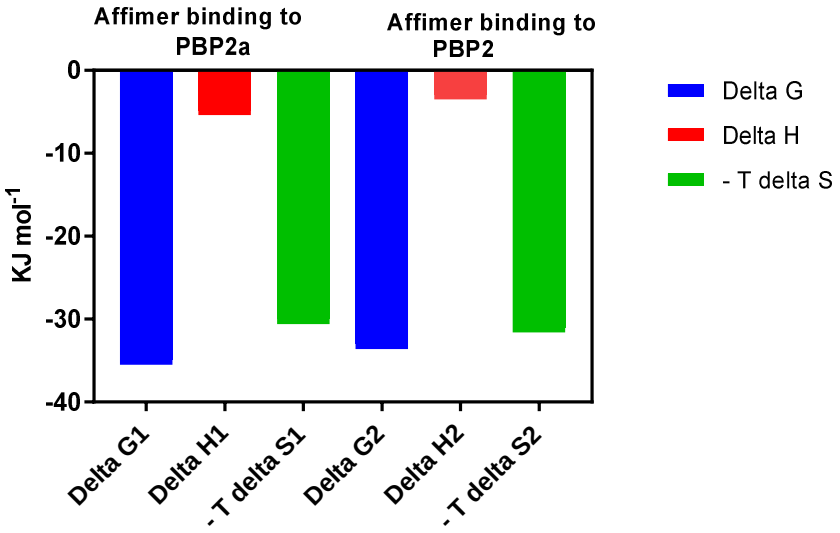


Figure 4.9. The Isotherms and the data fit for PBP2/PBP2a interactions with PBP2a (A) and PBP2 (B), determined by isothermal titration calorimetry.



Interaction	ΔH/kJ mol^{-1}	TΔS/kJ mol-1	ΔG/kJ mol-1
PBP2a•Affimer	- 4.8 ± 0.8	30.17 ± 1	- 34.97 ± 1
PBP2•Affimer	- 2.95 ± 0.6	31 ± 1	- 33.95 ± 1

Figure 4.10. Thermodynamic profile of PBP2/PBP2a Affimer binding to PBP2a and PBP2.

4.4.4.4 Crystallisation trials

In an attempt to gain structural insights into binding of the PBP2/PBP2a Affimer to PBP2 and PBP2a, crystallization trials for each complex were conducted. Protein complexes were purified by gel filtration chromatography as described in Section 2.5.3, and used to screen for crystal formation using four commercially available crystallization screens as described in 2.5.4. In this study, none of the crystallisation conditions were able to produce protein crystals.

4.4.5 *In vivo* toxicity and antibacterial efficacy of PBP2/PBP2a Affimer

Investigating toxicity and antibacterial efficacy of the dual Affimer into *Galleria mellonella* was performed by Charlotte Hind and Mark Sutton (Public Health England, Salisbury, UK). For a compound to be developed as a therapeutic agent for systemic administration it is essential to evaluate its efficacy in resolving infection (O'Neill and Chopra, 2004). Usually, this is accomplished using a mammalian model. However, such experiments are expensive, laborious, and necessitate full ethical consideration. Therefore, economically and ethically more acceptable invertebrate models of infection have been developed in recent years, involving use of the larvae of the greater wax moth *Galleria mellonella*. The *in vivo* efficacy of PBP2/PBP2a Affimer in protecting *G. mellonella* larvae from killing by *S. aureus* USA300 was tested. To determine the effect of this Affimer, a concentration of 24 mg/ml (which represents the solubility limit of this protein) was injected into *G. mellonella* larvae infected with *S. aureus* USA300, and the percentage survival was monitored over 120 hours (Figure 4.11). In this model, the dual Affimer showed some toxicity and no protection from *S. aureus* USA300 infection.

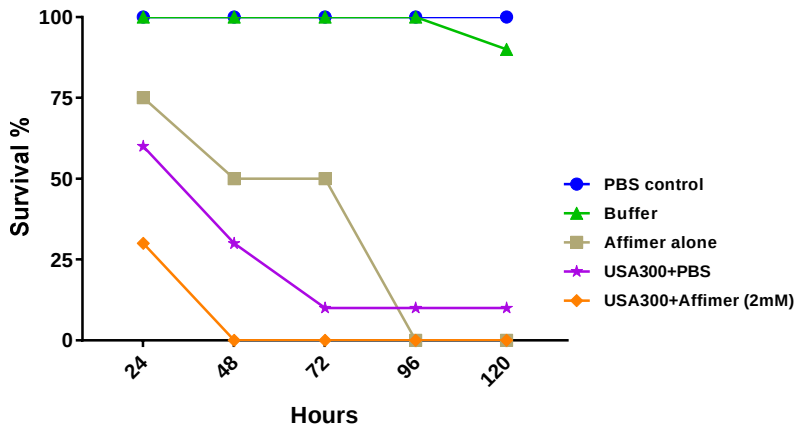


Figure 4.11. Treatment of *G. mellonella* larvae with the dual Affimer. 30 minutes after being infected by *S. aureus* USA300, ten larvae per group.

