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Biofilm Formation and Antibiotic
Resistance on Alginate Beads, of
Staphylococcus aureus and other
Health Care Associated Bacterial
Species

Anita Jean Wilkinson

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The University of Edinburgh

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Declaration

I declare that this thesis was composed by myself and all the research presented in is my own, unless otherwise stated.

Anita Jean Wilkinson

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Abstract

Health Care Associated Infections (HCAIs) are a concern especially in regards to antibiotic resistance and effective treatments. *Staphylococcus aureus* is often the main focus for eradication and prevention procedures, however, other bacterial species are also problematic. These include *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus epidermidis* amongst others. Chronic infections caused by these bacteria are often biofilm related, and include dental caries, otitis media, osteomyelitis, burns & chronic wounds, and device related & prosthetic joint infections.

Prosthetic joints and indwelling devices, such as catheters, are a prime environment on which biofilms can develop. This thesis aims to look at biofilms, investigating how they are established, the development of resistance against individual antibiotics and the antibiotic concentrations required to reduce biofilm load. A novel biofilm system – the alginate bead method will be used for these experiments, The alginate bead method was developed by a previous student in the Gallagher Laboratory, due to a need to have a reliable, robust and inexpensive technique to examine formation of biofilms and antibiotic resistance. There are devices and assays available, such as the Calgary Biofilm Device, which are extensively used for these purposes. However, the cost is prohibitive.

This thesis found that the development of biofilms occurs much earlier than expected, with stable, fixed formation after just four hours of growth. Depending upon the antibiotic, resistance can develop within the first two hours of growth and thereafter steadily increases. By 24 hours the biofilms are fully resistant to all the tested antibiotics. In mixed species biofilms, the two species act synergistically protecting each other against the antibiotics, resulting in a much higher antibiotic concentration required.

Common antibiotics used to treat staphylococcal infections are often combined to enhance their destructive effect and prevent the development of resistance. The effects of these antibiotics, when combined was explored. Biofilm resistance against gentamicin, one of the most common antibiotics used to treat staphylococcal infections develops quickly. However, when combined with other antibiotics gentamicin resistance is delayed.

As antibiotic concentrations have to be extremely high in order to have any effect on established biofilms, alternative methods need to be investigated. Any alternative approaches would be employed in conjunction with conventional therapies preventing stable biofilm formation and disrupting established biofilms. Such methods may include sugar metabolites, enzymatic disruption, D-amino acids and activation of the quorum sensing system.

The main conclusion which can be taken from this work are that firstly the alginate bead method of a viable, suitable alternative to the Calgary Biofilm Device and supports biofilm formation and testing. Secondly that biofilms form and are resistant to antibiotics much earlier than expected, and extreme concentrations of antibiotics are required to have an effect. Thus the inclusion of alternative methods which disrupt biofilms would be beneficial to clinical practice. However, the alternative methods investigated within this thesis (D-amino acids and sugar metabolites) failed to show any inhibition of biofilms. There are other possible choices which would need to be investigated.

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Abbreviations

ACCs	Aminoglycoside N-Acetyltransferases
AIP	Auto Inducing Peptide
ALACs	Antibiotic-Loaded Acrylic bone Cements
ANTs	Aminoglycoside O-nucleotidyltransferases
APHs	Aminoglycoside O-phosphotransferases
APS	Ammonium Persulfate
BMGY	Buffered Glycerol Complex Medium
BMMY	Buffered Methanol Complex Medium
CA-MRSA	Community Acquired Methicillin Resistant <i>Staphylococcus aureus</i>
CDI	<i>Clostridium difficile</i> Infection
CoNS	Coagulase negative Staphylococci
CPGRP	Camel Peptidoglycan Recognition Protein
ECL	Enhanced Chemiluminescence
eDNA	extracellular DNA
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extra Polysaccharide
ETTs	Endotracheal tubes
FnBPs	Fibronectin Binding Proteins
GlcNAc	N-acetylglucosamine
HCAI	Health-Care Associated Infection
IL	Interleukin
LB	Luria Bertani Medium
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
ManNAc	N-acetylmannosamine
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MBP	Mannose-Binding Protein
MH	Mueller-Hinton broth (cation-adjusted)
MIC	Minimum Inhibitory Concentration
MLSB	Macrolides, azalides, other lincoamides and group B streptogramins
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>

MSCRAMMs	Microbial Surface Components Recognising Adhesive Matrix Molecules
MSSA	Methicillin Sensitive <i>Staphylococcus aureus</i>
MurNAc	N-acetylmuramic acid
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
PAMP	Pattern Recognition Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGRP	Peptidoglycan Recognition Protein
PIA	Polysaccharide intercellular adhesion
PMF	Proton-motive Force
PNAG	Polymeric N-acetyl-glucosamine
PRP	Penicillin Resistant Pneumococci
PSM	Phenol Soluble Modulins
rClfA	Recombinant Clumping Factor A
rClfB	Recombinant Clumping Factor B
SCV	Small Colony Variants
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SP-D	Surfactant Protein D
SSRs	Suspended Substratum Reactors
TAE	Tris-Acetate-EDTA
TEMED	Tetramethylethylenediamine
TLR	Toll Like Receptor
TNF α	Tumour Necrosis Factor alpha
VRE	Vancomycin Resistant Enterococci
YPD	Yeast Peptone Dextrose
YPDS	Yeast Peptone Dextrose Sorbitol

Chapter 1: Introduction

Health Care Associated Infections

Health Care Associated Infections (HCAIs) are defined as 'an infection occurring in a patient during the process of care in a hospital or other health-care facility such as care homes and domestic environments where individuals are nursed, which was not present or incubating at the time of admission. This includes infections acquired in the hospital but appearing after discharge, and also occupational infections arising among staff of the facility' [WHO, 2002]. They can be acquired as a result of interventions, devices or procedures and are the most common adverse event in healthcare facilities [HPA, 2012; WHO, 2011].

HCAIs are a worldwide problem. However, the exact burden remains unclear, as reliable data collection is difficult, since standardized criteria, diagnostic facilities, and expert analysts are required. Surveillance in high-income countries (i.e. USA/UK) is good and control strategies are making a difference. Low- and middle-income countries are still working towards implementing similar systems [WHO, 2011]. Figures 1.1a and 1.1b display the prevalence of HCAIs in high income and low & middle income countries.

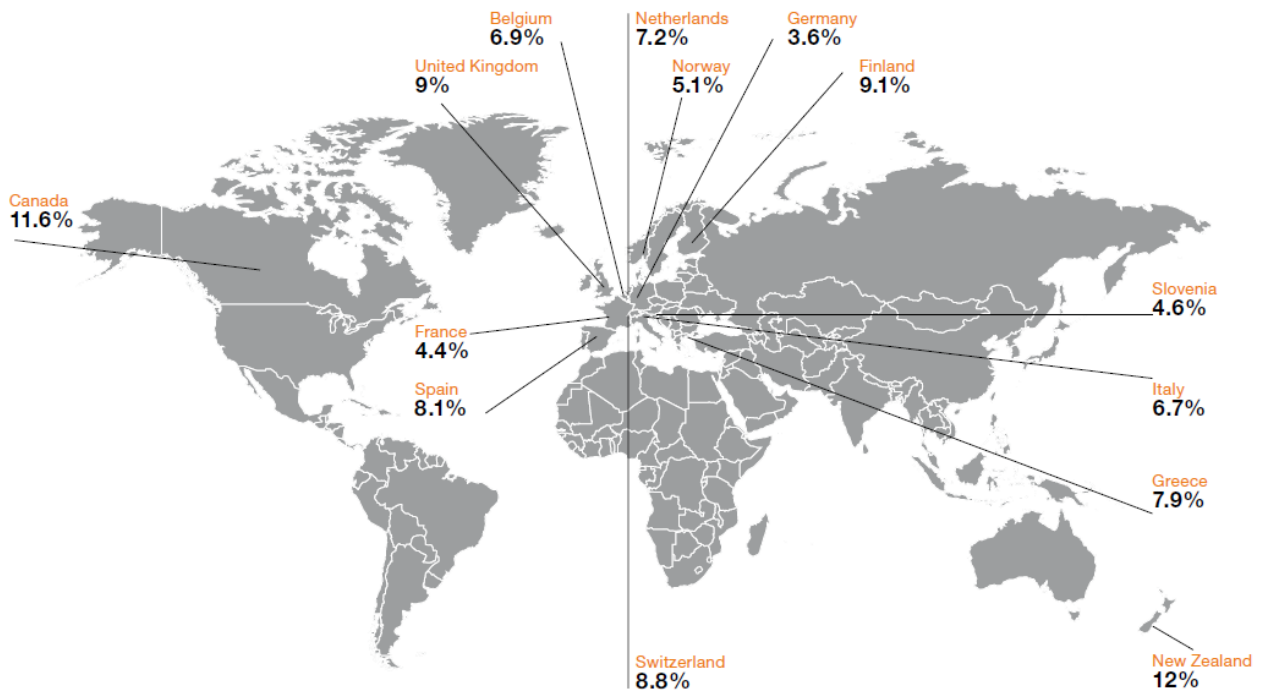


Figure 1.1a: Prevalence of HCAIs in High-income Countries (most recent data used) [WHO, 2011].

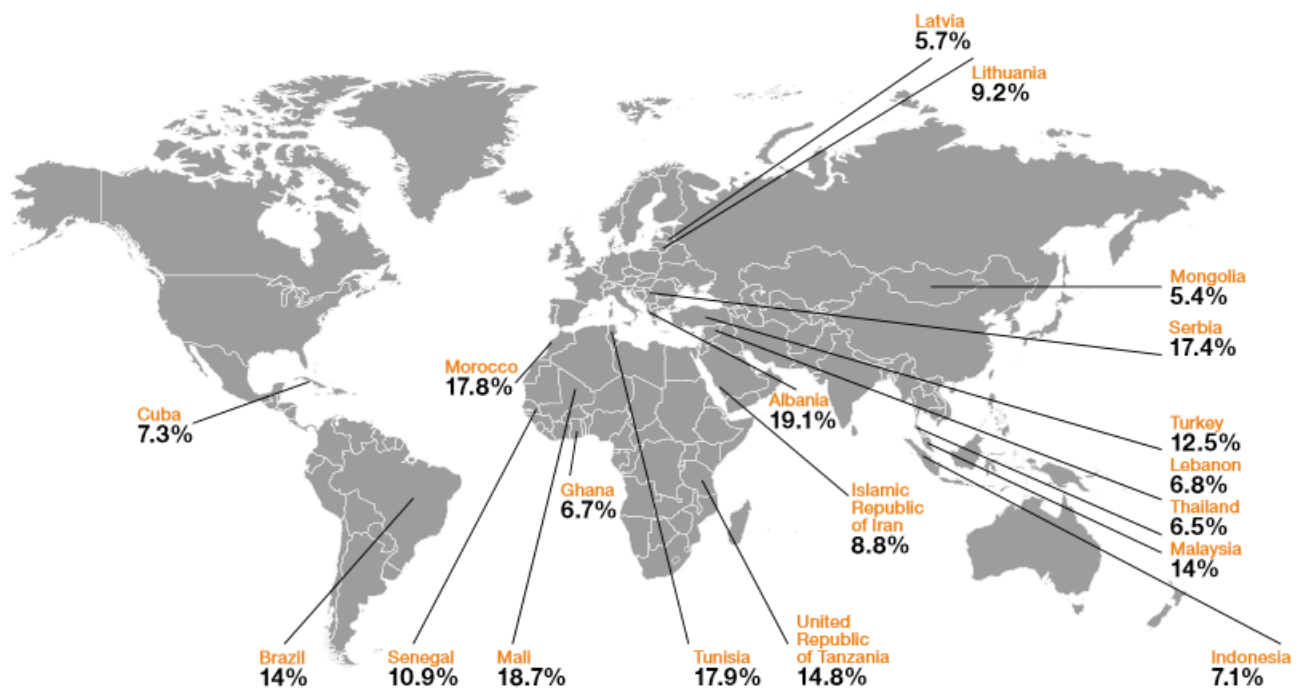


Figure 1.1b: Prevalence of HCAIs in Low- and Middle-Income Countries [WHO, 2011].

Within the UK, at the peak of the HCAI crisis, it was estimated that there were 300,000 HCAs annually with an estimated cost of £1 billion [NAO, 2004], 20% of these infections were considered preventable [Harbarth, 2003]. Consequently, due to the UK government prioritising infection control and pro-active reduction of HCAs over the last 15 years, infection rates have decreased from 9% in 2005 to 6.4% in 2011 [HPA, 2012; Postnote, 2005].

HCAI incidence is highest in patients aged 1-23 months (8.2%), followed by those aged 65-79 (7.4%) and 50-64 years (7.3%). Intensive Care Unit patients have the highest levels of HCAs (adults 23.4%, paediatric 14.7% and neonatal 13.1%). However, this only accounts for 9.4% of the total HCAs reported [HPA, 2012]. The most 10 common HCAs from the English Point Prevalence Survey in 2011 can be seen in Figure 1.2.

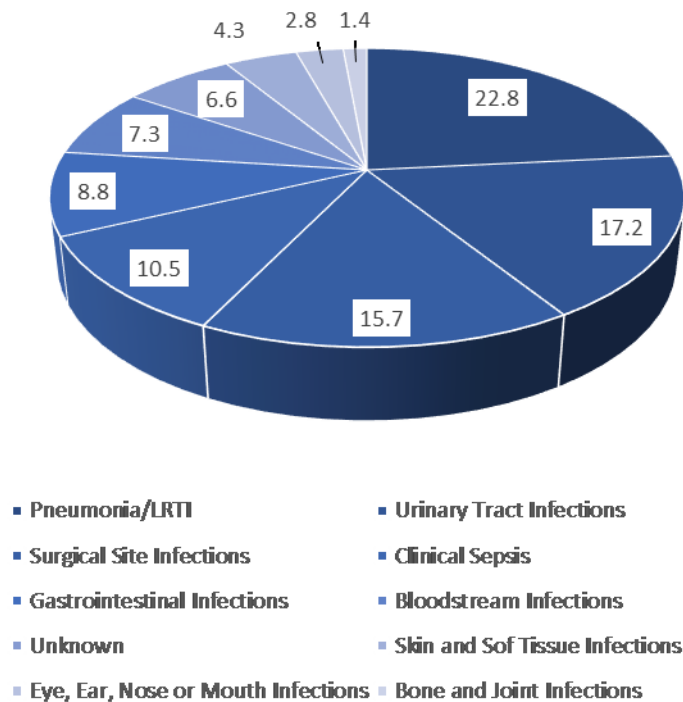


Figure 1.2: HCAI Percentage Distribution in 2011 in England [Adapted from HPA, 2012].

Types of Infections

In the past, focus has mainly been on two HCAs; Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* Infection (CDI). However, there is now mandatory government surveillance on Methicillin-sensitive *Staph. aureus* (MSSA), *Escherichia coli* bacteraemias, Glycopeptide-resistant *Enterococcus* (GRE) bacteraemias and orthopaedic surgical site infections (SSI) [HPA, 2012]. Other voluntary schemes, overseen by the HPA, exist for different micro-organisms and infections and their resistance profiles [HPA, 2012]. Table 1.1 shows the most commonly identified microorganisms associated with HCAs in England in 2011.

Table 1.1: Most Commonly Identified HCAI Organisms in England [HPA, 2012].

Microorganism	Percentage (%)
Enterobacteriaceae	32.4
<i>Staph. aureus</i>	15.5
<i>C. difficile</i> Infection	12.6
<i>Ps. aeruginosa</i>	6.0
<i>Enterococcus</i> spp.	5.8
<i>Staphylococci</i> , other/unknown	5.8
Other	21.9

In England, cases of MRSA and CDI have been decreasing over recent years (Figure 1.3) due to stringent control measures which have been implemented [HPA, 2012]. Reporting of other bacterial infections has shown that *E. coli* bacteraemias have been increasing with third generation cephalosporin resistance recorded at 9% in 2010 [ECDPC, 2011]. The increase in this type of infection may be due to the rigorous control measures and attention in place for MRSA and CDI.

SSIs reporting was established in 1997 however, only became notifiable in 2004. In 2010-2011 31% of these infections were observed to be caused by the Enterobacteriaceae, and a further 27% of infections due to *Staph. aureus* [HPA, 2011]. SSIs include abdominal and cardiac surgeries, limb amputations, bone prosthesis and coronary artery bypass grafts, amongst others [HPA, 2012].

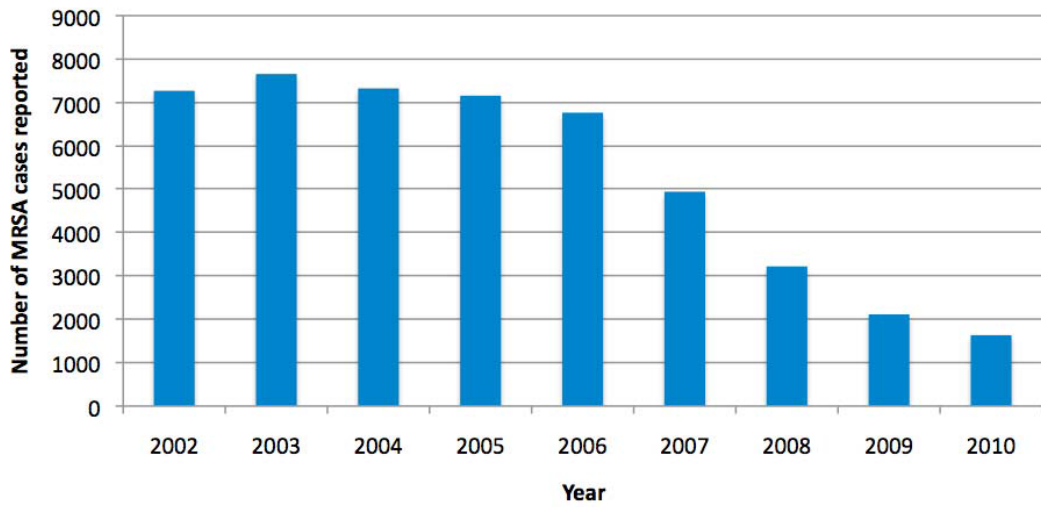
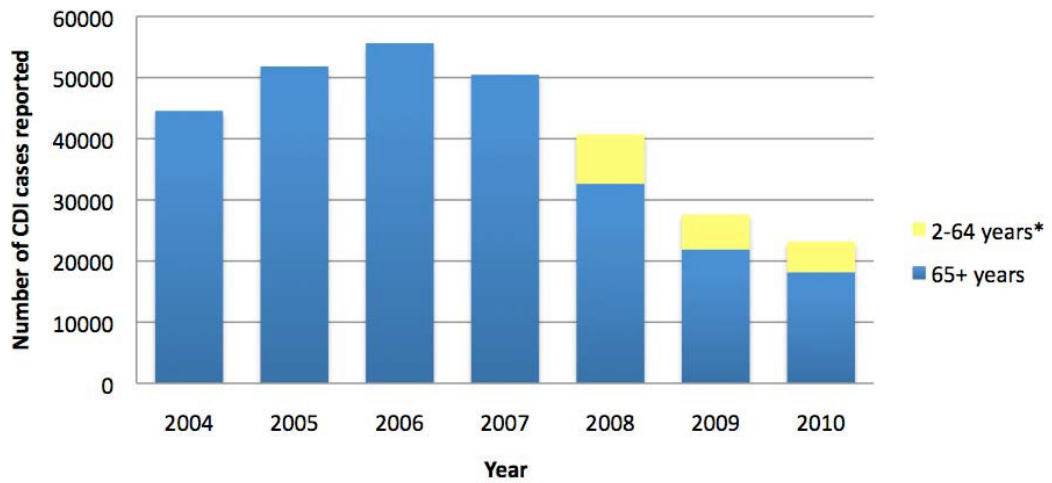


Figure 1.3a: MRSA Bacteraemia Cases in England 2002-2010 [HPA, 2012].



*Mandatory reporting of CDI in the under 65s started in 2008.

Figure 1.3b: CDI Cases in England 2002-2010 [HPA, 2012].

Risk factors for HCAs

Risk factors for HCAs depend upon the type of facility and the nature of the health care required, with more serious invasive procedures carrying a higher risk. Nonetheless, there are universal factors which predispose to infection including: age >65 years; emergency admission and to the ICU; insertion of catheters or endotracheal tubes; undergoing surgery; a hospital stay longer than seven days; trauma induced immunosuppression; neutropenia; impaired functional or coma status; and the presence of a potentially fatal disease [Gravel *et al.*, 2007; Klavs *et al.*, 2003; WHO, 2011].

Control Strategies for Dealing with HCAs

Prevention of HCAs is deemed the responsibility of all individuals and services providing healthcare. Cooperation and communication is required to reduce infection risk [WHO, 2002]. The most effective infection control programmes need to include surveillance, prevention activities and mandatory staff training [Godfrey *et al.*, 2013; Haley *et al.*, 1985].

Basic controls which should be adhered to by all personnel, include appropriate practices of hygiene (hand washing), providing patient care using practices which minimize infections, notifying the control committee of HCAI cases, isolating suspected HCAI cases, and advising visitors on preventing infection transmission [WHO, 2002].

Identification and control of suspected HCAs is the first line in breaking the chain of transmission. The source of the outbreak needs to be quickly identified, eliminated and preventative measures implemented to ensure there are no repeat infections [WHO, 2002]. Table 1.2 details the standard control measures for a HCAI outbreak management.

Table 1.2: Control Measures for Management of HCAI Outbreaks [WHO, 2002].

Type of Transmission suspected	Suggested Action
Cross-transmission (between individuals)	Patient isolation and barrier precautions determined by infectious agent(s)
Hand Transmission	Improvements in hand washing
Airborne Agent	Patient isolation with appropriate ventilation
Agent present in water, waterborne agent	Checking of water supply and all liquid containers. Use of disposable devices
Foodborne agent	Elimination of the food at risk

Antibiotic Use and Resistance Surveillance

With the advent of antibiotics in the 1940s, many severe and fatal diseases became curable. However, misuse and overuse led to the rapid spread of antibiotic resistance. Many different bacterial species are resistant to antibiotics, and in some cases to more than one class of antibiotic [WHO, 2002]. Within the context of HCAs this resistance is a significant issue as patients are more likely to have an underlying medical condition, and resistance can lead to increased morbidity and death [WHO, 2002].

The continual requirement for antibiotics within healthcare environments increases the likelihood of resistance developing. Resistant bacteria are easily spread between patients, and resistance markers between bacterial species. Every healthcare facility should be able to justify its antibiotic usage, as uncontrolled and inappropriate use amplifies this problem [WHO, 2002]. Other factors which may encourage resistance are under-dosing due to antibiotic shortage, empiric prescribing due to lack of microbiological identification and lack of suitable alternate agents [WHO, 2002].

For the first time, the 2011 point prevalence survey monitored Antimicrobial Usage (AMU) and it was found that prescription levels were 34.7% of patient admissions with the highest usage in paediatrics (44.7%) [HPA, 2012]. Usage was lowest in the over 80's suggesting that the policy of reducing antimicrobial usage to prevent CDI was effective [Fowler *et al.*, 2007]. The most common antibiotics prescribed were beta-lactams and enzyme inhibitors. Table 1.3 shows the 12 most frequently used antimicrobial classes in English hospitals [HPA, 2012].

Table 1.3: Most Frequently Used Antimicrobial Classes in English Hospitals [HPA, 2012].

Antimicrobial Class	Percentage Use (%)
Combinations of penicillins, including beta-lactamase inhibitors	23.1
Beta-lactamase resistance penicillins	7.4
Aminoglycosides	6.8
Macrolides	6.3
Penicillins with extended spectrum	4.9
Imidazole derivative	4.8
Glycopeptide antibacterials	4.5
Beta-lactamase sensitive penicillins	4.5
Carbapenems	4.5
Trimethoprim and derivatives	4.2
Second-generation cephalosporins	3.5
Fluoroquinolones	3.3
Other classes	22.2

As this was the first time AMU surveillance was carried out it will provide a baseline for future monitoring. Figure 1.4 shows the AMU by diagnosis for hospital acquired infections.

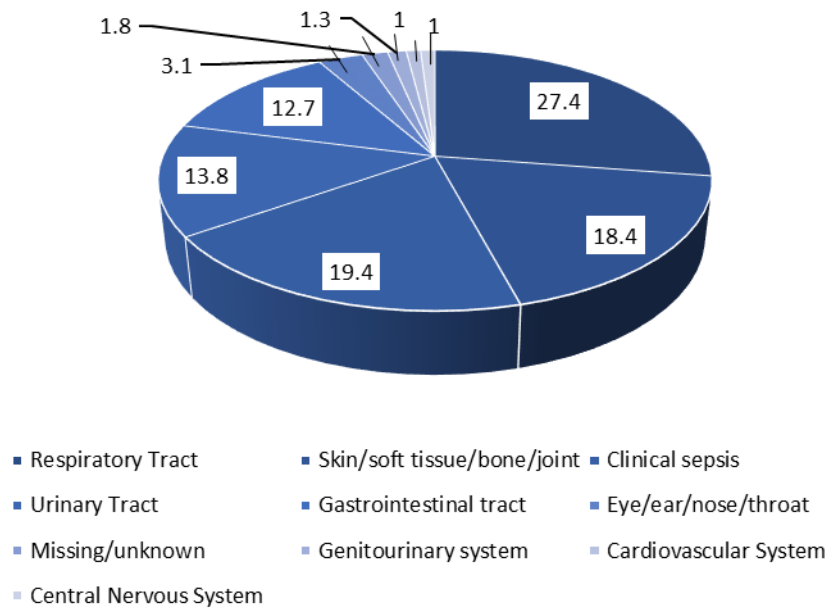


Figure 1.4: Percentage of Antimicrobial Usage by Diagnosis for Hospital Acquired Infections in England [Adapted from HPA, 2012].

***Staphylococcus aureus* as a HCAI**

Staph. aureus is one of the most prominent bacterial pathogens. Although found as a commensal on ~20% of the population, it causes a huge range of superficial to life threatening infections (Table 1.4) [Cassat *et al* 2007]. Carriage on skin allows easy access to open wounds, allowing rapid colonisation and subsequent biofilm formation. This may explain why so many diseases are caused by *Staph. aureus* [Archer *et al.*, 2011].

The ability of *Staph. aureus* ability to form biofilms on both natural tissues and indwelling medical devices [Cassat *et al.*, 2007] has elevated the species to a major concern especially when combined with its antibiotic resistance profile. Examples of biofilm related diseases are osteomyelitis, infections on indwelling devices, periodontitis and peri-implantitis, chronic wound infection, chronic rhinosinusitis, endocarditis, and ocular infections [Archer *et al.*, 2011].

Table 1.4: *Staphylococcus aureus* Diseases, Pathogenesis, Transmission, Treatment and Prevention Controls [Adapted from Goering *et al.*, 2008].

Diseases	Boils, Skin and soft tissue infections, Postoperative wound infections, Scalded skin syndrome, Catheter associated infections, Food borne infections, Septicaemia, Endocarditis, Toxic shock syndrome, Osteomyelitis, Pneumonia
Pathogenesis	Virulence is multifactorial. Present in all strains: mucopeptide and coagulase. Present in some strains: cell-associated: capsule, protein A, fibronectin-binding protein, collagen binding proteins. Extracellular: enterotoxins, epidermolysis toxin, membrane damaging toxins (haemolysins) leucocidin, staphylokinase Many strains have protein A bound to the cell wall which interacts with host IgG antibodies reducing opsonisation and causing local activation of complement.
Transmission	Normal habitat humans (and animals associated with them) skin especially nose and perineum (carriage rates higher in hospital patients and staff). Spread by contact and air borne routes. Organism survives drying and is tolerant of salt and nitrites.
Treatment/ Prevention	In susceptible strains beta-lactamase-stable penicillins however, vast majority of hospital isolates are resistant. Prevention of spread by isolation and/or treatment of carriers in high risk areas in hospitals.

Staph. aureus biofilms on indwelling devices cause millions of device infections and failures every year. Devices include prosthetic joints and related apparatus, stents, ventilators, infusion pumps, catheters, cosmetic surgical implants, stitch material and mechanical heart valves to name but a few. Treatment nearly always requires removal and replacement of the device to completely resolve the infection [Archer *et al.*, 2011].

Further difficulty in treating these infections arises from the development of antibiotic resistance. The most common resistance of *Staph. aureus* is against penicillin, methicillin and other beta-lactams [David and Daum, 2010]. However, there is

resistance against other antibiotic classes, most worrying vancomycin, which is one of the last effective antibiotics against MRSA [Courvalin, 2006]. Without effective antibiotics to treat otherwise minor diseases, worsened patient outcomes could result in corresponding increased costs and potential fatalities [Lauderdale *et al.*, 2010].

Whilst MRSA is often contracted within healthcare environments by patients with pre-disposing risk factors, there are increasing numbers of Community Associated MRSA (CA-MRSA) cases being reported [David and Daum, 2010]. Prevalence of CA-MRSA strains is not uniform geographically and has been reported at ~50% of total MRSA cases in parts of the USA [Popovich *et al.*, 2008]. CA-MRSA is considered a problem in most high income countries [DeLeo *et al.*, 2012]. CA-MRSA first appeared in the 1990s and tends to present in young, otherwise healthy individuals. Most cases are skin and soft tissue infections but necrotizing pneumonia and severe sepsis has also been recorded [Naimi *et al.*, 2003].

However, it is notable that control and prevention measures are implemented on both local and national scales with mandatory reporting of MRSA cases and these schemes have successfully decreased recorded MRSA cases (Figure 1.3a) [HPA, 2012].

Other Hospital Acquired Infections

Much attention has been paid to *Staph. aureus* and *C. difficile* in recent years however, there are other bacterial species which may cause HCAs.

Table 1.5 shows a few examples of relevant species, the diseases they cause, their pathogenesis, transmission routes and what treatments are commonly used [Goering *et al.*, 2008].

Table 1.5: Diseases, Pathogenesis, Transmission, Treatment and Prevention of some HCAs [Adapted from Goering *et al.*, 2008].

Species	Disease	Pathogenesis	Transmission	Treatment/Prevention
<i>Staphylococcus epidermidis</i> Coagulase Negative Staphylococci (CoNS)	Opportunist pathogen associated with device related sepsis (catheters, prosthetic valves, artificial joints, and shunt infections). UTIs, endocarditis sternal wound, osteomyelitis.	Extracellular slime production may be a virulence marker and aid in colonization of indwelling devices.	Normal habitat skin (100%) spread by contact with self, other patients or hospital personal. Almost all infections are from hospital but may be endogenous.	Often multi-resistant (including penicillin and methicillin). Presence within healthcare environments is increasing. Prevention of infection relies on device care esp. catheters.
<i>Staphylococcus saprophyticus</i> (CoNS)	Urinary tract infections in otherwise healthy women.	Ability to colonise periurethral skin and mucosa.	Normal habitat: skin and genitourinary mucosa. Endogenous spread.	Treatment usually with quinolones.
<i>Streptococcus pneumoniae</i>	Pneumonia (usually from capsular type III), septicaemia and meningitis. Otitis and related infections in children.	Capsule protects organism from phagocytosis. Pneumolysin may have a role as virulence factor. Viral infection may be precursor to pneumonia.	~5% of population may carry species in small numbers. Normal habitat is respiratory tract. Transmission via droplet spread.	Antibiotic of choice is penicillin but resistance is rapidly increasing. Tests should guide treatment. Vaccine available.

Table 1.5 continued.

Species	Disease	Pathogenesis	Transmission	Treatment
<i>Streptococcus mutans</i>	Dental caries but can cause endocarditis in immune compromised patients.	Largely unknown but able to survive environmental fluctuations.	Endogenous infections.	Most strains susceptible to penicillin but resistance has been recorded. Good oral hygiene prevent caries.
<i>Enterococcus faecalis</i>	UTIs, endocarditis, Septicaemia after surgery and in immune compromised (rare).	Plasmid mediated haemolysin may play a role. No other virulence factors recorded	Normal habitat is human and animal gut. Most infections are endogenous acquired but cross contamination may occur in hospitals.	Penicillin used in combination with aminoglycosides. Resistance to vancomycin is problematic. Patients with heart defects should be given prophylactic antibiotics.
<i>Escherichia coli</i>	UTI, diarrheal disease, neonatal meningitis, septicaemia. Possesses O (somatic), H (flagellar), F (fimbrial) antigens which are used for classification	Endotoxin (present in all strains). Adhesins: pili and colonization factors associated with GI tract infections. Capsule present in some strains: neonatal meningitis. Enterotoxins associated with diarrheal disease	Normal habitat is human and animal gut. Spread is by contact and through the faecal-oral route. May be food associated or endogenous.	Wide range of antibiotics can be used but resistance is variable, usage must be determined by susceptibility tests. Specific treatment of diarrheal disease not usually required.

Table 1.5 continued.

Species	Disease	Pathogenesis	Transmission	Treatment
<i>Klebsiella pneumoniae</i>	Opportunist UTI and respiratory infections in the compromised host, Distinction between colonization and infection can be difficult.	Usually capsular. Endotoxin and fimbriae or other adhesins. Capsules are important for inhibiting phagocytosis.	Normal habitat: human and animal gut, moist environment esp. soil and water. Infection can be endogenous or acquired by contact.	Multiple antibiotic resistance, usually plasmid mediated. Susceptibility must be determined. Prevention depends on aseptic techniques and hand washing
<i>Pseudomonas aeruginosa</i>	Opportunist pathogen which can infect almost any site providing the conditions are favourable. Causes skin and wound infections. Major pathogen in cystic fibrosis and burn wards and can cause pneumonia in intubated patients. Can also cause UTIs, septicaemia, osteomyelitis and endocarditis	Virulence factors: endotoxin and exotoxin A. extracellular polysaccharide capsule prevents phagocytosis (massive amounts of alginate produced by some strains). Pigments may have a role in virulence and pyoverdin acts as a siderophore.	Carriage occurs naturally in a small percentage of normal healthy individuals, this percentage increases in hospital inpatients. Infection may be endogenous. Widespread in moist environments. Patients usually become infected via direct or indirect contact spread from these sites.	Naturally resistant to many antibiotics and resistance can develop during therapy. Combination treatment (e.g. aminoglycoside and beta-lactam) based on susceptibility tests is required. Prevention depends on good aseptic practice, avoiding prolonged broad spectrum antibiotic treatment and prophylaxis use.

Difference in Cell Types – Persisters, Small Colony Variants and Biofilms

Infection, and resolution of infection happens continuously within a system, usually with no lasting adverse effects. However, on occasion especially within individuals with a compromised immune system, infections do not resolve even after treatment with antibiotics. There are various phenotypes and cell states which are considered responsible for underpinning these chronic infections: persister cells, small colony variants (SCV) and biofilms.

Persisters

Persisters were first recognised in 1944 after a small percentage of a *Staph. aureus* culture were found to survive treatment with penicillin [Bigger, 1944]. Persister cells are described as genetically identical to susceptible bacteria however, are in a non-growing state and thus able to tolerate antibiotics [Kint *et al.*, 2012]. The persister cell type can be triggered randomly or by specific environmental conditions [Dörr *et al.*, 2009; Vega *et al.*, 2012]. As the cells are genetically identical, once the selective pressure of the antibiotic has been removed the cells revert back to a metabolically active state, reseed the original population and regain sensitivity to antibiotics [Bigger, 1944; Lewis, 2005]. This provides a basis for recurrent infections [Kint *et al.*, 2012].

Persister concentrations depend upon the growth phase of the culture, amounts are very low in exponentially growing cultures but increase as the culture progresses towards stationary phase [Kint *et al.*, 2012]. Persisters are found in both biofilms and planktonic cultures and make up ~1% of the population [Lewis, 2007]. The basis behind persisters was thought to be their dormancy; that metabolic and biosynthesis pathways, energy production and non-essential genes were downregulated leading to inactive antibiotic targets [Kint *et al.*, 2012]. However, this passive dormancy cannot account entirely for the persister cell type [Allison *et al.*, 2011] and recent studies suggest that there is active suppression of oxidative stresses caused by bactericidal antibiotics [Grant *et al.*, 2012; Kint *et al.*, 2012; Nguyen *et al.*, 2011].

Small Colony Variants (SCV)

SCVs were first identified in 1910 as abnormal colony forms of *Salmonella enterica* serovar Typhi (then known as *Eberthella typhosa*) [Jacobsen, 1910; Proctor *et al.*, 2006]. They were subsequently discovered in *Staph. aureus* by Swingle [1935] and were thought to be a stage in the bacterial lifecycle.

Staph. aureus SCVs are characterised by small colonies ($1/10$ of a wild type colony size), reduced respiration, reduced pigmentation & metabolic activity (including coagulase) and decreased haemolysis on blood-containing agar which can lead to complications and misdiagnosis in routine laboratory tests [Kipp *et al.*, 2005]. Upon subculture the SCVs switch back to the wild type phenotype [Kahl, 2014].

The change from a normal colony phenotype to a SCV is likely due to a constitutive mechanism which generates an antibiotic tolerant subpopulation and can be considered a normal part of the *Staph. aureus* life cycle [Edwards, 2012].

Defects in metabolism may account for SCV formation. Two main groups have been identified from *Staph. aureus* clinical isolates, those deficient in electron transport and those deficient in thymidine or menadione biosynthesis [Acar *et al.*, 1995]. The electron transport defective SCVs cannot produce menadione or haemin and the addition of either of these two substances can return the bacterial strain to a wild type phenotype [Proctor *et al.*, 2006]. Occasionally other SCVs have been isolated which are CO₂ auxotrophs or for which the auxotrophism cannot be identified [Goudie and Goudie, 1955].

SCVs have been identified in a broad range of bacterial species including *Staph. epidermidis*, *Staph. capitis*, *Ps. aeruginosa*, *Burkholderia cepacia*, *Vibrio cholera*, *E. coli*, *Lactobacillus acidophilus* and *Salmonella* species [Proctor *et al.*, 2006].

Aggressive infections have been reported in both human and animals though it is more usual that a subacute, persistent, antibiotic resistant, infection is caused by SCVs. It has been suggested that intracellular SCVs may contribute to the difficulty in clearing *Staph. aureus* from patients leading to these recurrent infections [Proctor *et al.*, 1995; Proctor *et al.*, 2006].

As detailed above SCVs have defects in metabolism whereas persister cells have no specific resistance mechanisms and are considered genetically identical to susceptible bacteria [Lewis, 2005; Proctor *et al.*, 2006]. They are believed to be

individual cell types though they may act in the same way and produce a similar result, for example in antibiotic tolerance and contributing to biofilm formation and its resulting resistance [Singh *et al.*, 2009].

Biofilms

The third, and most important cell state which contributes to chronic, recurrent infections are biofilms. Biofilms may contain both SCVs and persister cells.

A biofilm can be defined as a microbially-derived largely sessile community with cells attached to a surface and each other, and embedded in a protective extracellular polymeric matrix [Costerton *et al.*, 1987]. Such cells have altered growth rates, gene expression, metabolic activity and protein production [Archer *et al.*, 2011; Lister and Horswill, 2014]. Biofilms are constructed by multiple different bacterial and fungal species, causing both individual and multispecies formations [Burmølle *et al.*, 2014]. Biofilms can be found in virtually every situation where there is an environmental interface [Costerton *et al.*, 1999].

Aqueous channels flow through the biofilm structure, allowing transport of nutrients and waste materials as the biofilms can be anything from one cell layer to a substantial community [Costerton *et al.*, 1995; Hogan *et al.*, 2015]. The shape of a Gram-positive biofilm usually differs from that of a Gram-negative biofilm, tending to be flatter due to the non-motile cells which make up the biofilm. However, fluid shear forces and other environmental conditions can lead to the development of tower-like structures [Costerton *et al.*, 1995; Mann *et al.*, 2009].

Differences in oxygen, nutrients and electron acceptor concentrations cause heterogeneous gene expression within a biofilm. Gene expression can even be different in neighbouring cells [Brady *et al.*, 2007]. This has led to the identification of different cell states. The cells on the outer surface of a biofilm are metabolically active and growing aerobically, cells further in the biofilm are either growing fermentatively, dormant (persister or very slow growing cells) or dead [Rani *et al.*, 2007].

The lifecycle of a biofilm can generally be split into four phases – 1) Attachment, 2) Cell Accumulation, 3) Biofilm Maturation and 4) Detachment & Dispersal (Figure 1.5).

Attachment

Initial attachment occurs when a planktonic cell settles on a surface, if not dislodged the cell will bind irreversibly to the surface [Lister and Horswill, 2014]. The surface becomes coated in proteinaceous components which encourages further cell adhesion [Francois *et al.*, 2000]. Production of extrapolymerized polysaccharide (EPS) and eDNA will start at this stage and begin to form the biofilm matrix [Gloag *et al.*, 2013]. Some of the most important protein components are the Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs); these proteins have major roles in attaching to host factors including fibrinogen, fibronectin and collagen, promoting cell accumulation and microcolony assembly [Patti *et al.*, 1994].

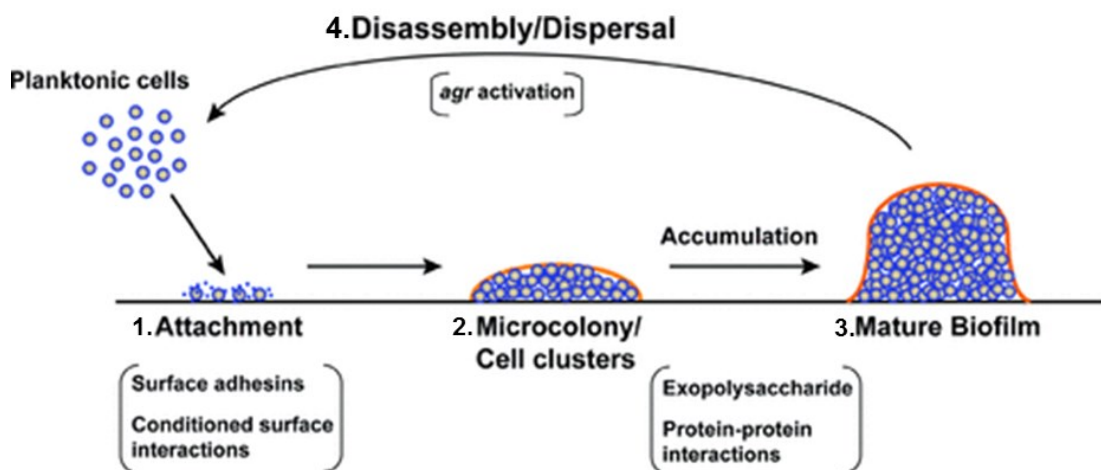


Figure 1.5: Construction and Dispersal of a Staphylococcal Biofilm [Kiedrowski and Horswill, 2011]. The lifecycle of a biofilm can be split into 4 phases: 1) Attachment of planktonic cells to a conditioned surface, 2) Accumulation of cells to form microcolonies which, under optimal conditions can, 3) Develop into a mature biofilm with an exopolysaccharide matrix and protein-protein interactions. The final stage 4) The biofilm breaks down leading to the dispersal of cells which regain their planktonic characteristics allowing dissemination to new sites.

Cell Accumulation

After initial attachment cells start to accumulate, forming microcolonies. Some sources consider this stage to be the early part of biofilm maturation, as there are no specific properties associated with it. However, *in vivo* microscopy studies on staphylococcal biofilms have found evidence of these small microcolonies [Stoodley *et al.*, 2008]. With optimal growth conditions these microcolonies, along with further cells accumulating, develop into a mature biofilm with all the corresponding properties.