4.5 Discussion

Using PBP2 and PBP2a from *S. aureus* as a model target proteins, the potential of the Affimer library as a source of dual targeted Affimers with inhibitory effect towards two essential target proteins was investigated. Results presented in this chapter emphasized the utility of the Affimer library; from a single phage display, in which the two target proteins were alternatively biopanned with the library, three distinct PBP2/PBP2a Affimers were selected.

Microbiological characterisation of these Affimers confirmed that a PBP2/PBP2a binding Affimer that is cross reactive and inhibitory towards PBP2 and PBP2a was successfully selected. Whole bacterial cell antibacterial activity of this Affimer against *S. aureus* was demonstrated by either intracellular expression or exogenous addition of this Affimer to bacterial cells. The results suggested that this Affimer is species specific for *S. aureus*; it completely inhibited bacterial growth of methicillin sensitive and methicillin resistant *S. aureus* strains (MIC 62µM), while no effect of this Affimer was seen on growth of *S. epidermidis*, even at four fold that of the *S. aureus* MIC. There is an increasing agreement that species-specific agents are the future of antimicrobial discovery; specificity of a drug for a particular species is an important factor in reducing the disruption of normal flora (Fischbach and Walsh, 2009). From this perspective, Affimers would be useful for rapid development of narrow-spectrum antibacterial drugs. On the other hand though, and in the aid of genomic information that facilitate the identification of shared targets in many bacterial species, Affimers may also

be selected against these targets to develop broad-spectrum antibacterial agents.

The ITC results established that this Affimer was able to bind its partner proteins (PBP2a and PBP2) with comparable Kd values of 730 and 950 nM, respectively, and similar mechanism of interactions. An alignment of amino acid sequences of PBP2 and PBP2a (PDB codes 2OLU and 1MWS, respectively) (Lovering et al., 2007, Lim and Strynadka, 2002), shows that these proteins are structurally similar and have 18% shared amino acid sequence identity (Madej et al., 2014). These results suggested that dual targeted Affirmer can be selected against structurally similar protein targets despite relatively low sequence identity.

Although a relatively high concentration of this Affimer is needed to inhibit bacterial growth, which may result in some toxicity, further optimisation of this Affimer may come over this issue. The potency of this Affimer may be optimised by improving its affinity for its target proteins, which may be achieved via site directed mutagenesis through random substitution of several residues (Simon et al., 2013).The affinity of this Affimer for its target proteins could be also improved using phage display-based optimization, by applying second library to select for Affimer variants with high affinity to both target proteins. Using this approach, the affinity of binding proteins can be improved by 380-fold (Pearce et al., 1999).

Although crystallisation trials did not produce crystals, structure studies may, in future, be performed using cryo-EM technique by fusing Affimers to homo-oligomeric scaffold to allow cryo-EM analysis for the complex (Coscia et al., 2016).

This study confirmed the utility of Affimers for multi-targeting extracellular proteins, emphasising their potential use in the field of antibacterial drug discovery.

5. Discussion and Conclusions

There is no doubt that the increasing incidence of antibacterial resistance combined with a failure to develop new antibiotics creates a need for novel strategies to address this problem.

One method being explored is the use of monoclonal antibodies (mAbs) to prevent infections or to cure an infection adjunctively with antibacterial drugs (DiGiandomenico and Sellman, 2015). However, the limitation of antibodies, Section (1.4.4.5), has led to development of alternative proteins with improved properties. Although there are some studies exploring the use of these proteins as therapeutic agents, mainly to treat cancer, no work has been done to investigate their potential as agents for antibacterial chemotherapy. One of these artificial binding protein scaffolds is the recently developed Adhirons (Tiede et al., 2014), now known as Affimers (Tiede et al., 2017), Section (1.5.2.1).