Biofilm Maturation

After the cells have accumulated, and with the exopolysaccharide (EPS) matrix becoming more established, protein-protein interactions complete the maturation of the biofilm formation. Most reports indicate that the EPS matrix is composed primarily of polysaccharides [Cramton *et al.*, 1999], proteins [O'Neill *et al.*, 2008] and extracellular DNA (eDNA) [Rice *et al.*, 2007]. However, the exact composition of the matrix is subject to change and is species and strain dependent [Kiedrowski and Horswill, 2011].

There are two distinct biofilm forms which have been identified from clinical isolates of *Staph. aureus* and *Staph. epidermidis* [Knobloch *et al.*, 2001; Rohde *et al.*, 2005]. The first is mediated by the intercellular adhesion operon (*icaADBC*) which encodes the enzymes involved in the synthesis of polymeric *N*-acetyl-glucosamine (PNAG) and polysaccharide intercellular adhesin (PIA), these are considered *ica*-dependent biofilms [Cramton *et al.*, 1999]. The second form is facilitated by the fibronectin-binding proteins (FnBPs) which are *ica*-independent biofilms [Houston *et al.*, 2011; O'Gara, 2007].

ica-dependent Biofilms

It has been reported that 85% of *Staph. epidermidis* blood culture isolates contain the *ica* genes [Ziebuhr *et al.*, 1997]. The gene cluster (*icaADBC*) can be considered a marker to discriminate between clinically significant infecting strains or contaminating

isolates [Frebourg *et al.*, 2000]. It has also been shown that PNAG/PIA has a role as a virulence factor for *Staph. epidermidis* [Vuong *et al.*, 2004].

The majority of *Staph. aureus* strains contain the *icaADBC* operon which is upregulated under *in vivo* conditions. However, its expression is highly regulated in an *in vitro* setting, requiring stringent conditions such as low oxygen [Fluckiger *et al.*, 2005].

The *icaADBC* encodes four genes: *icaA*, *icaB*, *icaC*, and *icaD*. *icaA* and *icaD* which collectively produce PIA which facilitates cells binding together and forming into biofilms [Oliveira and Cunha, 2008].

PIA is produced from UDP-N-acetylglucosamine through the enzyme N-acetylglucosamine transferase which is encoded by *icaAD* within the cellular membrane (Figure 1.6). This forms oligomers with between 10 and 20 sugar residues [Vuong *et al.*, 2004]. When *icaAD* is co-expressed with *icaC* chains of up to 130 sugar residues can be synthesised [Gerke *et al.*, 1998]. *icaC* also exports the growing oligomer to the cell surface where it is deacetylated [Vuong *et al.*, 2004].

The final gene *icaB* produces a surface bound protein which is responsible for the deacetylation of the poly-N-acetylglucosamine molecule. It has been proven that non deacetylated poly-N-acetylglucosamine in an *icaB* mutant strain cannot bind to a bacterial cell surface or facilitate biofilm maturation [Vuong *et al.*, 2004].

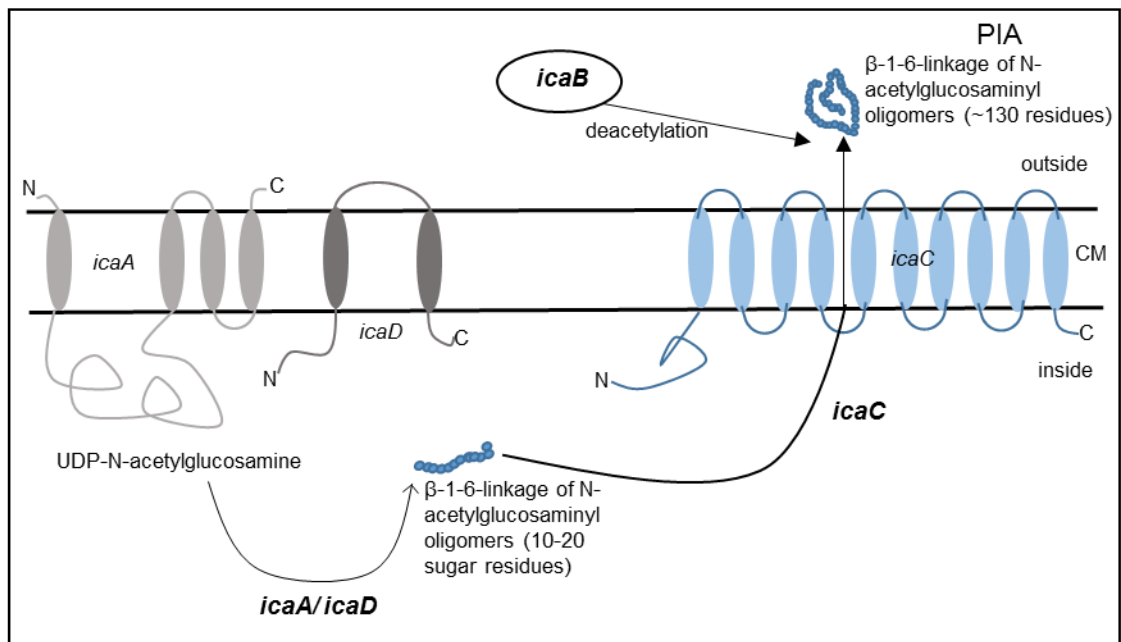


Figure 1.6: Model of *ica*-dependent Staphylococcal Biofilm Formation [Adapted from Oliveria and Cunha, 2008]. *IcaA* and *icaD*, work together to produce oligomers of 10- 20 sugar residues which are derived from UDP-N-acetylglucosamine. Only in the presence of *IcaC* can longer oligomers (of up to 130 sugar residues) be synthesised. *IcaC* also translocates the growing oligomer to the cell surface where it deacetylated by the surface bound protein *icaB*.

ica-independent Biofilms

Although the vast majority of clinical *Staph. aureus* biofilms carry the *icaADBC* gene cluster, there have been growing reports of the species producing *ica*-independent biofilms [Boles and Horswill, 2008; Fitzpatrick *et al.*, 2005]. These biofilms are mediated by FnBPs, the cell wall components MSCRAMMs and teichoic acids [Houston *et al.*, 2011]. Mutations in the two FnBPs (A and B), which have been identified to be important in biofilm development, can completely prevent biofilm formation [O'Neill *et al.*, 2008]. Single mutations do not impair formation as the proteins are independently transcribed [Jönsson *et al.*, 1991]. The FnPBs are large functional proteins binding not only to fibronectin but also elastin and fibrinogen. The subdomain N2N3 of FnBPA plays an essential role in binding to fibrinogen and promoting bacterial adhesion [Geoghegan *et al.*, 2013; Keane *et al.*, 2007]. This is a two step process, the first step is FnBPA proteins encourage attachment to a suitable surface (1 on Figure 1.7). The second step involves biofilm accumulation through cell

aggregation (2 on Figure 1.7). Whether homophilic bonds or ligand binding is responsible for cell-cell attachment and biofilm formation is yet to be determined as can be seen in Figure 1.7 (3) [Herman-Bausier *et al.*, 2015].

The same process can be found in *Staph. epidermidis* [Rohde *et al.*, 2005] though the mechanism of biofilm formation differs slightly. The proteins which have been strongly linked to biofilm formation are the accumulation associated protein (Aap) [Rohde *et al.*, 2006] and a biofilm associated protein (BAP) homologue; Bap-homologue protein (Bhp) [Tormo *et al.*, 2005]. Mutation of the *bap* gene destroyed the *Staph. epidermidis* strains ability to develop a biofilm, and transforming a biofilm-negative *Staph. aureus* strain with the Bap protein enabled biofilm formation [Tormo *et al.*, 2005].

Once fully developed a biofilm matrix is composed of polysaccharides, proteins and extracellular DNA [Gloag *et al.*, 2013; Mann *et al.*, 2009], though there is significant variation between strains. Other suggested components of the matrix include cell wall-associated components [Wu *et al.*, 2003], and teichoic acids [Sadovskaya *et al.*, 2006]. These and other, as yet unknown, components could be contributing to the dynamic matrix structure, however, more investigation is needed to clarify the exact composition [Kiedrowski and Horswill, 2011].

No strict guidelines exist on what a fully established biofilm is however, the ability to resist antimicrobial treatment is considered a good guide [Kiedrowski and Horswill, 2011].

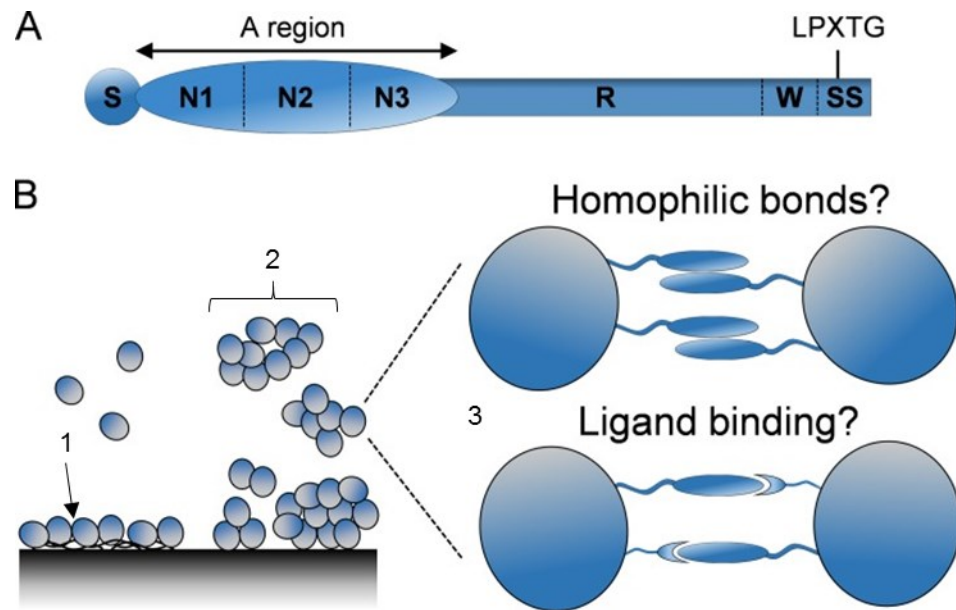


Figure 1.7: *ica*-Independent Biofilm Formation in *Staphylococcus aureus* [Herman-Bausier *et al* 2015]. FnBPA is responsible for biofilm formation in strains which do not contain the *icaADBC* gene cluster. It is the subdomain N2N3 which is the functional part of the FnBPA molecule responsible for fibrinogen and elastin binding and cell-cell aggregation during biofilm formation. A) Schematic representation of the FnBPA protein: S, secretory signal sequence; the A region comprising N1, N2 and N3 subdomains; R, tandem repeats of fibronectin-binding domains; W, proline-rich cell wall spanning region; SS, sorting signal comprising the LPXTG motif, membrane-spanning domain and positively charged tail. B) FnBPA encourages attachment to a surface (1) followed by cell accumulation and biofilm formation (2). The role of FnBPA in biofilm formation is thought to be to promote cell-cell attachment though either homophilic bonds or ligand binding (3).

Detachment and Dispersal

Once the biofilm is fully developed it provides protection and advantages over a planktonic cell state (detailed below) and is extremely difficult to dislodge. Shear forces may rarely have an effect, however, specific bacterial mechanisms need to be activated for the biofilm to fully disperse [Cassat *et al.*, 2007].

The best characterised mechanism in staphylococcal species is the accessory gene regulator (*agr*) of the quorum sensing system. Unlike in *Pseudomonas aeruginosa* [O'Loughlin *et al.*, 2013], an active quorum sensing system in *Staph. aureus* inhibits the development and encourages the detachment and dispersal of a biofilm [Davis *et*

al., 1998; Vuong *et al.*, 2000]. Recsei *et al* [1986] first described the *agr* gene locus and since then ~150 genes have been shown to be regulated by it. At least 16 of these are involved in virulence [Gospodarek *et al.*, 2009; Lyon and Novick, 2004].

As can be seen from Figure 1.8, activation of the *agr* system increases the production of extracellular proteases which affect biofilm development, indeed bacterial cells from a recently dispersed biofilm show very high levels of *agr* activity [Boles and Horswill, 2008]. The extracellular proteases degrade the protein adhesins which bind the individual cells within the biofilm matrix thereby releasing the cells back to their planktonic state [Boles and Horswill, 2011; Vuong *et al.*, 2000].

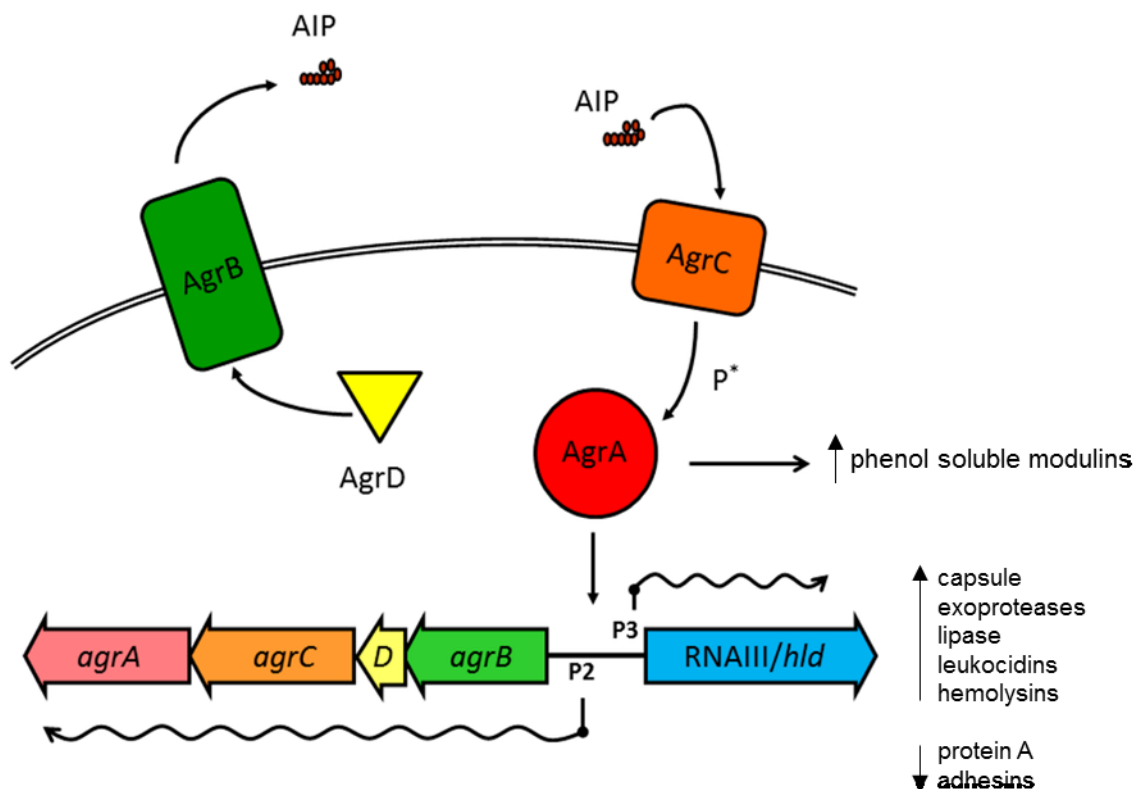


Figure 1.8: The Function of AutoInducing Peptide (AIP) and the *agr* Quorum Sensing System in *Staphylococcus aureus* [Gray *et al.*, 2013]. The cyclic AIP activates the AgrCA two-component system increasing expression of virulence factors including exoproteases, lipase, leukocidins and hemolysins. Biofilm disassembly is also triggered. AgrB acts as a chaperone protein and is a multifunctional endopeptidase. AgrD is processed by AgrB into AIP. AgrC is the integral membrane sensor and AgrA is the transcription factor regulator companion to AgrC. This molecule acts to upregulate *agr* and RNAIII expression. RNAIII operates to modulate gene expression through post-transcriptional control.

Advantages of Biofilms

There are a number of advantages biofilms confer over the planktonic cell phenotype. The major ones are resistance to antimicrobials, antiseptics & disinfectants, prevention of clearance by immune responses from the host and protection from environmental conditions [Archer *et al.*, 2011; Mann *et al.*, 2009]. Clearance methods for biofilms include use of antifouling agents, shear stress, host phagocytic elimination, host radical and protease defences [Archer *et al.*, 2011].

The polymeric matrix provides a structural and protective physical barrier against immune responses, environmental conditions such as desiccation and extended periods of reduced nutrients, and is a contributing factor to antibiotic resistance as it slows the inward diffusion of the antibiotics [Lewis, 2005; Hogan *et al.*, 2015; Mann *et al.*, 2009; Xu *et al.*, 2000].

The low nutrient and oxygen concentrations within a biofilm leads to a semi-dormant cell phenotype with low metabolic activity and cell division rates which forms part of the basis for antibiotic resistance within biofilms [Lewis 2005]. Persister cells begin to dominate the population. However, once the selective pressure of the antibiotic is removed the persisters regain a normal phenotype and can re-ignite infection [Lewis, 2005].

Molecular Mechanisms of Biofilm Resistance

Resistance of biofilms is multifactorial, with the different factors acting in concert with each other [Burmølle *et al.*, 2014]. These factors include changes in the chemical microenvironment, reduced diffusion rates of compounds into the matrix, altered gene expression patterns and low or stalled growth rates [Mah and O'Toole, 2001; Van Acker *et al.*, 2014]. The biofilm matrix, as it may consist of up to 97% water, provides protection from desiccation and other environmental stresses [Flemming and Wingender, 2010]. Horizontal gene transfer rates are higher than in planktonic

cultures, due to the close proximity of the cells, leading to increased resistance due to the mobile genetic elements which can be transferred. These mobile elements can be shared between bacteria of the same species or between different compatible species [Madsen *et al.*, 2012].

A class of genetic element which may be transferred in this way are efflux pumps. Efflux pump genes and proteins are present in all organisms [Gillis *et al.*, 2005] with the genes located on the chromosome or on transferable genetic elements [Piddock, 2006]. Efflux pumps can transport either a specific substrate or a group of structurally dissimilar compounds such as different classes of antibiotics (Figure 1.9). Efflux pumps are relevant in the health care environment as they can confer reduced antibiotic susceptibility and clinical levels of antibiotic resistance to multiple species [Hooper, 2005].

There are five families of efflux pumps: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family [Piddock, 2006]. The classifications of the efflux pumps into these families are based on the number of components a pump has, the number of transmembrane-spanning regions the transporter protein has, the energy source a pump uses and the types of substrates a pump exports (Figure 1.9) [Piddock, 2006].

Efflux pumps prevent antibiotics from entering the cell and also actively export antibiotics out of the cell. The development of antibiotic resistance may be a side effect of the normal physiological role of the pumps in transporting molecules produced by the host organism (e.g. bile) out of the cell allowing the bacteria to survive [Piddock, 2006].

Other mechanisms which may contribute to biofilm resistance are matrix polysaccharides which limit the penetration of antibiotics [Van Acker *et al.*, 2014], presence of persister cells [Lewis, 2010] and protection against oxidative stresses [Dwyer *et al.*, 2009].

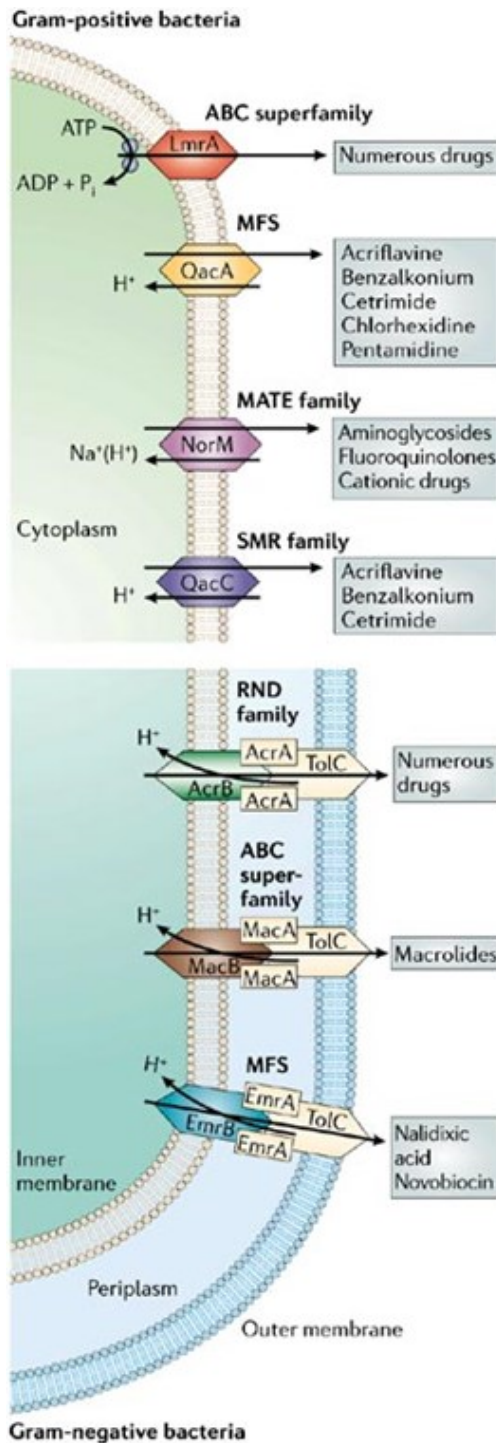


Figure 1.9: Multidrug Resistance Efflux Pumps in Bacteria [Pidcock, 2006]. There are five families of multidrug resistance efflux pumps in bacteria: The ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family. A diagrammatic representation is shown, common examples of the proteins which form each class and examples of transported substrates (including antibiotics) are indicated.

Multispecies Biofilms

Multispecies biofilms have been described for bacterial species found in clinical and human situations such as the oral environment [Kolenbrander *et al.*, 2010]. They have also been identified in all of non-host environments including soil, sea water, boat hulls and dairy production processes [Burmølle *et al.*, 2014; Schwering *et al.*, 2013].

In an example of organisms of medical importance, mixed species biofilms consisting of *Staph aureus* and *Candida albicans* have been studied and they were found to exhibit enhanced virulence and resistance when co-infecting mouse models [Shirliff *et al.*, 2009]. Other examples of where mixed species biofilms of importance occur in healthcare facilities are in combat acquired and chronic wounds with *Staph. aureus* and *Ps. aeruginosa* [Dean *et al.*, 2015], diabetic ulcers [Dowd *et al.*, 2008], colonisation of urinary catheters, dental plaque [Yang *et al.*, 2011] and cystic fibrosis associated infections [Elias and Banin, 2012].

Multispecies biofilms act together in a symbiotic way [Burmølle *et al.*, 2014]. The mechanisms of biofilm protection and resistance mentioned before are often enhanced [Burmølle *et al.*, 2006]. Therefore, this information should be taken into account when deciding treatment regimens and cleaning procedures [Burmølle *et al.*, 2014] as species may cross-protect each other leading to treatment failure [Bridger *et al.*, 2012].

Biofilm Infections

Biofilms contribute to persistent chronic infections, eradication is arduous, often requiring physical intervention such as surgical debridement or replacement of the affected device [Archer *et al.*, 2011]. Table 1.6 details a few examples of medical infections caused by biofilms. This is by no means an exhaustive list. Estimates suggest that up to 65% of HCAs are a direct result of biofilms [Costerton *et al.*, 1999].

Removal and long term antibiotic treatment are the current first options when dealing with biofilms on indwelling devices however, attempts are being made to move away

from this as it is costly, time consuming and reduces the patient's quality of life [Archer *et al.*, 2011]. New and different therapy methods are required to treat biofilms and alternative approaches are currently under investigation.

Table 1.6: Examples of Medical Infections Caused by Biofilms [Adapted from Costerton *et al.*, 1999].

Causative Organism	Infection/Disease
<i>Streptococcus</i> spp.	Dental caries
<i>Haemophilus influenzae</i>	Otitis media
Gram-positive cocci (<i>Staphylococcus</i>)	Musculoskeletal infections
Group A strep	Necrotizing fasciitis
Enteric Bacteria	Biliary tract infection
Various bacteria and fungi – often mixed	Osteomyelitis
<i>E. coli</i> and other Gram-negatives	Bacterial prostatitis
Viridians group streptococci	Native valve endocarditis
<i>P. aeruginosa</i> and <i>Burkholderia cepacia</i>	Cystic fibrosis pneumonia
<i>Pseudomonas pseudomallei</i>	Melioidosis
<i>Staph aureus</i> and <i>Staph epidermidis</i>	Sutures
<i>Staph aureus</i> and <i>Staph epidermidis</i>	Arteriovenous shunts
<i>Ps. aeruginosa</i> and Gram-positive cocci	Contact lens
<i>E. coli</i> and other Gram-negative rods	Urinary catheter cystitis
Variety of bacteria and fungi	Peritoneal dialysis (CAPD) peritonitis
Variety of bacteria and fungi	Endotracheal tubes
<i>Staph epidermidis</i> and others	Central venous catheters
<i>Staph aureus</i> and <i>Staph epidermidis</i>	Mechanical heart valves
Gram-positive cocci	Vascular grafts
Enteric Bacteria and Fungi	Biliary Stent blockage
<i>Staph aureus</i> and <i>Staph epidermidis</i>	Orthopaedic devices

Antibiotics

One of the major categories of biofilm infections in hospitals is device related infections such as orthopaedic implants. A common practice to try to prevent prosthetic joint infections is the use of Antibiotic-Loaded Acrylic bone Cements (ALACs). Gentamicin is the most common antibiotic in use in bone cement in Europe because of its broad antibacterial spectrum and stability at the high temperatures which are required to polymerize the polymethylmethacrylate [Neut *et al.*, 2005]. Due to resistance to gentamicin the bone cements may also contain another antibiotic such as clindamycin (Refobacin Revision®/Copal G+C®) or vancomycin (Vancogenx®) [Gallo *et al.*, 2013].

A third antibiotic, such as linezolid, daptomycin or ciprofloxacin, is sometimes mixed into the bone cement, though there is little evidence for increased effectiveness of individual or combination antibiotic treatments. Thus the antibiotics chosen are common ones in current practice which have proven effective [Nandi *et al.*, 2009; Taggart *et al.*, 2002]. Detailed below are the usual routes of administration, modes of action, effect on biofilms and resistance mechanisms of gentamicin, rifampicin, vancomycin, ciprofloxacin, clindamycin, linezolid and daptomycin.

Gentamicin

Gentamicin is a bactericidal member of the aminoglycoside family of antibiotics whose mode of action involves protein mistranslation through the incorporation of incorrect amino acids into peptides. A secondary effect of this is the inclusion of these mistranslated proteins into the cytoplasmic membrane resulting in increased permeability and uptake of the antibiotic into the cell [Davis *et al.*, 1986].

Aminoglycosides are primarily used for the treatment of staphylococci, other Gram-positives and Gram-negative aerobic bacilli infections [Ramirez and Tolmasky, 2010]. When used against Gram-positives, another antibiotic (β -lactam or vancomycin) is often combined with a synergistic effect [Ramirez and Tolmasky, 2010]. Anaerobic species are naturally resistant as the uptake of aminoglycosides into the bacterial cell requires oxygen [Byran *et al.*, 1979].

Administration of aminoglycosides is through injection; intramuscular or in the most severe cases, intravenously. Oral administration is not possible due to the low adsorption levels. Side effects of these antibiotics are nephrotoxicity and ototoxicity which can limit the dose given [Ramirez and Tolmasky, 2010]. Kidney function needs to be monitored especially in older patients or those with impaired renal functions [NHS, 2010].

There are several recognised bacterial resistance mechanisms against aminoglycosides; more than one can be present in the same cell [Houghton *et al.*, 2010]. The mechanisms include modification of the antibiotic binding target by mutation of the ribosomal proteins [Galimand *et al.*, 2005], reduced membrane permeability [Hancock, 1981], active export (usually Gram-negatives) [Aires *et al.*, 1999], sequestration of the antibiotic (usually Gram-negatives) [Magnet *et al.*, 2003] and most importantly enzymatic inactivation of the antibiotic. This last mechanism is the most common in the clinical environment [Ramirez and Tolmasky, 2010].

Enzymatic inactivation of aminoglycosides was first reported in 1977 [Davies and Courvalin, 1997] and involved two plasmid mediated enzymes. Since then many more aminoglycoside modifying enzymes have been recorded. These can broadly be split into three groups; the aminoglycoside N-acetyltransferases (AACs), the Aminoglycoside O-nucleotidyltransferases (ANTs) and the Aminoglycoside O-phosphotransferases (APHs). All these enzymes can modify different –OH or –NH₂ groups of the 2-deoxystreptamine nucleus or sugar moieties of the antibiotic [Ramirez and Tolmasky, 2010].

The effectiveness of gentamicin on both biofilms and planktonic cultures has been found to be nutrient dependent [Henry-Stanley *et al.*, 2014] which has serious implications in the clinical environment especially with biofilms found in hard to treat areas such as prosthetic joints. In fact the use of aminoglycosides for treating biofilms may be counter indicated as there have been reports that they promote biofilm formation in both Gram-negative and Gram-positive species [Hess *et al.*, 2011; Hoffman *et al.*, 2005]. Hess *et al.*, [2011] found that biofilm biomass increased when using both sub-inhibitory and inhibitory concentrations of gentamicin in established *Staph. aureus* biofilms. Another aminoglycoside, tobramycin, has been found to have the same effect on *Ps. aeruginosa* and *E. coli* biofilms [Hoffman *et al.*, 2005]. It is thought that the eDNA and extracellular matrix of the biofilm is primarily responsible for this [Hess *et al.*, 2011; Hoffman *et al.*, 2005; Chiang *et al.*, 2013]

Rifampicin

Rifampicin is fairly unusual as it has activity against a wide range of both Gram-positive and Gram-negative bacteria, targeting the bacterial RNA polymerase and inhibiting RNA synthesis [Campbell *et al.*, 2001]. Studies have shown that rifampicin is extremely effective against staphylococci in various growth modes including biofilms, especially when combined with other antimicrobials [Kiedroski and Horswill, 2011; Raad *et al.*, 2007].

The causes behind the efficiency are unknown. However, rifampicin has excellent pharmacokinetic properties, and is lipid soluble which improves penetration into multiple tissue sites [Zavasky and Sande, 1998]. Rifampicin's bactericidal activity remains active under low pH conditions, and it accumulates to high concentrations in neutrophils which are among the first responders to infections [Lam and Mathison, 1983].

Single point mutations or the accumulation of such mutations in the *rpoB* gene which encodes for DNA-dependent RNA polymerase confers a high level of resistance [Aubry-Damon *et al.*, 1998]. Resistance to rifampicin develops quickly, therefore it is always used as part of a combination treatment and never on its own [Zimmerli *et al.*, 2004].

When used to treat biofilm based infections rifampicin has been shown to effectively and uniformly penetrate *Staph. epidermidis* and *Staph. aureus* biofilms, however has a limited effect on cell viability [Croes *et al.*, 2010; Zheng and Stewart, 2005]. Jones *et al* [2001] confirmed this with regards to MRSA biofilms in that rifampicin had no significant effect on biofilm thickness over 24 hours when compared to control cultures, even when the antibiotic concentrations used were well over the MIC and minimum bactericidal concentration (MBC). However, in spite of its limited activity rifampicin is the most common combination drug used to treat biofilms [Jacqueline and Caillon, 2014]

Vancomycin

Vancomycin is a glycopeptide produced by *Amycolatopsis orientalis*, which binds to the peptidoglycan side chains in the cell wall, and is the treatment of choice for invasive staphylococcal infections [Kiedrowski and Horswill, 2011].

During cell wall synthesis, cross linking occurs; vancomycin prevents this leading to a weakened cell wall, reducing growth and eventually causing death of the cell [Kiedrowski and Horswill, 2011]. Long hospital stays are associated with this antibiotic, due to its slow bactericidal activity (significantly only acts on growing cells), and the need for intravenous administration [Chamber and DeLeo, 2009].

Vancomycin monotherapy on biofilms is not effective; though damage to the biofilm does occur the cells are still remain viable [Dunne *et al.*, 1993]. However, combining with other antibiotics, such as tetracycline or rifampicin, leads to increased cell death [Monzón *et al.*, 2002]. Vancomycin has been shown to penetrate effectively into biofilms [Darouiche *et al.*, 1994] and vancomycin concentrations close to the MIC have been shown to prevent the initiation of biofilm formation however, when added to a established biofilm very little effect is seen even at over 100 times the MIC value [Hajdu *et al.*, 2009; Jacqueline and Caillon, 2014; Jones *et al.*, 2001].

There are two major variations for vancomycin resistance, the first is complete resistance due to a plasmid which encodes VanA and accessory proteins. This decreases the cell's binding affinity for vancomycin [Courvalin, 2006]. The second results in intermediate resistance and is caused by regulatory mutations that increase cell wall thickness and teichoic acid D-alanylation [Howden *et al.*, 2010]. For *Staph. aureus* these are termed Vancomycin Resistant (VRSA) or Vancomycin Intermediate (VISA).

Resistance to vancomycin was identified in *Enterococcus* species in the mid-1980s and has since spread rapidly. Resistance remains at a reasonably low level. However, it is transferrable between Gram-positive species thus, there are significant concerns about its emergence and the impact it would have on treating staphylococcal infections [Cetinkaya *et al.*, 2000].

Ciprofloxacin

This is a second generation fluoroquinolone which is active against a wide range of aerobic Gram-positive and Gram-negative bacteria but with limited use against anaerobes [Card *et al.*, 2015]. Ciprofloxacin's mode of action is to target DNA replication and repair by binding to DNA gyrase and inhibiting its action. This induces double strand DNA breaks and causes cell death [Drlica and Zhao, 1997]. A second essential enzyme which is targeted is DNA topoisomerase IV [Drlica and Zhao, 1997]. As eukaryotic cells do not contain DNA gyrase, this antibiotic is selective for bacterial cells.

There are three major, currently recognised, resistance mechanisms. These are mutations that affect the antibiotic targets, mutations that impede concentrations reaching effective levels within the cells, and plasmids encoding genes which protect the bacterial cell from the lethal effects of the quinolones [Jacoby, 2005].

The most commonly seen resistance mechanism are mutations of either the DNA gyrase or topoisomerase IV genes; generally the more resistant a strain the more mutations it contains [Lindren *et al.*, 2003]. In *Staph. aureus* increased expression of *norA*, encoding a broad spectrum transporter keeps ciprofloxacin from reaching effective levels within the cell. This resistance mechanism is also active against a number of other antibiotics, antiseptics, and detergents [Jacoby, 2005]. Plasmid mediated resistance has three variations. In the first the plasmids encode for proteins of the pentapeptide repeat family which protect the DNA gyrase and topoisomerase IV. The second involves acetylation of the antibiotic and the third is plasmid encoded genes which produce enhanced efflux pumps for exporting the antibiotic out of the cells [Jacoby *et al.*, 2014].

The effect of ciprofloxacin on biofilms formed by *Staph. aureus*, *Staph. epidermidis*, *E. coli*, *K. pneumoniae*, *Ps. aeruginosa* and *Proteus vulgaris* was investigated by El-Feky *et al* [2009] who found that ciprofloxacin in combination with N-acetylcysteine inhibited biofilm formation by $\geq 60\%$ compared to the controls. Disruption of established biofilms with ciprofloxacin only has also been found to be effective [El-Feky *et al.*, 2009; Kwiecińska-Piróq *et al.*, 2013]

Clindamycin

Clindamycin is a member of the lincosamide class of antibiotics and has a bacteriostatic effect on the cell through inhibition of protein synthesis [Kohanski *et al.*, 2010]. The mode of action involves blocking the access of peptidyl-tRNAs to the ribosome, subsequent blocking of the peptidyltransferase elongation reaction by steric inhibition and triggering dissociation of the peptidyl-tRNA, [Tenson *et al.*, 2003].

Clindamycin is active against many aerobic Gram-positive cocci, Gram-positive and Gram-negative anaerobes and is available in both intravenous and oral formulations [Lewis and Jorgenson, 2005]. Distribution is good into skin and the antibiotic itself may be able to reduce virulence in staphylococcal species by suppressing production of extracellular virulence factors [Stevens *et al.*, 1988].

Resistance in Gram-negative species is due to mutations in the 23S rRNA encoding gene and alterations in efflux of the drug [Card *et al.*, 2015], whereas resistance in Gram-positives is due to modification of the antibiotic binding site on the ribosome; this also confers resistance to macrolides, azalides, other lincoamides and group B streptogramins and is referred to as 'MLSB resistance' [Roberts *et al.*, 1999]. This inducible resistance has led to uncertainly over prescribing practices. However, with the emerging concern of CA-MRSA, clindamycin is a decent alternative for treatment of various CA-MRSA diseases including skin and soft-tissue, pneumonia and musculoskeletal infections [Lewis and Jorgensen, 2005].

The effect clindamycin has on biofilms varies depending upon the species forming the biofilm. In *Ps. aeruginosa* biofilms sub-inhibitory MICs suppressed biofilm formation [Ichimiya *et al.*, 1994] whereas in MRSA biofilms mean cell survival was as high as 62% [Smith *et al.*, 2008]. For the 12 different clinical isolates of MRSA examined by Smith *et al* [2008] clindamycin's MIC varied from 0.125-1024 µg/ml demonstrating that the effectiveness of the antibiotic can be strain dependent. Of the five antibiotics tested by Smith *et al* [2009], which included clindamycin, daptomycin, linezolid, tigecycline and vancomycin, clindamycin was the least effective against biofilms. Clindamycin's effect may be enhanced when used for combination treatment, for example with tetracycline [Monzón *et al.*, 2001].

Linezolid

This bacteriostatic synthetic oxazolidinone was first approved for use in the UK in 2001 [Ager and Gould, 2012]. Linezolid has proven activity against a broad range of Gram-positive aerobic bacteria including antibiotic resistant species such as MRSA, Vancomycin Resistance Enterococci (VRE) and Penicillin Resistance Pneumococci (PRP). It is also active against some Gram-positive and Gram-negative anaerobes, Mycobacterial species and *Nocardia* species [Mouton and Jansz, 2001].

Protein synthesis is inhibited by linezolid binding to domain V of the 23S ribosomal RNA of the 50S subunit and to the peptidyltransferase centre of the bacterial ribosome [Colca *et al.*, 2003]. Activity against biofilms has been reported in both *in vivo* and *in vitro* models, with better success rates linked to longer antibiotic exposure [Gander *et al.*, 2001]. When combined with rifampicin in prosthetic joint infections, with implant removal the infection was cleared in ~70% of cases [Gomez *et al.*, 2011]. When tested against 12 different clinical isolates of MRSA the MIC range was found to be between 1 and 2 µg/ml with mean survival rates of cells from biofilms of 45% [Smith *et al.*, 2009]. Linezolid can inhibit initial biofilm formation of *Staph. aureus* and *Staph. epidermidis* when added simultaneously at concentrations close to the MIC [Jacqueline and Caillon, 2014].

Resistance has been reported in *E. faecium*, *Staph. aureus*, *E. faecalis*, CoNS and viridans Streptococci though cases remain rare [Ager and Gould, 2012]. Resistance is due to point mutations in the 23S rRNA target site and only develops slowly as there are multiple copies of the 23S rRNA gene in every cell [Prystowsky *et al.*, 2001]. Resistance usually occurs after prolonged linezolid treatment, although there have been cases of nosocomial acquisition reported in patients with no prior linezolid treatment [Dobbs *et al.*, 2006]. Combination treatment with either rifampicin or fusidic acid could postpone the development of resistance [Millar *et al.*, 2008].

Advantages of linezolid are the option of oral route of administration, good tissue distribution, penetration and bioavailability. Another more unusual benefit of this antibiotic is the ability to suppress toxin production in *Staph. aureus*, thus improving disease outlook [Bernardo *et al.*, 2004].

Daptomycin

This is a calcium-dependent bactericidal antibiotic which was developed in response to the increasing resistance of staphylococcal strains; it has been shown to be effective against most Gram-positive organisms including those with resistance to multiple antibiotics [Streit *et al.*, 2004].

Daptomycin is a 13 member amino acid cyclic lipopeptide with a decanoyl side chain [Steenbergen *et al.*, 2005]. The bactericidal effect of daptomycin is thought to be achieved through the insertion of the lipophilic tail into the bacterial cell membrane causing membrane depolarization and potassium ion efflux. DNA, RNA and protein synthesis is rapidly terminated leading to cell death [Silverman *et al.*, 2003]. The calcium required by daptomycin triggers a conformation change into an active state, which allows the antibiotic to penetrate deeper into the bacterial cell membrane. [Straus and Hancock, 2006].

The effects of daptomycin on biofilms have reported with a MIC range of 0.06-0.25 µg/ml and a mean cell survival rate of ~4% when tested against 12 clinical isolates of MRSA [Smith *et al.*, 2009]. Other studies have also shown that compared to linezolid, rifampin, and vancomycin, daptomycin was the fastest at clearing the majority of the biofilm [Raad *et al.*, 2007]. In fact this antibiotic may offer valuable alternatives for the treatment of antibiotic resistant organisms through its effective action [Petersen *et al.*, 2002].

Resistance was first reported in 2005 in patients with MRSA osteomyelitis [Hayden *et al.*, 2005] and this is thought to be due to point mutations in phospholipid biosynthesis genes. These mutations lead to changes in membrane composition and charge which either directly affects the daptomycin molecule binding or causes electro-repulsion of the calcium-complexed daptomycin [Cameron *et al.*, 2015]. With the increasing resistance to vancomycin, daptomycin is used as an alternative first line therapy against both log and stationary phase bacterial cells [Mascio *et al.*, 2007]. Monotherapy of biofilms is effective though total killing is not achieved [Smith *et al.*, 2009] though, clinical success has been seen in staphylococcal bone and joint infections [Falagas *et al.*, 2007].

Combining Antibiotics

Combination therapy potentially has many benefits compared to individual antibiotic therapy specifically in the cases of severe infections. Combinations would increase the effectiveness of the antibiotics through synergism, increase the range of bacteria targeted in empirical therapy through affecting different targets, limit virulence factors expression, and prevent the development of antibiotic resistance.

However, factors to be aware of when combining antibiotics are the possible antagonism between antibiotic classes, the detrimental effect of rapid bacteriolysis on the host and the fact that dual treatment might be no more beneficial than using a single antibiotic [Hagihara *et al.*, 2012].

The most well-known system for antibiotic combination therapy for biofilms is the use of bone cement. Initially lower infection rates were reported when using antibiotic loaded cement compared to plain cement [Thierse, 1978]. However, the long term release of gentamicin promoted the development of antibiotic resistance bacterial strains [van der Belt *et al.*, 1999]. Thus the decision to add a second antibiotic to the cement was made. The second antibiotic can vary including clindamycin, vancomycin and fusidic acid [Gallo *et al.*, 2013; Neut *et al.*, 2005]. As mentioned above in the individual antibiotic sections combinations of antibiotics for the treatment of biofilms can have a beneficial effect.

Combining antibiotics improves treatment outcome though the way in which this is done is not clearly understood and more work needs to be conducted on this study especially in regards to biofilm infections. An alternative option which is currently being explored is combining the antibiotic with adjuvants as detailed in the next section.