This study investigated the potential of these proteins as antibacterial agents and as modulators of bacterial resistance, with a focus on generating dual targeted Affimers rather than Affimers for a single target protein. The experimental findings of this thesis reported for the first time proof of concept that Affimers may be isolated for dual target proteins. Recognition and binding of a single Affimer to different target proteins could therefore have direct advantage on the use of Affimers in antibacterial chemotherapy. Dual targeted Affimers generated in this study are capable not only of interfering with antibacterial resistance but also exhibiting antibacterial activity by inhibiting multiple essential bacterial target proteins. It is now known that

multi-targeting is fundamental for a lower rate of resistance, which is an important measure for proceeding a lead into clinic for development of a perfect antibiotic regardless of spectrum (Butler et al., 2017). Hence, Affimer proteins provide an important approach to antibacterial drug discovery. Even though these proteins could not enter the cell, this study strongly suggested that they are of beneficial use as antibacterial agents through targeting of extracellular target proteins such as PBPs.

Results from this work showed that five out of eight (62.5%) isolated dual targeted Affimers were able to inhibit their target proteins, which suggests that they bind to their targets at regions crucial for biological activity. In contrast, only 0.001% of the new compounds identified every year by HTS approach exhibit antibacterial activity, with extremely complex synthesis schemes and massive production costs (Fernebro, 2011) (Ojala et al., 2013). This, together with the cost-effective and ease of their production, indicates that dual targeted Affimers provide an excellent approach to antibacterial drug discovery.

Evaluating the antibacterial activity of selected Affimers via intracellular expression of these proteins prior to being taken for further biochemical characterisation eliminates an important doubt at the beginning of the discovery process. Nuisance Affimers or those that exhibit no antibacterial activity inside bacterial cells are rapidly eliminated prior to proceeding with more complex biochemical characterisation, which provides an opportunity for saving time and effort.

Biochemical studies of both Affimers (FusB/FusC Affimer and PBP2/PBP2a Affimer) using purified protein identified that they bind their dual targets with

1:1 soichiometry and high affinity in the range of nanomolar. For both tested dual targeted Affimers, the affinity of Affimer for its target proteins was comparable and in the nanomolar range. These results along with specificity studies indicate that even when an Affimer binds its target with a high affinity it is still possible to achieve binding to another target protein from the same family with high affinity and specificity. Comparable affinity of a dual targeted Affimer for its target proteins is another advantage of this Affimer. This may prevent reduced potency that result from big differences in affinities of a selected binder for its target proteins (Tkaczyk et al., 2017).

This work suggests that Affimers may provide immediate opportunities for developing antibacterial agents against essential cell surface proteins. These agents may work via interfering with signalling cascades by triggering conformational changes in the target protein, or competing for binding by normal ligands. Ideally, the surface proteins to be targeted should be well conserved amongst clinical isolates of a certain pathogen, in order to ensure efficacy and reduce the possibility of drug resistance.

6. Future work

An important step to enable further characterisation of the isolated dual targeted Affimers is the understanding of how these Affimers crossinteract with their target proteins. In order for this to be achieved, the dual targeted Affimers need to be fused with a linker to achieve a size that allow analysis of Affimer : target protein complexes by cryo-EM (Coscia et al., 2016).

To investigate if the teichoic acid hinders PBP2/PBP2a Affimer getting to its target proteins, antibacterial activity of this Affimer combined with teichoic acid inhibitors need to be tested, similar experiments have previously been used to identify the effect of combining teichoic acid inhibitors with β-lactames (Lee et al., 2016)

PBP2-binding Affimers may be tested for their ability to bind monofunctional glycosyltransferase (MGT) from *S. aureus*, a protein that take over the synthesis of PG in the absence of functional GT domain of PBP2, in this organism (Reed et al., 2011) Affimers with crossreactivity towards both target proteins will then be tested for their antibacterial activity.

I addition to targeting PBPs, Affimers could be directed to many extracellular target proteins, in various drug-resistant bacteria. For instance, the biogenesis of outer membrane (OM) proteins in gram negative bacteria represents an attractive target for developing antibacterial Affimers. Inserting proteins into the outer membranes of bacteria is one of the most fundamental

processes taking place in these microorganisms, so it offers a significant target for therapeutic development. An example is Bam A protein, an essential protein, which catalysis the insertion of β barrel membrane proteins (Noinaj et al., 2013). The two component signal transduction system (TSS) is another example that may provide a potential target for Affimers. This system typically involves a sensor Histidine kinase for receiving external input signals and a response regulator that makes a proper change in the bacterial cell physiology (Tiwari et al., 2017). For numerous reasons, TCSs have emerged as convincing targets for antibacterial drug design. Several studies have shown that TCSs are essential for the coordinated expression of virulence factors and, in some cases, for bacterial viability and growth. TCSs proteins are absent in animals, drugs targeting these proteins can potentially have less toxicity (Gotoh et al., 2010).

7. References

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