Disruption to Staphylococcal Biofilms

With the difficulty of treating, and the increasing development of antibiotic resistance, alternative approaches need to be investigated to inhibit and disrupt biofilm formation Table 1.7 highlights a number of alternative approaches which have been proposed

to be effective. A few of these are examined in more detail below [Hogan *et al.*, 2015; Kiedrowski and Horswill, 2011].

Table 1.7: Alternative Approaches to Inhibiting Staphylococcal Biofilms [Adapted from Hogan *et al.*, 2015; Kiedrowski and Horswill, 2011].

Agent	Target	Mode of Action
Enzymatic disruption		
Lysostaphin	Cell wall	Cleaves pentaglycine bridges in cell wall causing cell lysis
DNase	Extracellular DNA	Cleavage of eDNA in the biofilm matrix directly or indirectly
Dispersin B	Exopolysaccharide	Glycoside hydrolase enzyme which degrades PIA/PNAG polymer
V8	Peptide bonds	Disrupts protein mediated biofilms by cleaving peptide bonds
Attachment		
Charge Modification	Negatively charged surfaces prevent attachment and repel bacteria	
Metals e.g. Silver	Prevent bacterial adhesion	
Quorum Sensing Targets		
Autoinducing peptide (AIP)	<i>agr</i>	Activation of the <i>agr</i> system leading to dispersal of the biofilm
Phenol Soluble Modulins (PSMs)	Unknown	Surfactant properties promote dispersal of biofilms
Bacteriophages		
Bacteriophages	Cell Wall	Degrades cells wall causing cell lysis
Chemical Molecules		
<i>N</i> -acetyl-L-cysteine	Disrupts disulphide bonds and inhibits amino acid use	Inhibition of biofilms
<i>Cis</i> -2-decanoic acid	Unknown	Dispersal of biofilms

Chelators e.g. EDTA	Unknown	Chelator of metals especially magnesium
Natural Molecules		
D-amino acids	Incorporation in the peptidoglycan of the cell wall	Inhibition of Protein Synthesis in <i>B. subtilis</i> Unknown in <i>Staph. aureus</i>
Sugar metabolites	Reactivation of metabolic systems allowing antibiotics to affect cells	
Immunisation		
rClfA/rCLFB	Stimulates antibody production to clumping factors A and B (rClfA/rClfB) and the surface protein	
cna-FnBP	Recombinant protein creation of fibronectin binding protein and collagen to prevent biofilm adhesion	
rIsdB	Vaccine competes with the iron sequestering protein	
Photodynamic treatment		
Chlorin (e6)	Produces free radicals and cytotoxic reactive oxygen species	
Antimicrobial peptides		
Cathelicidin	Cytoplasmic membrane	Disrupts biofilm formation

Enzymatic Interference: Lysostaphin

An enzyme upon which a fair amount of investigation has been carried out is lysostaphin [Hogan *et al.*, 2015]. Lysostaphin is a 27kDa glycyl-glycine endopeptidase which acts by cleaving the cross-linking pentaglycine bridges in the staphylococcal cell wall, lysing and killing the bacteria [Wu *et al.*, 2003; Kokai-Kun *et al.*, 2009]. The enzyme is also able to destabilise biofilms by disrupting the extracellular matrix through the rapid lysis of the component cells leading to detachment [Wu *et al.*, 2003].

It has been shown to have activity against *Staph. epidermidis*, MSSA, and most importantly MRSA [Dajcs *et al.*, 2000; Wu *et al.*, 2003]. Wu *et al* [2003] also investigated the effects of lysostaphin against *Ps. aeruginosa* with no change in the biofilm formation, it may be that this enzyme is purely for use with staphylococcal

biofilms. This property is an advantage over conventional antibiotics as it should not affect the host's normal gut microbiota.

Additionally, lysostaphin does not have any effect on human cells and little toxicity though repeated treatments can possibly cause an immune reaction to the protein [Kokai-Kun *et al.*, 2009]. An alternative to treating the entire system can be using lysostaphin coated catheters which have been shown to prevent colonization of both *Staph. aureus* and to a lesser extent *Staph. epidermidis*. The catheters retain their antimicrobial properties for several days after coating [Shah *et al.*, 2004].

A major concern with using enzymes to disrupt biofilms is the potential dispersal and seeding of bacteria to other organs and parts of the body. Nonetheless, the use of enzymes is promising, although, only when used in conjunction with systemic antibiotics [Hogan *et al.*, 2015].

Other enzymes which have been shown to have activity against biofilms include Dispersin B, a glycoside hydrolase enzyme which degrades the surface polymer PIA/PNAG; DNase I which inhibits biofilm formation via the cleavage of cell surface nucleic acids which act as surface adhesins; and V8, a protease which severs peptide bonds, such as glutamic acid, to disrupt protein formation within the biofilm [Hogan *et al.*, 2015].

Bacteriophage Therapy

Bacteriophages were originally recognised in 1915 in a culture of dysentery bacilli [Twort, 1915] and were researched extensively until the 1940s however, with advent of antibiotics they were side-lined in the majority of countries [Yilmaz *et al.*, 2013]. Therapy involves using lytic bacteriophages, or components thereof, to treat bacterial infectious diseases [Gutiérrez *et al.*, 2015].

Bacteriophages act on bacterial cells by attaching to the cell wall and translocating their DNA into the cell where it undergoes replication, subsequently producing the new phage particles. These are released when the cell bursts, through the actions of the phage proteins endolysin and holing. The new phages then infect neighbouring cells repeating this cycle. As the phages replicate in the system a low initial dose will rapidly be increased [Matsuzaki *et al.*, 2005].

Successful treatment has been reported against both Gram-positive and Gram-negative species, including *E. coli*, *Ps. aeruginosa*, *K. pneumoniae*, *Salmonella* spp., *E. faecium* and *Staph. aureus* [Matsuzaki *et al.*, 2005]. Purified phage-encoded peptidoglycan hydrolase (lysin) is active against *Strep. pyogenes*, *Strep. pneumoniae*, and *Bacillus anthracis* with immediate effect when used topically [Matsuzaki *et al.*, 2005].

The main advantage of bacteriophage treatment over conventional antibiotic therapy is that the bacteriophages are able to affect drug resistant species, as the phages have a completely different mode of action from antibiotics. The emergence of phage resistant bacteria may also be prevented by the mutation of the bacteriophage in order to maintain its infectivity [Matsuzaki *et al.*, 2005].

Additionally, as bacteriophages are highly species specific, there is no broad disruption to the host microbiota, and consequently, only prokaryotic cells are targeted. Thus the adverse side effects and complications from antimicrobial treatment are drastically reduced. However, this could prove to be a disadvantage for a clinical setting as the specific bacterial strain causing the infection would need to be identified before treatment could commence [Pincus *et al.*, 2015].

There are conflicting studies regarding the effectiveness of bacteriophages against biofilms; Yilmaz *et al* [2013] found that a combination of bacteriophage with antibiotic reduced a *Staph. aureus* biofilm (5000 CFU/ml for phage and antibiotic compared to 17,165 CFU/ml for antibiotic only and 30,788 CFU/ml for phage only, 50,586 CFU/ml for the control group) and to a lesser extent a *Ps. aeruginosa* implant infection whereas Seth *et al* [2013] found that disruption though debridement was required before bacteriophage therapy was effective.

The site of infection and state of the host immune system may also be relevant to the efficiency of bacteriophage treatment [Pincus *et al.*, 2015]. Duration of treatment, route of administration, potential side effects and appropriate dose ranges would need to be investigated as this information is currently unknown [Hogan *et al.*, 2015; Pincus *et al.*, 2015; Yilmaz *et al.*, 2013]. A definite decision can only be made regarding the effectiveness of bacteriophages against biofilm infections after this information has been analysed.

Natural Molecules: D-amino acids

The effect of D-amino acids on biofilms was first discovered in cultures of *Bacillus subtilis* where it was found that increased incubation times in spent medium dissolved pellicles which had previously formed within the culture [Kolodkin-Gal *et al.*, 2010]. The factor which triggered the dissolution of the biofilm was found to be D-amino acids which are naturally produced during late stationary phase cultures, primarily D-tyrosine, D-leucine, D-tryptophan and D-methionine. Other D-amino acids and L-enantiomers did not have any effect [Kolodkin-Gal *et al.*, 2010].

L-enantiomers are predominantly used rather than the D-enantiomers within cells naturally [Lam *et al.*, 2009] therefore, incorporating D-amino acids within the cell wall will modulate peptidoglycan synthesis and inhibit attachment to the biofilm matrix proteins. This results in reduced intercellular adhesion thus having an effect on the stability of the biofilm [Lister and Horswill, 2014]. The morphology of cells is not altered by this inclusion [Lam *et al.*, 2009].

D-amino acids were also shown to be effective against other bacterial species including *Staph. aureus* and *Ps. aeruginosa* strains [Kolodkin-Gal *et al.*, 2010]. The three amino acids which have the greatest effect on *Staph. aureus* are D-tyrosine, D-phenylalanine and D-proline. A mixture of these three was apparently more effective than using them individually [Hochbaum *et al.*, 2011].

Ramón-Peréz *et al* [2014] has demonstrated that clinical and commensal strains of *Staph. epidermidis* are affected by D-amino acids, though the sensitivity/resistance differs depending on the strain. The amino acids used were D-Met, D-Phe, D-Ala, D-Pro, D-Tyr and D-Leu, with D-Met and D-Ala having the greatest effects unlike in *Staph. aureus*.

A possible way to utilise this information could be to coat medical devices, such as catheters, with D-amino acids to prevent initial biofilm formation as it has been suggested that the D-amino acids have to be present from the initial stages of biofilm formation in order to have an effect [Ramón-Peréz *et al* 2014].

The viability of D-amino acids use to disrupt/inhibit bacterial biofilms is debatable as the inhibitory effect of the compounds is strain dependent, and there have been some recently published papers which have contradicted the effectiveness of D-amino acids on biofilm dispersion [Leiman *et al.*, 2013; Sarkar and Pires, 2015].

The first paper raising doubts about the D-amino acids was Leiman *et al* [2013] who found that the inhibitory effects of D-amino acids on *B. subtilis* biofilms reported by Kolodkin-Gal *et al* [2010] was caused by a strain-specific mutation. This mutation was in the *dtd* gene. This gene encodes D-tyrosyl-tRNA deacylase and is responsible for preventing the misincorporation of D-amino acids into proteins. When this mutation was repaired the strain's biofilm became resistant to the effects of the D-amino acids [Leiman *et al.*, 2013].

Sarkar and Pires [2015] found that the D-amino acids do not have any effect on the inhibition of biofilm formation in *Staph. aureus*. The original paper by Hochbaum *et al* [2011] used *Staph. aureus* SC01 which was shown to be sensitive to D-amino acids. However, when Sarkar and Pires [2015] used this strain and the published D-amino acid concentrations no effects on the biofilms was observed. Even with a 100-fold increase in the D-amino acid concentrations no inhibition was seen [Sankar and Pires, 2015]. Repeating the experiment with *B. subtilis* and *Staph. epidermidis* yielded the same results suggesting that in fact D-amino acids have no effect on biofilm formation and dispersal.

Natural Molecules: Sugar Metabolites

The influence of sugar metabolites was first reported by Allison *et al* [2011] who discovered that after adding specific carbon metabolites to persister cells, the cells revert back into a state against which antibiotics, specifically aminoglycosides, are once again effective.

The way in which this works is that the metabolites are taken up by proton-motive force (PMF) into the cell which then also takes up the aminoglycosides. The sugar metabolites are taken up by the bacterial cell and enter glycolysis resulting in the production of NADH. The NADH is oxidised by electron transport chain enzymes which contribute to PMF. The revival of PMF enables aminoglycoside uptake into the cell leading to cell death. This effect is only seen with the aminoglycosides and not with the other classes of antibiotics tested (β -lactams and quinolones) [Allison *et al.*, 2011].

The experiment was conducted on both Gram-negative (*E. coli*), and Gram-positive (*Staph. aureus*) bacteria and the effect was found in both species. Interestingly in

Staph. aureus, only fructose induces the reversion to aminoglycoside sensitivity whereas in *E. coli*, the relevant sugars are mannitol, glucose and pyruvate [Allison *et al.*, 2011].

Sugar metabolites have also been reported to block adhesion, reduce bacterial load (by 38% compared to the control) and inhibit biofilm formation by *Staph. aureus* on endotracheal tubes. Once again fructose was used [Durmus *et al.*, 2012].

In *Ps. aeruginosa* pulmonary infections caused by both mucoid and non-mucoid strains, a combination of three sugars, mannose, fructose, and galactose was found to have a synergistic effect when combined with conventional antibiotics such as β -lactams and quinolones. Bucior *et al* [2013] demonstrated that a mixture of these sugars and antibiotics blocked bacterial adhesion and diminished bacterial induced cell damage in a murine model of acute pneumonia.

Side effects would seem to be minimal as the bacterial cells do not revert to a metabolically active and thus pathogenic type [Allison *et al.*, 2011; Bucior *et al.*, 2013] and the sugars used are naturally found within the body. Adhesion to indwelling medical devices could also be reduced, thereby preventing biofilm formation [Durmus *et al.*, 2012].

Therefore, it seems as the use of sugars, either individually as with *Staph. aureus*, or in combination as with *Ps. aeruginosa*, could provide an inexpensive simple therapy, which could prove a valuable adjunctive to conventional treatment.

Quorum Sensing Targets: Autoinducing Peptides (AIP)

Regulation of staphylococcal biofilm formation and dispersal occurs through the *agr* quorum sensing system (Figure 1.8) which is activated in response to the extracellular concentration of an AutoInducing Peptide (AIP) [Boles and Horswill, 2011; Kiedrowski and Horswill, 2011].

AIP is a cyclic thiolactone-containing peptide which varies slightly in composition depending on the strain which produces it. At low concentrations AIP binds to the membrane bound receptor domain of the AgrC histidine kinase activating the AgrCA two-component system [Hogan *et al.*, 2015]. Once activated the global gene expression is adjusted increasing virulence factor expression and biofilm disassembly

[Kiedrowski and Horswill, 2011; Thoendel *et al.*, 2011]. The virulence factors include proteases and phenol-soluble modulins (PSMs) which are small pore forming toxins [Boles and Horswill, 2011]. Within cells that make up biofilms, *agr* expression is repressed naturally whereas in newly dispersed cells *agr* activity levels are high [Boles and Horswill, 2008].

It has been shown that addition of exogenous AIP leads to complete disassembly of a biofilm within 20 hours and reversion to a planktonic state with a restoration in antibiotic sensitivity [Lauderdale *et al.*, 2009]. However, the activation of the *agr* system with resulting increased expression of virulence factors may trigger the conversion of the staphylococcal strain into a more invasive pathogen, with associated tissue damage and reactive host immune response. This would be a major concern. A combination treatment of *agr* activation and antibiotic could alleviate this concern by eliminating the planktonic cells [Kiedrowski and Horswill, 2011].

Other concerns include the AIP composition as different staphylococcal species' recognise structurally unique AIPs [Thoendel *et al.*, 2011]. A universal *agr* activator would have to be carefully selected in order to be effective against the majority of staphylococcal biofilms. The impact on the host's innate and adaptive immune system function would also have to be considered [Hogan *et al.*, 2015].

Current Biofilm Models

In order to study biofilm formation, dispersal, antibiotic resistance and effects of disruptive molecules, an easy, reproducible and reliable model is required. However, the complexity of a biofilm makes reproduction of one, in a laboratory situation, a challenge. There are various models which have been developed, these are either open (continuous culture) or closed (batch culture) [McBain, 2009].

The agar plate is considered an example of a closed system biofilm model. This model reproduces some of the biofilm properties such as high cell density and gradients within the biofilm; it is useful but simplistic. Other closed systems are 24 well cell culture plates into which a disc has been placed; in this way a variety of bacteria and

materials can be investigated [McBain, 2009], and the Calgary Biofilm Device [Ceri *et al.*, 1999], this will be discussed in detail further on.

Open system biofilm models include suspended substratum reactors (SSRs) where the biofilms are grown inside fermentation vessels at the solid-liquid interface on colonisable materials which can be removed from the vessel at required sampling times [McBain, 2009]. SSRs are used to examine dental microbiology [Bradshaw *et al.*, 1996], colonic microbiology [McFarlarlane and McFarlarlane, 2006], and to investigate gene expression [Whiteley *et al.*, 2001].

Rotating reactors for control of shear stress, The Robbins device and flow cells, drip fed biofilms, and perfused biofilm fermenters are other examples of open system biofilm models and are comprehensively detailed in McBain [2009].

The advantages of all these systems are production of high population density biofilms, large biomass yields and controlled fluid dynamics, however, the one disadvantage they all have in common is that only a few samples can be processed or investigated at any one time [Harrison *et al.*, 2010]. The Calgary Biofilm Device and MBEC (minimum biofilm eradication concentration) assay was developed to enable large numbers of samples to be processed [Ceri *et al.*, 1999].

The Calgary Biofilm Device

The rationale behind the development of this device was that the standard Minimum Inhibitory Concentration (MIC) testing was designed for planktonic cultures and acute infections [Andrews, 2001] and was not effective against chronic or device related infections [Ceri *et al.*, 1999]. As biofilms cause a significant number of clinical infections an assay was required which could provide relevant and effective antibiotic dose recommendations. This is the Minimum Biofilm Eradication Concentration (MBEC) [Ceri *et al.*, 2001]. The Calgary biofilm device utilises such an assay and involves a standard 96 well plate with a lid which has plastic removable pegs (Figure 1.10) upon which biofilms can form [Ceri *et al.*, 1999].

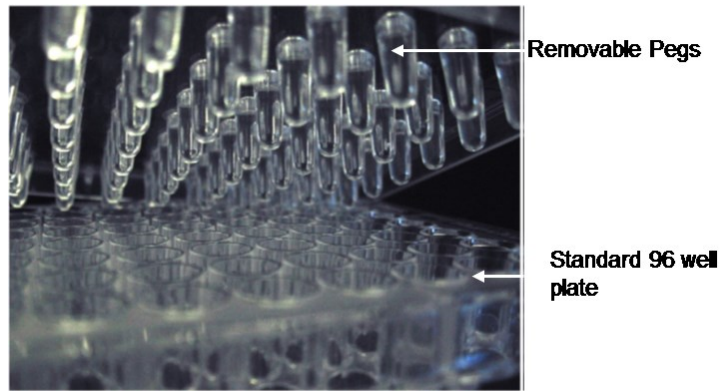


Figure 1.10: Photograph of a Calgary Biofilm Device Plate [Parker *et al.*, 2014]. A standard 96 well plate is used as the base and can be filled with medium, antibiotics, antiseptics or biocides. The lid contains pegs which slot into the base well and upon which the biofilms can form.

Once formed, washed and transferred these biofilms can then be exposed to antibiotics, antiseptics and biocides [Allan *et al.*, 2011]. Studies have been conducted for various Gram-positive and Gram-negative bacterial species [Olson *et al.*, 2002]. Large numbers of different antibiotics (types and concentrations) can be investigated at any one time [Ceri *et al.*, 1999]. A comprehensive protocol has been published by Harrison *et al.*, [2010] detailing the method and the parameters and controls required to obtain reliable results. Figure 1.11 gives a summary of an example experiment.

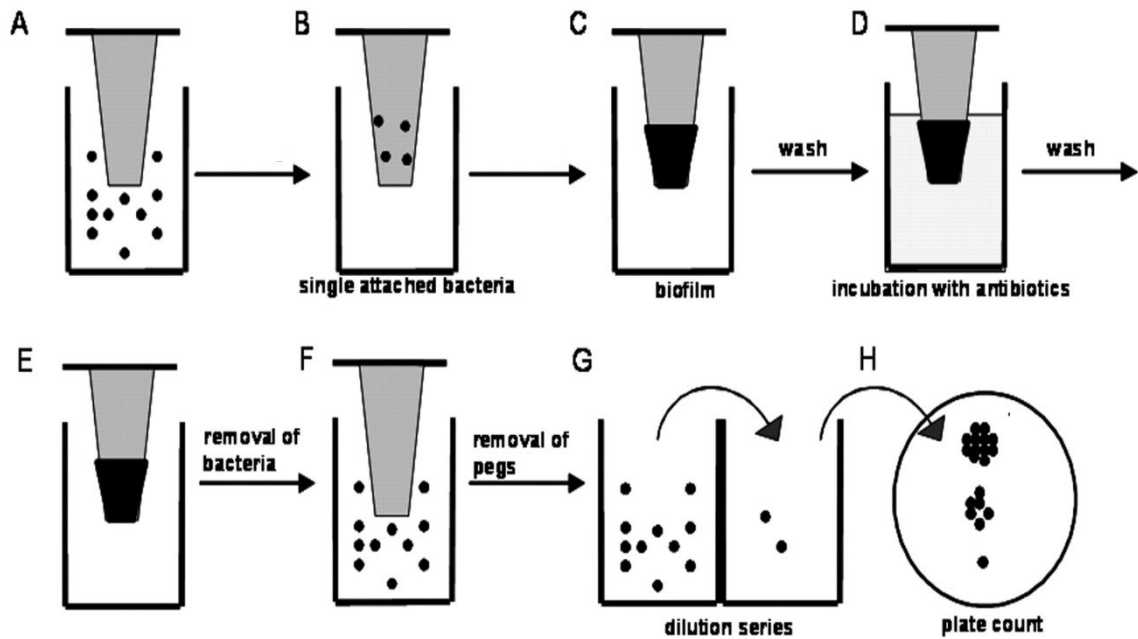


Figure 1.11: Formation and Challenge of Biofilms using the Calgary Biofilm Device [Adapted from Herrmann *et al.*, 2010]. The formation and challenge of a biofilm on the Calgary Biofilm Device involves introducing the pegs into a planktonic culture (A) and incubating during which time the biofilm forms (B/C). After washing, the biofilms are transferred to fresh medium and challenged with antibiotics (D) and washed again (E). Removal of the biofilm from the peg by sonication (F) followed by serial diluting (G) and plating to determine the CFU/ml count (H) and biofilm sensitivity.

However, with all the benefits of the Calgary Biofilm Device there are significant disadvantages namely, the difficulty in achieving reproducible results. In fact, only minor deviations from the protocol such as using a rotating rather than a tilting platform during incubation of the biofilms can majorly affect results [Dall, 2013]. Another disadvantage is the removal of the biofilm from the pegs by sonication [Harrison *et al.*, 2010]. Sonication is superior to other removal methods such as scraping [Bjerkkan *et al.*, 2009], but has its own drawbacks, specifically incomplete removal of biofilms, and possible damage to cells especially Gram-negative and anaerobic bacterial species [Monsen *et al.*, 2009].

A significant factor to contemplate when considering using the Calgary Biofilm Device is the cost. Variations of the original 96 well plate lid with pegs and base are available. These are a trough base or hydroxyapatite coated pegs which facilitate biofilm growth

by fastidious microorganisms. The cost of these range from \$672.50 to \$985 (for 25) [Innovotech]. A sonicator would also have to be acquired further increasing the cost of this procedure.

The Alginate Bead Method

In order to develop a cost effective alternative to the Calgary Biofilm Device, a previous student in the Gallagher laboratory developed the alginate bead method [Dall, 2013]. This method involves using sodium alginate solidified with 2M CaCl₂ and formed into a bead (Figure 1.12).



Figure 1.12: An Alginate Bead [Dall, 2013]. The beads are formed with 210 μ l 4% Sodium Alginate within a 96 well plate producing the characteristic oval shape and size. The bead is approximately 5.4 mm by 2.7 mm with a surface area of 160.31 mm².

It is on this surface that the biofilm forms; once the experiment/antibiotic challenge has been completed, the bead is dissolved ensuring that the entire biofilm is recovered. Full details of this method can be found in Chapters 2 and 3.

Statement of Need

Investigating biofilms and the resulting antibiotic resistance is important in light of the increasing bacterial resistance these days especially in health care environments. Determining an effective antibiotic range for biofilm infections is essential in order to treat patients successfully. Even though the Calgary Biofilm Device is well characterised and useful there are still drawbacks to it. The primary ones are the cost of the equipment and the difficulty in reproducibility. It is hoped that the alginate bead method developed by members of the Gallagher lab and fully characterised in this study will provide a cost-effective and simple alternative.

Aims of This Work

To Investigate:

- The alginate bead biofilm method and compare it to the established Calgary Biofilm Model.
- The differences in antibiotic resistance between planktonic and biofilm *Staphylococcus aureus* cultures.
- The development of resistance and consequently the amount of antibiotic required to reduce biofilm load of *Staphylococcus aureus* biofilms.
- The effects of different antibiotic combinations on *Staphylococcus aureus* biofilms and whether synergy or antagonism between the antibiotics develop.
- The development of biofilms and resulting antibiotic resistance of different health care associated bacterial species.
- The development of mixed species biofilms and their effect on antibiotic resistance.
- Disruption of biofilms with natural molecules (sugars metabolites/ D-amino acids).

Chapter 5 relates to bacterial binding proteins and different expression systems and is a discrete chapter.

Chapter 2:
Materials and Methods

Strains, Plasmids and Oligonucleotides

Table 2.1: Bacterial and Yeast Strains.

Strain	Details	Source
<i>Escherichia coli</i> strains for protein expression		
BL21 (DE3)	<i>F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</i>	Lab Stock
DH5α	<i>F⁻ Φ80 lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK⁻, mK⁺) phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	Lab Stock
Top10	<i>F mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ⁻</i>	Lab Stock
Rosetta-gami 2	<i>Δ(ara-leu)7697 ΔlacX74 ΔphoA Pvull phoR araD139 ahpC galE galK rpsL (DE3) F'[lac⁺ lacI^q pro] gor522::Tn10 trxB pRARE2 (Cam^R, Str^R, Tet^R)</i>	Loake Lab
EHPT1	Produces a His-tagged, arabinose inducible protein	Lab Stock
Yeast		
<i>Pichia pastoris</i> KM71wt	Mut ^s , Arg ⁺	Barlow Lab
<i>Pichia pastoris</i> G1107C	Positive Control for <i>P. pastoris</i> expression	Barlow Lab
Bacterial strains for biofilms		
<i>Staphylococcus aureus</i>	ATCC# 29213	Lab Stock
<i>Streptococcus mutans</i>	NCTC#10923	Lab Stock
<i>Klebsiella pneumoniae</i>	Clinical Isolate	Lab Stock
<i>Staphylococcus saprophyticus</i>	Clinical Isolate	Lab Stock

<i>Staphylococcus epidermidis</i>	Clinical Isolate	Lab Stock
<i>Pseudomonas aeruginosa B</i>	Constitutive AlgL producer - mucoid	Lab Stock
<i>Pseudomonas aeruginosa G</i>	Clinical Isolate from patient with bronchiectasis	Govan Lab
<i>Escherichia coli</i>	ATCC#25922	Lab Stock
<i>Escherichia coli K12</i>	K12, MG1655	Lab stock
<i>Streptococcus pneumoniae</i>	Clinical sample	Glaxo Verona, Italy
<i>Enterococcus faecalis</i>	Clinical Isolate from heart valve	Lab Stock

Table 2.2: Plasmids.

Plasmid	Relevant Features	Source
pPICZαB	Vector containing inducible promoter AOX1 for secretable protein expression	Barlow Lab
pCPGRP	Contains CPGRP gene in pPICZαB. Optimised for <i>Pichia pastoris</i>	Gene Art, Invitrogen
pNirFP-C	Allows fusions to the NirFP C-terminus	Evrogen
pCPGRP-NIR	pPICZαB containing fusion of CPGRP and NirFP	This Study
pCPGRP1 (Optimised for bacterial growth)	Contains CPGRP gene in pBAD/HisA.	Gene Art, Invitrogen
pSP-D	Contains SP-D gene in pBAD/HisA. Optimised for bacterial growth	Gene Art, Invitrogen

Table 2.3: Oligonucleotides.

	Name	Sequence (5'-3')	Relevance
A	5'AOX <i>Pichia</i> sequencing primer	GACTGGTCCAATTGACAAGC	Used to sequence construct
B	3'AOX <i>Pichia</i> sequencing primer	GCAAATGGCATTCTGACATCC	Used to sequence construct
C	α -factor <i>Pichia</i> sequencing	CTACTATTGCCAGCATTGCTGC	Used to sequence construct
D	NIR Plasmid forward	GTGGGAGGTCTATATAAGCA	Used to sequence construct
E	NIR Plasmid reverse	GGGAGGTTTTTTAAAGCAAG	Used to sequence construct
F	NIR sequence Middle Reverse	AGATGAGGCAGCCGTTCTGG	Used to sequence construct
G	CPGRP sequence middle reverse	AATGTTCCAACCTCTACCCT	Used to sequence construct
H	CPGRP Reverse	GGT CCC ACT ACA GAG CTT AAT CTA GAA	Used to sequence construct
I	<i>Pichia</i> Primer for NIR Forward	AGAAGGGGTATCTCTCGAGA	Used to sequence construct
J	NirFP Forward	CACCATCATCATGGTACCATGG GAGAAGGATAGCGAGCTGATC	For insertion of NirFP into pCPGRP
K	NirFP Reverse	TCATGCGGCGCGCGCGGCCG CTGTGCCCCAGTTTGCTAGGC A	For insertion of NirFP into pCPGRP
L	CPGRP Forward	GCACAAATAACGGGTTATTG	Used to sequence construct
M	CPGRP Reverse	CAACTTGAAGTGAAGAACAG	Used to sequence construct
N	SP-D Forward	GGGCTAACAGGAGGAATTAAC C	Used to sequence construct
O	SP-D Reverse	GAATTTGCCTGGCGGCAGTAG	

Bacterial Growth and Storage Conditions

Bacterial and yeast strains were stored long term at -80°C , taken from a 5 ml overnight culture and mixed at a ratio of 1:1 with 20% glycerol. The cultures were revived by streaking onto appropriate agar, with antibiotic if required. Short term storage on an appropriate agar plate stored at 4°C for no more than 10 days.

Unless otherwise stated, overnight cultures were prepared by inoculating 5 ml of appropriate medium, with antibiotics if required, with a single colony from a plate. Incubation at 37°C (bacteria) or 30°C (yeast) overnight at 200rpm.

Stock solutions

TAE (Tris-acetate-EDTA): 242 g Tris base (FW121.14), dissolve in 750 ml dH_2O . Add 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA. Adjust to final volume of 1l. Store at room temperature. Working concentration of 1xTAE.

Biofilm solutions were:

Gelating agent: to make a 2M CaCl_2 solution mix 29.4 g into 100 ml dH_2O and autoclave to sterilise.

To make the alginate beads mix 4 g of sodium alginate into 100 ml dH_2O and autoclave to sterilise.

Dissolving buffer: To make a 10x stock solution dissolve 5.3 g NaCO_3 and 5.2 g citric acid in 100 ml dH_2O and filter sterilise.

Universal neutraliser: Dissolve 1 g histidine, 1 g cysteine, and 2 g reduced glutathione in 20 ml dH_2O . Filter sterilise and store at -20°C in 1 ml aliquots. This is added to 40 ml 1x dissolving solution when required.

Biofilm Set up

Preparation of alginate beads: Using a 96 well plate pipette 20 μ l 2M CaCl₂ into each well, 210 μ l 4% sodium alginate followed by 20 μ l 2M CaCl₂. The sodium alginate must be pipetted slowly due to its viscous consistency. Replace the lid and incubate for 4 hours at 60°C to set the beads. Store at 4°C for up to a week.

Growing the biofilms: Using a 48 well plate and an overnight culture, 600 μ l LB containing 1/10 000 overnight culture, diluted in fresh medium ($\sim 10^4$ CFU/ml), was pipetted into the wells. Alginate beads, after been washed in sterile water using flamed wire and forceps were added to the wells. The plate was incubated at 37°C, 150 rpm for the required time. Triplicate beads are used for each condition.

After incubation the beads were transferred, using flamed forceps, into 600 μ l dH₂O in a 48 well plate for washing. They were then placed into fresh medium with antibiotic if required by the experiment. Incubate at 37°C for the determined challenge time. At the required sampling time the beads were removed and rinsed in 600 μ l dH₂O in a 48 well plate before being dissolved in 2 ml dissolving solution in a 15 ml falcon tube (with universal neutraliser if required). The beads were crushed with a sterile loop handle. Controls of beads in LB and plain LB were always included to show no contamination. If there was any growth on these plates the experiments were repeated.

An example plate is shown: Testing of antibiotic against biofilm growth time. The beads were incubated for the shown time, after which the beads were washed and placed into fresh medium with the antibiotic at the appropriate concentration added to wells 1-3 for 2 hours challenge. The beads were then washed and dissolved. Control beads (wells 4-6) were not supplied with antibiotics.

	A	B	C	D	E	F	G	H
1	0.5hrs	1hr	2hrs	3hrs	4hrs	6hrs	8hrs	24hrs
2								
3								
4	0.5hrs	1hr	2hrs	3hrs	4hrs	6hrs	8hrs	24hrs
5								
6								

For some experiments the beads were not washed and placed into fresh medium between incubation and antibiotic challenge. Instead the antibiotic was added directly into the wells.

Planktonic cultures were set up in the same manner, antibiotic was added directly to the wells and samples were taken and diluted as with the biofilm cultures.

Counting the organisms: In order to assess the level of growth on the beads, they were placed in 2 ml of dissolving solution, with universal neutraliser as required, in a 15 ml falcon tube crushed with the bottom of a sterilised metal loop and rotated until dissolved. After vortexing for 10 seconds, a sample was taken and 10 fold diluted, 6-8 times, with PBS in a 96 well plate. Using the Miles Misra method the cultures were spotted onto plates (20 μ l spots) and incubated at 37°C overnight or until colonies grew. The plates were not incubated for more than 48 hours.

To calculate the CFU/ml bacterial load of each bead $N = (C.V)/D$ was used. Where C is the number of colonies, spots were counted if they contained between 2 and 30 colonies. V is 250 (to account for the 2 ml dissolving solution) and D is the dilution factor. For planktonic culture CFU calculations V is 50.

For the graphs, CFU/ml was plotted against growth time, challenge time, antibiotic concentration or bead condition depending upon the experiment. Error lines of standard deviation were used. The detection limit of the assay is ≤ 50 CFU/ml and is plotted as a black dashed line on the graphs.

Sonicating Probe and Water Bath

For dislodging the biofilms from the beads using the sonicating probe (Ultrasonic Processor) the following protocol was used. The beads, after washing were placed in a short plastic container in 2 ml dissolving solution. The container was placed on ice while sonication was carried out. The sterile probe was inserted into the solution and the bead sonicated for the required time. The probe was sterilised by wiping with 70% ethanol. The solution was vortexed before a sample was taken. From that point the samples were treated in the same manner as described above.

When using the sonicating water bath (Decon F5 Minor, Ultrasonic Ltd) the beads were placed in a 48 well plate containing 600 μ l dissolving solution, after the

experiment had been concluded. The plates were sealed with Parafilm to prevent leakage and/or contamination. The well plate was placed in the water bath under the water line but not completely submerged. After the required sonicating time the bead and surrounding liquid was transferred to a 15 ml falcon containing 1.4 ml solution. This was vortexed and sampled. The sample was treated as described above.

Determination of Minimum Inhibitory Concentration (MIC)

The antibiotic concentrations required were determined either by growth on an agar plate or in liquid medium. For either determination a range of concentrations were used based around a commonly used or manufacturers recommended concentration. LB agar was used which had been melted and cooled before the antibiotic was added. The plates were poured and allowed to set before being streaked with the test organism. The plates were incubated overnight at 37°C. The inhibitory concentration was determined by growth or no growth on the plates.

For determining the MIC in liquid culture, a 48 well plate was used (triplicate wells per concentration). 600 µl of a diluted overnight culture in fresh LB (~10⁴ CFU/ml) was pipetted into the wells and the plate incubated for 3 hours at 37°C, with shaking (150rpm). After three hours growth the different antibiotic amounts were added to the wells. The plates were incubated for a further 2 hours before samples were taken, serially diluted and plated onto LB plates which were incubated overnight (37°C). The antibiotic's inhibitory concentration was determined by growth/no growth on the agar plates.

Media

Yeast Peptone Dextrose (YPD): 10 g yeast extract, 20 g peptone, 20 g dextrose (D-glucose) and 20 g agar if required for solid medium. Dissolve in 1 litre dH₂O. Final pH 6.5± 0.2 at 25°C. Autoclave to sterilise.

Yeast Peptone Dextrose Sorbitol (YPDS): 10 g yeast extract, 20 g peptone, 20 g dextrose (D-glucose), 182.2 g sorbitol and 20 g agar if required for solid medium. Dissolve in 1 litre dH₂O. Final pH 6.5±0.2 at 25°C. Autoclave to sterilise.

Luria Bertani (LB): 10 g Tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar if required for solid medium. Dissolve in 1 litre dH₂O. Autoclave to sterilise.

Low salt LB: 10 g Tryptone, 5 g NaCl, 5g Yeast extract, 15 g agar if required for solid medium. Dissolve in 1 litre dH₂O. Autoclave to sterilise. For use with Zeocin to culture *E. coli* containing pPICzαB, pCPGRP or pCPGRP-NirFP.

Stationary Phase Medium: LB inoculated with *Staph. aureus* and grown at 37°C, 200rpm overnight. Spin down and remove cell pellet. Filter sterilise supernatant and use as normal. Prepare fresh before every use.

Mueller-Hinton (MH) Broth 2 (cation-adjusted): Commercial mix containing 17.5 g casein acid hydrolysate, 3 g Beef Extract, 1.5 g Starch. Dissolve in 1 litre dH₂O. Final pH 7.3 ± 0.2 at 25°C. Autoclave to sterilise according to the manufacturer's instructions.

Buffered Glycerol/Methanol complex medium (BMGY/BMMY). For *P. pastoris* expression.

- 1% yeast extract
- 2% peptone
- 100 mM potassium phosphate
- 1.34% Yeast Nitrogen Base (YNB)
- 4x10⁻⁵% biotin
- 1% glycerol or 0.5% methanol (use methanol when expressing)

Dissolve 10 g yeast extract and 20 g peptone in 700ml dH₂O. Autoclave and cool to room temperature. Add 100 ml 1M potassium phosphate, 100ml 10xYNB, 2ml 500x biotin and 100 ml 10x glycerol or 10x methanol. Store at 4°C, shelf life 2 months. More details can be found in the *Pichia pastoris* Expression Kit User manual by Invitrogen [2010b].

Chemicals, Enzymes and Antibiotics

Unless otherwise stated, all chemicals and antibiotics were purchased from Sigma-Aldrich Co. Zeocin was purchased from InvivoGen, ciprofloxacin and daptomycin from

LKT Laboratories Inc. Clindamycin and linezolid were bought from the Cayman Chemical Company. SYBR safe DNA gel stain was purchased from Invitrogen. Restriction enzymes, T4 DNA ligase, high fidelity DNA polymerase and molecular weight markers were purchased from New England Biolabs (NEB).

Dinucleoside triphosphates (dNTPs) were purchased from Roche. All kits including plasmid mini prep, midi prep, gel purification and PCR purification kits were from Qiagen and used as per manufacturer's instructions. For extraction of yeast DNA a Wizard Genomic DNA Purification Kit (Promega) was used. Agarose for DNA gels and acrylamide for SDS PAGE gels was purchased from Severn Biotech Ltd.

Molecular Weight Markers

For agarose gel electrophoresis 100 bp and 1 kb DNA ladders were used. For the SDS-PAGE gels a prestained protein marker, broad range (7-175 kDa) was used.

D-Amino Acids

D-Phenylalanine, D-Proline and D-Tyrosine were purchased from Sigma-Aldrich. They were dissolved in dH₂O to 0.1M stock. D-Tyrosine requires NaOH to fully dissolve. Store stock at 4°C.

Enzymes

For insertion of CPGRP into pPICZαB EcoRI and XbaI were used

KpnI and SacII used for the digestion of pCPGRP and NirFP for ligation.

SalI to digest pCPGRP-NirFP for chromosomal insertion into *P. pastoris*

SacI and EcoRI were used to insert SP-D and CPGRP into pBAD/HisA

Antibiotics

Table 2.4: Antibiotics.

Antibiotic	Solvent	Stock concentration	Working concentration
Carbenicillin	dH ₂ O	50 mg/ml	100 µg/ml
Ciprofloxacin	Dilute HCl	10 mg/ml	*
Clindamycin	EtOH	0.5 mg/ml	*
Daptomycin	dH ₂ O	1 mg/ml	* (supplement with 50 µg/ml CaCl ₂)
Gentamicin	dH ₂ O	10 mg/ml	*
Kanamycin	dH ₂ O	50 mg/ml	50 µg/ml
Linezolid	EtOH	1 mg/ml	*
Rifampicin	MeOH	17 mg/ml	*
Vancomycin	dH ₂ O	10 mg/ml	*
Zeocin	dH ₂ O	100 mg/ml	25 µg/ml for <i>E. coli</i> (low salt LB)
			100 µg/ml <i>P. pastoris</i> (YPD)

*The working concentrations of these antibiotics are dependent upon the bacterial strain used. Specific concentrations used are stated with the results.

Dissolved in solvent and filter sterilised through a 0.22 µm filter.

Preparation of DNA

Kits were purchased from Qiagen and used according to the manufactures instructions. Plasmid mini-preps from the *E. coli* strains were prepared from 5ml overnight cultures in LB broth, with antibiotics as required. DNA was stored at -20°C until required. PCR clean up and gel extraction kits were used as required and according to the manufacturer's instructions.

DNA was extracted from *P. pastoris* using the Wizard Genomic DNA Purification Kit from Promega according to the manufactures instructions.

To confirm correct insertion of constructs a DNA sample to was prepared to between 500-600 ng in 30 μ l dH₂O (20ng/ μ l) and sent with suitable primers to Dundee Sequencing Services at the University of Dundee.

Nucleic Acid Manipulation

Polymerase Chain Reaction

For PCR amplification of DNA fragments, a standard reaction was as follows

	Volume per reaction
5x PCR buffer	5 μ l
dNTPs (200 μ M)	0.5 μ l
Forward primer (0.5 μ M)	1 μ l
Reverse primer (0.5 μ M)	1 μ l
DNA	1 μ l
dH ₂ O	16.5 μ l
Final volume	25 μ l

When using Phusion (NEB) high fidelity polymerase, the following programme was used (with modifications depending upon the melting temperature of the primers)

Initial denaturation	98°C	1min	
Denaturation	98°C	10sec	} 30 cycles
Annealing	62°C	20sec	
Extension	72°C	15sec	
Final extension	72°C	5 min	

To confirm transformation success colony PCR was carried out. A small amount of bacterial colony was added to 100µl of dH₂O, and vortexed. 1µl of this mixture was added to the PCR mixtures.

To visualise DNA, agarose gels were used. The agarose was dissolved in 1xTAE buffer with SYBR Safe added at a concentration of 1:10 000. Gels were run at 80-110V (depending on gel size) and imaged using an Epi Chemi II Darkroom (UVP Laboratory Products). For the *P. pastoris* experiments a 0.8% gel was used and for all other experiments a 1% gel was used.

Restriction Digestion

The following standard mixture was used for restriction digestions

Enzyme	0.5 µl
Buffer	2 µl
DNA	10 µl
dH ₂ O	8.5 µl
Final volume	20 µl

Reactions were incubated at the temperature and time recommended by NEB. High fidelity enzymes were used when possible. Reactions were either heat denatured or PCR purified depending on the nature of the enzyme.

Ligation

A ratio of 1:5 (vector: insert) was used as standard, however 1:7 ratio mixtures were occasionally set up to maximise chances of ligation. Reactions were incubated at room temperature for 2 hours or 16°C overnight. The T4 Ligase buffer was aliquoted into 10 µl volumes and only taken through one freeze-thaw cycle to reduce loss of ATP.

The following standard mixture was used for ligations

T4 DNA Ligase	0.5 μ l
Buffer	1 μ l
Vector DNA	2 μ l
Insert DNA	10 μ l
dH ₂ O	6.5 μ l
Final volume	20 μ l

Transformations

To make electrocompetent bacterial cells: 1000 ml LB broth was inoculated with a 1/100 dilution of an overnight culture and grown to OD_{600nm} of 0.4 before transferring to ice for 30 minutes. Cells were pelleted at 12 000 rpm and washed three times in 200 ml ice-cold 10% glycerol. Cold conditions were maintained throughout. After the last wash the pellet was re-suspended 1:1 in 10% ice cold glycerol and aliquoted into 50 μ l volumes before snap-freezing in an ethanol and dry ice bath. Cells were stored at -80°C until required.

For electroporation the cells were thawed on ice, DNA was added and left to incubate for 15 minutes on ice before being transferred to a chilled electroporation cuvette (Molecular BioProducts). The cells were shocked using a Bio-Rad Gene Pulser at 1.5V. 450 μ l LB broth was added to the cells and incubation at 37°C, 150 rpm for 1 hour was carried out before 100 μ l was plated. The remaining culture was pelleted and re-suspended in 100 μ l LB broth, this was also plated, onto LB agar containing the appropriate antibiotic.

To make chemically competent bacterial cells, 100 ml of LB was inoculated with a 1/100 dilution of an overnight and grown to OD_{600nm} of 0.4 and centrifuged at 12 000 for 1 minute. The pellet was re-suspended in ice-cold 0.1M CaCl₂ and incubated on ice for 30 minutes. Cells were pelleted and re-suspended in 100 μ l 0.1M CaCl₂. 5-10 μ g of DNA was added and mixture was incubated on ice for 30 minutes. Cells were

heat-shocked in a 37°C water bath for 5 minutes. 900 µl of LB was added before incubation for 1 hour at 37°C. Plated as above.

In order to transform *P. pastoris* the cells were made electrocompetent through the use of sorbitol. An overnight culture was grown and used to inoculate 500ml of fresh YPD. Cells were grown overnight to an OD₆₀₀ of 1.3-1.5. In order to measure this accurately, if the culture sample was over OD₆₀₀ 1, it was diluted. This was done for all OD readings.

Cells were pelleted at 1500 g for 5 minutes at 4°C and washed with 500 ml ice cold dH₂O. They were centrifuged again and re-suspended in 250 ml ice cold dH₂O followed by two more spins to re-suspend in 20 ml and 1 ml ice cold 1M sorbitol for a final volume of ~1.5 ml. The cells were mixed with 5-20 µg linearised DNA and shocked at 2.5V (Bio-Rad Gene Pulser) to transform. 1 ml ice cold 1M sorbitol was added and the solution was transferred to a 15 ml falcon tube. Incubate without shaking for 2 hours. Plate 50-200 µl aliquots onto YPDS plates containing 100 µg/ml zeocin. Incubate at 30°C for 2-3 days until colonies appear. Screen for insertion. Full details of all *Pichia pastoris* methods can be found in the *Pichia* Expression Kit User manual [Invitrogen, 2010b] and the pPICZα A, B, and C User Manual by [Invitrogen, 2010c].

Expression from *Pichia pastoris*

The standard protocol was used initially however, as that did not yield any protein the method was altered in an attempt to express/detect expression.

This is the protocol taken from the User manual [Invitrogen, 2010b]. Baffled flasks should contain no more than 30% culture of the total flask volume to ensure adequate aeration. Cultures used were KM71wt (control), pCPGRP and pCPGRP-NIR. From a single colony inoculate 25 ml BMGY, grow at 30°C until OD₆₀₀ 2-6. Once the correct OD was reached the cells were harvested (1500xg, 5 minutes) and resuspended in BMMY to induce expression. 100% methanol was added to a final concentration of 0.5% (v/v) every 24 hours to maintain expression. 1 ml samples were taken and spun

down at 14000 rpm for 2 minutes, both the supernatants and the pellets were retained and stored at -80°C. Samples were analysed by SDS-PAGE gels and Western Blots.

This protocol was supplied by the lab which supplied the strains. Inoculate 5 ml BMGY with a single colony and grow at 30°C for 24hrs. Add 95 ml BMGY and grow for a further 60 hours. Harvest cells and resuspend in 25 ml BMMY, incubate at a reduced temperature of 18°C for 5 days. Add 125 ml methanol once on the second and third days, then twice on the fourth day (morning and evening). Harvest on the fifth day. In later cultures sorbitol was added with the methanol to encourage expression

Larger culture volumes were set up with 50 ml samples taken to purify on Ni-NTA Magnetic Agarose Beads using the poly-His tag on the plasmid.

Expression from *Escherichia coli*

CPGRP and SP-D were cloned into pBAD/His A to test for expression in different *E. coli* strains. Once transformed, expression was as follows. A colony was used to inoculate 5 ml LB (with 50 µg/ml ampicillin) and grown overnight at 37°C until OD₆₀₀ = 1-2. Inoculate 10 ml LB (containing antibiotic) and grow until OD₆₀₀ = 0.5. Remove 1 ml as a 0 hour sample. Add arabinose to induce expression and sample at set times. Centrifuge, remove supernatant and store pelleted samples at -20°C until required.

Refer to the pBAD/His A manual [Invitrogen, 2010a] for more information.

Purification on Ni-NTA Magnetic Agarose Beads (Qiagen)

Wash Buffers

Wash 1: 0.606 g Tris, 1.75 g NaCl, 0.1 ml Tween, 0.034 g imidazole, 20 µl β-mercaptoethanol. Dissolve in 50 ml dH₂O and filter sterilise.

Wash 2: 0.606 g Tris, 0.3 g NaCl, 0.1 ml Tween, 0.034 g imidazole, 20 µl β-mercaptoethanol. Dissolve in 50 ml dH₂O and filter sterilise.

Elution Buffer: 0.606 g Tris, 0.3 g NaCl, 0.1 ml Tween, 1.7 g imidazole, 20 µl β-mercaptoethanol. Dissolve in 50 ml dH₂O and filter sterilise.

Add samples to beads and rotate overnight at 4°C in a falcon tube. Using a magnetic rack draw off supernatant, resuspend beads in wash or elution buffers. Use 50-100 µl bead resin to 5 ml sample, 400 µl for the washes and no less than 50 µl for the elution. Buffers should be made fresh for every use. Used beads can be stored in 30% EtOH at 4°C until regenerated.

Centricon Plus-70 (10K) Centrifugal Filter Devices

The Centricon Plus-70 (10K) Centrifugal Devices were purchased from Millipore. Duplicate supernatants from expression using the Barlow Protocol were used to give 50ml to spin down. Before the supernatant was passed through the columns it was filter sterilised (0.45 µm) to remove any remaining cell debris. A swinging bucket centrifuge was required and the columns were pre-rinsed using 70 ml dH₂O. The supernatant was spun down using 3500 xg for 30 minutes to give ~400 µl. Aliquot and freeze at -80°C until required. Further information can be found at http://kirschner.med.harvard.edu/files/protocols/Millipore_centricon70.pdf.

Lysis of *Pichia pastoris* Cell Pellet

Buffer: 0.44g NaCl,

40µl β-mercaptoethanol,

50µl leupeptin,

50µl peptatin,

50µl AEBSF

} From a 5mg/ml stock

Dissolve in 50 ml TrisHCl, pH 7.8

To make TrisHCl dissolve 0.303 g Tris in 50 ml dH₂O, pH to 7.8 with HCl

Method:

1. Add 1 volume ice cold lysis buffer and 3 volume zirconia beads to the cell pellet
2. Vortex the cells for 5x1 min intervals with 1 minute on ice between each vortex
3. Add 3 volumes of ice cold buffer and vortex briefly. Centrifuge for 20 minutes at 4000rpm to collect cell debris and zirconia beads.
4. Transfer the supernatant to Eppendorf tubes and centrifuge at 14000 rpm for 20 minutes
5. Pool supernatant for further use.

SDS-PAGE gels and Western Blots

Acrylamide gels were prepared as set out in Laemmli [1970]. Gels were prepared with a final concentration of 5% w/v (stacking) and 10% w/v (separating) bis-acrylamide.

The gels were prepared to the following recipe. APS and TEMED were the last ingredients to be added to the mixture to prevent premature setting.

Separating gel (10%) – makes 2 gels

dH ₂ O	2.8 ml
40% bis-acrylamide	2 ml
Separating buffer	3 ml
10%SDS	80 µl
10%APS	80 µl
TEMED	8 µl

Stacking gel (5%) – makes 2 gels

dH ₂ O	2.5 ml
40% bis-acrylamide	0.4 ml
Separating buffer	1 ml
10%SDS	40 µl
10%APS	40 µl
TEMED	4 µl

Solutions Required:

Separating buffer (1.5M Tris HCl at pH 8.8): 90.8 g Tris in 450 ml dH₂O. Adjust pH using HCl and bring volume up to 500 ml.

Stacking Buffer (0.5M Tris HCl at pH 6.6): 30.5 g Tris in 450 ml dH₂O. Adjust pH to 6.8 and bring volume up to 500 ml.

APS: Ammonium persulfate. Make up a 100 mg/ml solution. Store at 4°C for no more than 14 days.

5x Running Buffer: 30 g Tris, 144 g Glycine, 10 g SDS in 2 l dH₂O

Coomassie Brilliant Blue Stain: 25 ml methanol, 35 ml acetic acid in 500 ml dH₂O. Pinch of Coomassie Brilliant Blue.

Coomassie Blue Destain: 100 ml methanol, 140 ml acetic acid in 2 l dH₂O.

Western blot transfer buffer: 3 g Tris, 200 ml methanol, 14.4 g glycine in 1 l dH₂O.

Blocking Solution: 5 g Marvel skimmed milk powder and 0.25 ml Tween20 in 100 ml 1xPBS.

PBS-Tween: 250 µl Tween20 added to 500 ml 1x PBS.

Antibody: His-probe (H-3): sc-8036 HRP, Santa Cruz Biotechnology. 1:500 dilution in blocking solution.

ECL kit: Immobilon western Chemiluminescent HRP Substrate kit (Millipore).

Method:

Pelleted samples were re-suspended in 1xSDS-PAGE loading buffer before boiling for 10 minutes. 1xSDS-PAGE loading buffer was added to the supernatant samples before boiling for 10 minutes. Electrophoresis was carried out in 1x Running Buffer. Gels were run at 120-180 V depending on level of separation until the loading dye was at the bottom of the glass plates. Proteins were transferred from SDS-PAGE gels onto nitrocellulose membrane (GE Healthcare) using a semi-wet transfer system (BioRad).

The gel was placed on three pieces of blotting paper pre-soaked in Western Blot Transfer buffer. The nitrocellulose membrane was equilibrated by dipping in transfer buffer and placed onto of the gel. A further three layers of pre-soaked blotting paper was placed on top of the membrane and the stack rolled flat to remove any air bubbles. Run at 200 mA (10 V) for ~90 minutes.

Post transfer, membranes were incubated for one hour in blocking solution to prevent non-specific binding. After this, membranes were washed in PBS-Tween (4x5 minutes) and incubated with the antibody solution (1:500 dilution) for 2 hours. Wash again in PBS-Tween (4x5 minutes).

Western blots were developed with ECL chemiluminescence kit (Millipore). Equal amounts of the reagents were mixed together and poured over the entire surface of the nitrocellulose membrane. This was left for no more than three minutes, based on the manufacturer's instructions. The blot was imaged with no light, long exposure using an Epi Chem II Darkroom (UVP Laboratory Products).

Chapter 3:

Investigating the Alginate Bead Method as an Alternative to the Calgary Biofilm Device and Antibiotic Resistance in *Staphylococcus aureus*.

Introduction

The Calgary Biofilm Device and MBEC Method

The Calgary Biofilm Device [Ceri *et al.*, 1999] was developed in response to the need to have an accurate measurement of the concentration of antibiotic required to significantly affect a biofilm. Before this, the Minimum Inhibitory Concentration (MIC) was the standard measurement used for treatment of infections.

However, this measurement is obtained by determining the concentration of antibiotic effective against actively growing planktonic cultures and is used as the recommended dosage in the treatment of many acute infections [Andrews, 2001].

As has been detailed in Chapter 1, biofilms are communities of cells attached to a surface and each other, and embedded in a protective extracellular polymeric matrix [Costerton *et al.*, 1987; Lister and Horswill, 2014]. Biofilms have many protective properties including prevention of clearance by the host immune response, protection against harsh environmental conditions and most importantly, resistance to antibiotics [Archer *et al.*, 2011].

Due to these properties antibiotic MIC values, when used for the treatment of chronic and device related infections, often have little to no value, resulting in treatment failure [Costerton *et al.*, 1995].

There are various other models and techniques which replicate biofilms to some extent, the most significant of which is the modified Robbins device (MRD) which has been used for investigating biofilm physiology and antibiotic and disinfectant susceptibility. The main disadvantages of this device are the small number of samples that can be processed at any one time, the nutrient gradient which develops along the length of the device, and its unsuitability for rapid testing in a clinical environment [Ceri *et al.*, 1999; Hall-Stoodley *et al.*, 1999].

These are the reasons the Calgary Biofilm Device was developed and why it is used to determine the Minimum Biofilm Eradication Concentration (MBEC) of bacterial biofilms.

Chapter 1 details the Calgary Biofilm Device and MBEC Assay but the procedure is summarised here. The Calgary Biofilm Device consists of a lid with 96 pegs (Figure 1.10) which fits into a standard 96 well plate. A bacterial inoculum is introduced to the pegs which are then incubated on a rocking table at 35°C for the required time to allow the biofilms to form. The lid can then be transferred to other bases containing antibiotics, antiseptics or disinfectants for set times to challenge the biofilms [Allan *et al.*, 2011]. The pegs are washed before and after exposure to the challenge medium to remove any non-adherent cells and residual antibiotics [Harrison *et al.*, 2010].

The biofilms are then removed from the pegs either individually by breaking them from the lid and sonicating, or from all the pegs at once though sonication. The viability of the biofilms is determined either by plate counts or by reading the turbidity at 650nm after a period of growth in a plate reader.

There are many advantages to the Calgary Biofilm Device, the main one is the ability to process multiple samples at any one time. However, there are also disadvantages including the difficulty in reproducibility and the cost of the device [Dall, 2013; Harrison *et al.*, 2010].

A cost effective alternative which is based on the submerged substratum model has been developed by a previous student in the Gallagher laboratory [Dall, 2013]. This method consists of sodium alginate beads (Figure 1.12) on which the biofilms form, and which can subsequently be dissolved to recover viable bacteria. Formation of stable biofilms have been confirmed on the beads though cryo-scanning electron microscopy (Figure 3.1). The results from the electron microscopy show that within 2 hours ~10% of the alginate bead surface was colonised with *Staph. aureus* biofilms 5-6 cells thick [Dall, 2013].

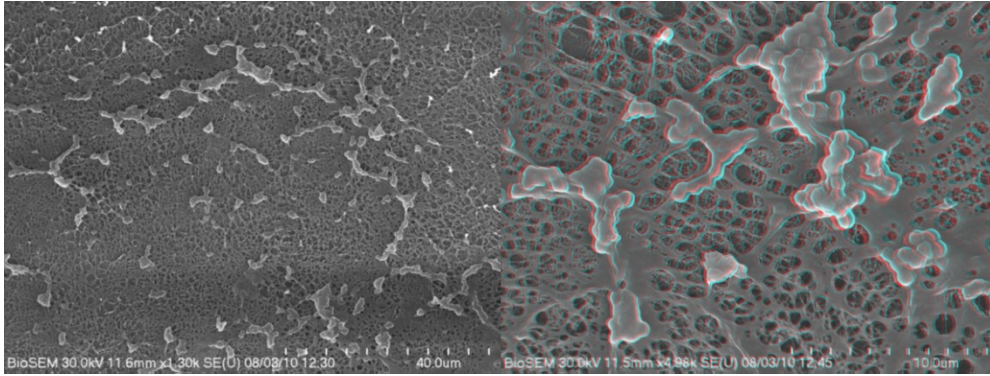


Figure 3.1: Cryo-SEM Images of Stable Biofilms on Alginate Beads [Dall, 2013]. The images were taken perpendicular to the alginate bead and show EPS secreted by and encasing the *Staph. aureus* colonies. The image on the right is in 3-D.

The Alginate Bead Method

The beads are formed by solidifying sodium alginate with 2M CaCl₂ through pipetting 20 µl 2M CaCl₂ into each well of a 96 well plate, adding 210 µl of sterile 4% sodium alginate (w/v), followed by 20 µl 2M CaCl₂, under aseptic conditions. The plate is covered and incubated for 4 hours at 60°C to set the beads. These beads can be stored at 4°C for up to a week. The basic shape of the bead is determined by the 96 well plate in which it is formed (Figure 1.12). The beads have an average surface area of 160.31 mm² [Dall, 2013].

For the following experiments, the beads were removed from the 96 well plate using a sterile wire and flamed forceps, individually washed in dH₂O (using a 48 well plate) and placed in a 48 well plate. Overnight bacterial cultures were diluted in fresh medium to ~10⁴ CFU/ml and 600 µl was pipetted into the wells containing the beads. The plates were incubated at 37°C, with shaking (150 rpm) for the required time to allow the biofilms to form. Triplicate beads were used for each different condition and controls to test for sterility were always included.

There are both advantages and disadvantages with this method. The major advantages are the low cost and easy procedures. Most of the reagents and equipment required are cheap, and present in many laboratories or easily obtainable. A disadvantage would be that the process is labour intensive. However, levels of reliability and reproducibility are high.

Comparisons are made between the two methods in terms of recovery of biofilms, medium used and dissolving solutions.

Rationale behind Antibiotic Choices Tested against *Staphylococcus aureus* Biofilms

The antibiotics used for the following experiments are detailed in Chapter 1 and are based on those in use in current clinical practice for the treatment of prosthetic joint infections [Nandi *et al.*, 2009; Zilberman and Elsner, 2008]. A previous student in the Gallagher laboratory had investigated them in regards to orthopaedic infections specifically antibiotic loaded acrylic bone cements [Dall, 2013].

Aims of this Chapter

- To determine whether the alginate beads influence biofilm formation and the length of time it takes for biofilms to develop.
- To compare the Calgary Biofilm Device method to the alginate bead method using different biofilm recovery techniques, media, and bead dissolving solutions.
- To examine the different gentamicin concentrations required to affect planktonic cultures and biofilms of the same age.
- To investigate the development of gentamicin resistance over 24 hours of *Staphylococcus aureus* biofilms.
- To examine how *Staphylococcus aureus* biofilms are affected by commonly used antibiotics used to treat staphylococcal infections.
- To explore what the effects are when gentamicin is combined with other antibiotics commonly used to treat *Staphylococcus aureus* infections.

Stable Formation of Biofilms Occurs Within Four Hours

The use of alginate beads for the formation of biofilms has already been demonstrated [Dall, 2013]. However, like most publications which report on biofilm related investigations, Dall [2013] examined biofilms which had been grown for 24 hours or more. The initial formation (within a few hours of incubation) of biofilms needs to be examined in terms of how quickly the bacterial cells attach and how long after incubation the biofilm becomes firmly established.

The biofilms were developed using overnight cultures diluted to $\sim 10^4$ CFU/ml to inoculate triplicate alginate beads for each time point in 48 well plates. The well plates were incubated at 37°C, with shaking (150rpm) for the required time (0.5, 1, 2, 3, and 4 hours). After removal under aseptic conditions, the beads were washed and dissolved in 2ml dissolving buffer. This solution was sampled, serially diluted and plated. The CFU/ml counts were calculated as previously described. Controls of beads in LB and plain LB were always included. If any contamination was present in these, the experiments were repeated.

It can be seen from Figure 3.2 that *Staph. aureus* biofilms form quickly on the alginate beads and that attachment is established by four hours growth, as judged by the fact the organisms remained attached during the wash stages.

A preliminary experiment to this was to determine how adherent the biofilm was, whether the bacteria were loosely attached or whether attachment was firm. To this end three sets of triplicate beads were incubated for two hours in $\sim 10^4$ CFU/ml diluted overnight culture. The beads were then washed once, twice or three times. The CFU/ml count of the beads after 1, 2 or 3 washes was approximately the same indicating that the cells firmly attached and starting to form a biofilm (Data not shown).

As this demonstrates that the alginate beads are a suitable base for biofilm formation, comparison between the alginate beads and the Calgary method can be carried out.

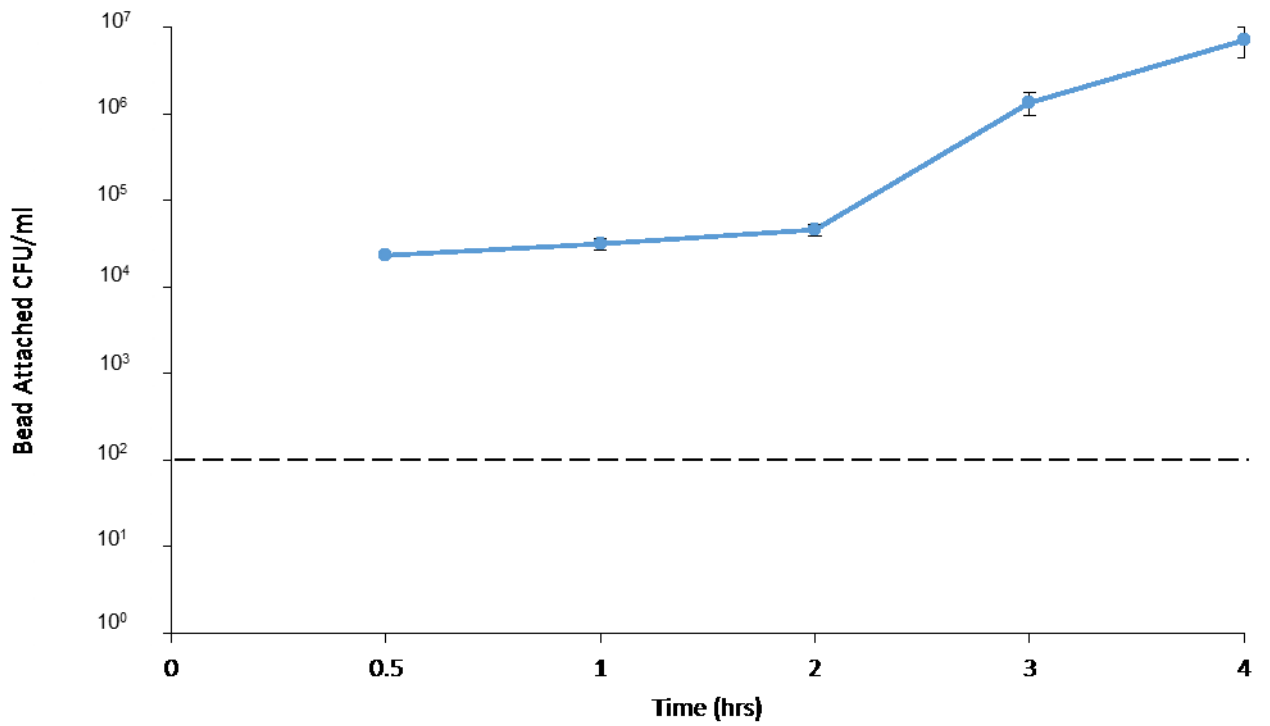


Figure 3.2: Stable Formation of Biofilms by 4 hours. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. Cultures were set up from diluted overnight cultures (~10⁴ CFU/ml) with triplicate beads sampled at 0.5, 1, 2, 3 and 4 hours. Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml)

Investigating the Alginate Bead Method as an Alternative to the Calgary Biofilm Device

One of the perceived points of variation in the Calgary Method is the recovery step. When the biofilms are sonicated off the pegs it is unlikely that the entire biofilm is removed. Indeed Dall [2013] had considerable difficulty regarding the reproducibility of this step. This was one of the reasons why the alginate bead method was developed.

As the beads are crushed and dissolved in the alginate bead method, the entire biofilm is recovered; however, the crushing and dissolving is a labour intensive process and fairly time consuming. Thus, it was decided that sonication would be experimented with to examine how it differed in removal of the biofilm from the bead compared to the original crushing method.

Beads were incubated for 24 hours with diluted overnight cultures ($\sim 10^4$ CFU/ml) at 37°C, 150 rpm, (as described in Materials and Methods), before being washed and transferred into short plastic universals in 2 ml dissolving solution. Triplicate beads were used for each condition. Initially, an Ultrasonic Processor (Thermo Scientific) was used for different times to sonicate the beads. During sonication the universal was placed on ice to reduce the heating effect of the machine. After sonication the solution was sampled, serially diluted and plated using the Miles Misra method (20 μ l spots). Spots were counted if they contained between 2 and 30 colonies.

The CFU/ml was calculated using $N = (C.V)/D$, where C is the number of colonies, V is 250 and D is the dilution factor. Error bars indicate standard deviation from the mean of the triplicate beads. Figure 3.3 shows the effect of high intensity sonication on the biofilms.

When examining the graph (Figure 3.3) the bacteria did dislodge but at higher sonication times there was reduced recovery. This type of sonicator is commonly used to lyse cells which could account for the reduced bacterial counts. The bead structure was affected by the sonication with condition 2 beads (1.5 min sonication) dissolving by about 80%. Condition 3 beads (1 min sonication) dissolved by about 50% and for condition 4 (30 sec sonication) the beads broke up and dissolved by about 30%.

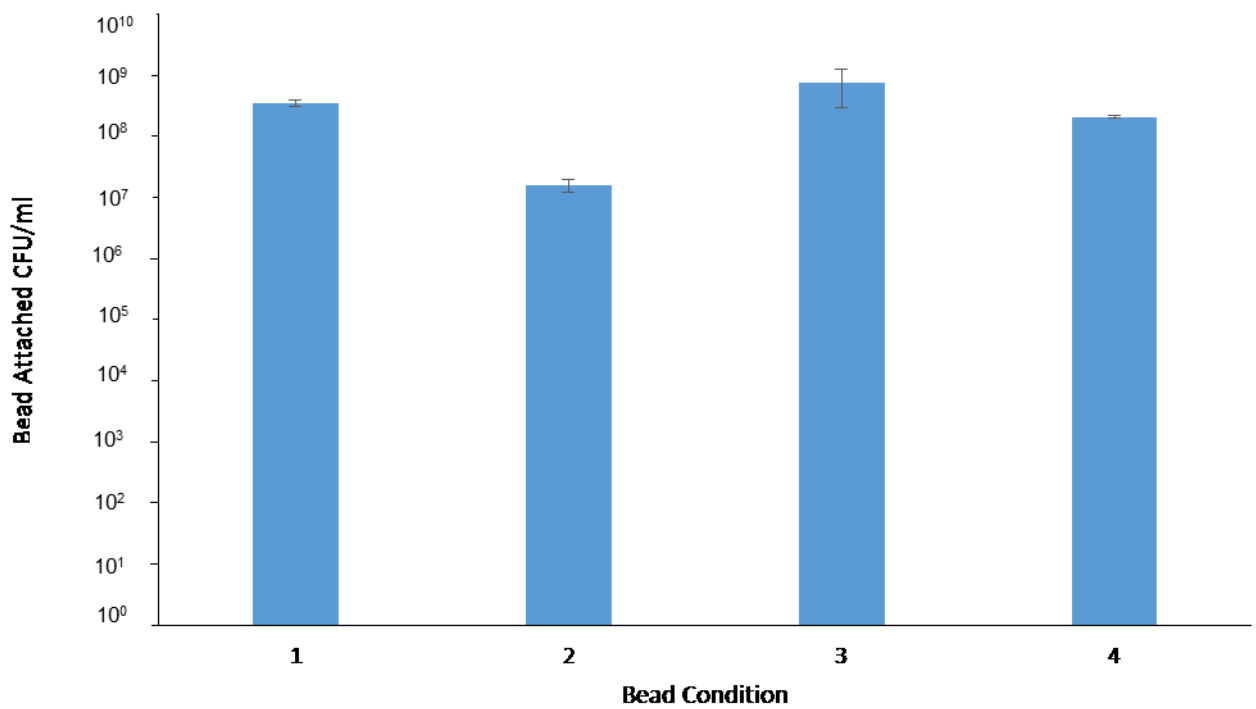


Figure 3.3: The Effect of High Intensity Sonication on 24 hour old *Staphylococcus aureus* Biofilms. Mean CFU/ml counts are plotted against different bead conditions with error bars of standard deviation. Triplicate beads were incubated for each time points for 24 hours at 37°C, 150 rpm. Beads were washed and transferred into 2 ml dissolving solution. Condition 1: normal method of crushing and dissolving, Condition 2: 1.5 min sonication at 1kHz, Condition 3: 1 min sonication at 1kHz and Condition 4: 30 sec sonication at 1kHz. After treatment the bead solutions were sampled, serially diluted and plated as described in Materials and Methods.

Ceri *et al* [1999] sonicated the pegs using an Aquasonic machine for 5 minutes on high. The specifications of that machine are 120V, 60Hz, 4 Amps, whereas the lowest setting on the Ultrasonic Processor is 1 kHz; so there is a huge increase in the amount of power applied to the cells. A lower powered machine was therefore explored to allow a more accurate comparison of the Calgary Biofilm Device and alginate bead methods.

Additionally, using the Ultrasonic Processor is time consuming as only one sample can be treated at any one time. The use of a sonicating water bath (Decon F5 Minor, Ultrasonic Ltd, 240V/40kHz) would solve this issue.

When using the sonicating water bath, the biofilms were grown for 24hrs at 37°C, 150 rpm before being washed and transferred into a standard 48 well plate containing 600 µl dissolving solution which was then placed into the water bath. Triplicate beads were removed at 5, 10, 15, 20, 25, and 30 minutes (Figure 3.3). The beads and surrounding solutions were transferred to 1.4 ml dissolving solution, sampled, serially diluted and plated as described in Materials and Methods.

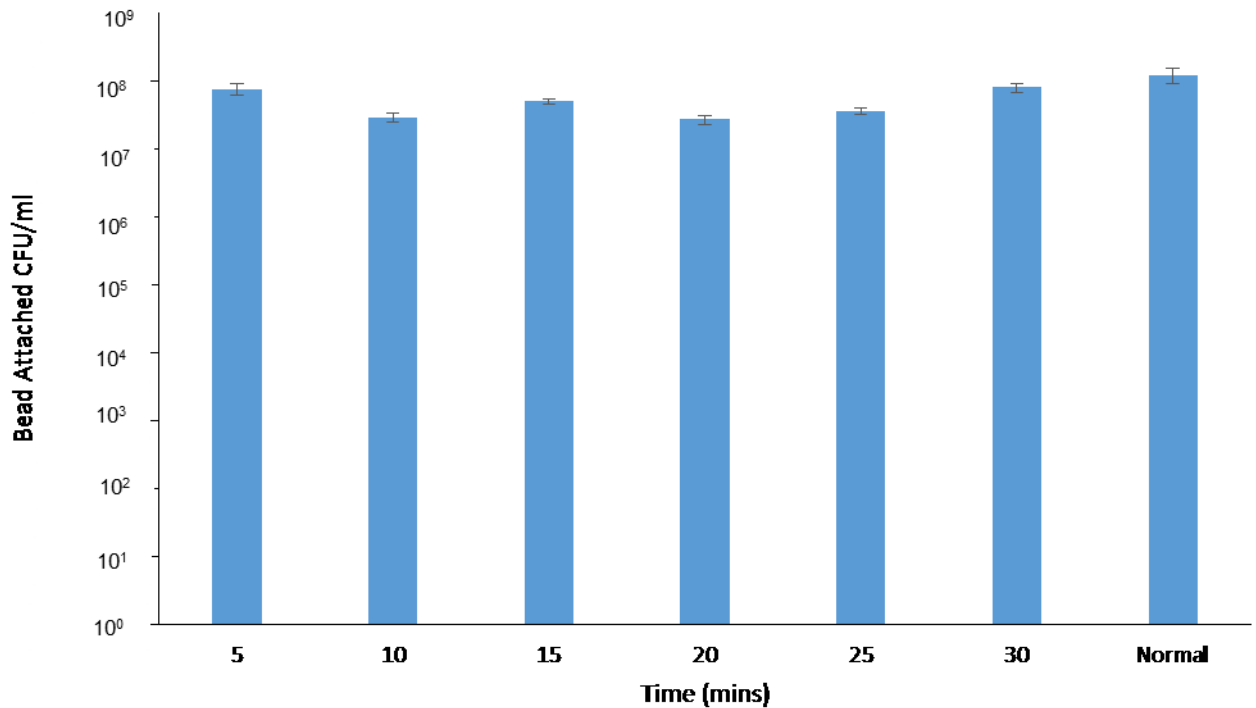


Figure 3.4: The Effect of Different Times in a Sonicating Water Bath on 24 Hour Old *Staphylococcus aureus* Biofilms. Mean CFU/ml counts are plotted against different bead conditions with error bars of standard deviation. Triplicate beads were incubated for each time point for 24 hours at 37°C, 150 rpm. Beads were washed and transferred into 600 µl dissolving solution in a 48 well plate which was then placed in the sonicating water bath for 5 (1), 10 (2), 15 (3), 20 (4), 25 (5), or 30 (6) minutes at 40kHz. For condition 7, the beads were treated in the normal way. After sonication, the beads and surrounding solution were transferred to 1.4 ml dissolving liquid. This solution was sampled, serially diluted and plated as described in Materials and Methods.

Figure 3.4 illustrates that with the lower powered machine, the different sonication times do not seem to affect whether the entire biofilm is recovered, with 5 minutes sonication (condition 1) as effective as the normal method of crushing and completely dissolving the bead (condition 7). The beads remained intact, however, longer sonication times softened their structure. It appears that 5 minutes sonication is an acceptable alternative to the original method of crushing.

The use of the water bath is a viable alternative to crushing and dissolving the beads reducing the time and labour required to recover the biofilms. The experiments which produced Figures 3.3 and 3.4 used pure dissolving solution as the liquid in which the biofilms are recovered. However, this solution is fairly acidic and investigations were carried out to examine whether this solution has an effect on cell viability.

Figure 3.5 shows the effect of different mixtures of recovery liquid for both the original method (crushing and dissolving) and for 5 minutes sonication. Two different bacterial species were used for these experiments, *Staph. aureus* (A) and *Ps. aeruginosa* B (B). This *Ps. aeruginosa* strain is extremely mucoid and a constitutive alginate producer; it was included to examine whether this property has any effect on the bead composition.

The beads were incubated at 37°C, 150 rpm for 24 hours in diluted overnight cultures ($\sim 10^{-4}$ CFU/ml) before being washed and placed into the recovery solution. For conditions 1-3 the normal method of crushing and dissolving the bead was carried out. For conditions 4-6 the beads were sonicated for 5 minutes as previously described. For conditions 1 and 4 2 ml dissolving buffer was used, conditions 2 and 5 used 1ml dissolving buffer and 1 ml medium and conditions 3 and 6 used 2 ml medium. LB, which had been used in all previous experiments was used, as was cation-adjusted Mueller-Hinton (MH) broth (Figure 3.5). The choice of this medium was based on recommendations by the Clinical Laboratory Standards Institute (CLSI) for antibiotic assays and determination of MICs [Harrison *et al.*, 2010; Koeth *et al.*, 2000].

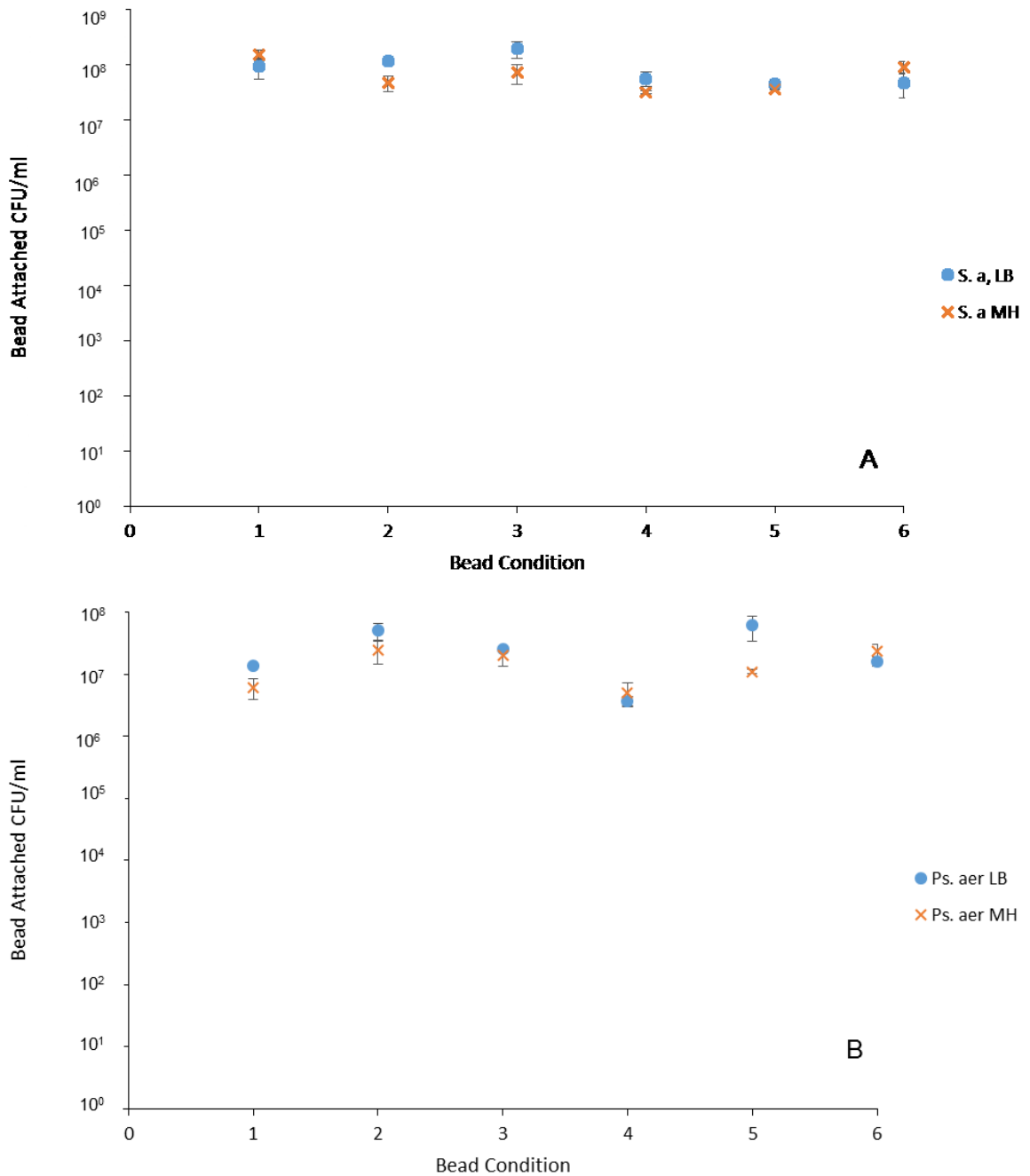


Figure 3.5: Comparison of Recovery Solutions and Medium for *Staphylococcus aureus* (A) and *Pseudomonas aeruginosa* (B) Biofilms. Mean CFU/ml counts are plotted against different bead conditions with error bars of standard deviation. Triplicate beads were incubated for each time points for 24 hours at 37°C, 150 rpm. Beads were washed and transferred into either 2ml liquid and treated in the normal manner (conditions 1-3) or 600 µl liquid in a 48 well plate and sonicated for 5 minutes (Conditions 4-6). Conditions 1 and 4: 2 ml dissolving solution, Conditions 2 and 5: 1 ml dissolving solution, 1ml medium. Conditions 3 and 6: 2 ml medium. After sonication the beads and surrounding solution were transferred to 1.4ml dissolving liquid. This solution was sampled, serially diluted and plated as described in Materials and Methods.

From Figure 3.5 A, it can be seen that there is no great difference between the two medium types. However, the fact that the CFU/ml for condition 3 is slightly higher than the CFU/ml for condition 1 is a bit of an anomaly. There is next to no difference between the different recovery solutions for *Staph. aureus* (Figure 3.5 A).

Regarding Figure 3.5 B the dissolving solution used for recovery (conditions 1 and 4) has a lower recovery of CFU/ml than the other conditions. This could be due to the acidic nature of the dissolving solution having an effect on cell viability. It seems that a 1:1 mixture of dissolving solution and medium is the best recovery solution. The fact that sonication was also able to remove the *Ps. aeruginosa* biofilms from the alginate beads show that even an alginate producing bacterial strain is able to form biofilms and be removed from the alginate beads.

As it has now been established that the alginate beads are a suitable alternative to use to examine biofilm formation, the development of antibiotic resistance can be investigated.

Inhibition of *Staphylococcus aureus* Biofilms by Gentamicin

Gentamicin is a member of the aminoglycoside class of antibiotics which are commonly used in the treatment of staphylococcal infections [Ramirez and Tolmasky, 2010]. One of gentamicin's main uses is in the bone cement used for prosthetic joint surgeries. A major component of prosthetic joint infections is based biofilm based with long term sub-clinical infections as the most common presentation [Stoodley *et al.*, 2011]. Resolution of infection often requires surgical intervention and prolonged antibiotic treatment which severely reduces a patient's quality of life. In a step to prevent infections, Antibiotic-Loaded Acrylic bone Cements (ALACs) are used, these contain gentamicin because of its broad antibacterial spectrum and stability at high temperatures [Neut *et al.*, 2005].

The Differences in Gentamicin Concentrations which Affect Planktonic Cultures and Biofilms

The ineffectualness of an antibiotic MIC on biofilm infections is well documented [Andrews, 2001; Ceri *et al.*, 1999]. A direct comparison between the antibiotic concentrations required to affect either a planktonic culture or a biofilm is shown in Figure 3.6.

In order to investigate this, overnight cultures were diluted to $\sim 10^4$ CFU/ml in fresh LB and 600 μ l were pipetted into a 48 well plate. Triplicate wells were set up for each different concentration. The 48 well plate was incubated at 37°C, 150 rpm. After 3 hours incubation the designated gentamicin concentrations were added to the wells and the plate was incubated for a further two hours. After the challenge the wells were sampled, serially diluted and plated.

The same diluted overnight culture was used to inoculate the alginate beads. Triplicate beads were set up for each antibiotic concentration. The beads were incubated for 3 hours before being washed and transferred into fresh LB broth with the appropriate antibiotic concentration for the 2 hour challenge. Once challenged, the beads were washed, crushed, dissolved and sampled as described in the Materials and Methods.

There is a significant difference between the antibiotic concentrations required to kill a three hour old planktonic culture compared to a 3 hour old biofilm. Examining Figure 3.6, it requires 125 μ g/ml gentamicin to kill a 3 hour old planktonic culture whereas it requires 225 μ g/ml to kill a 3 hour old biofilm culture suggesting that the biofilm affords protection against the antibiotic action.

150 μ g/ml gentamicin was over the concentration required to eliminate an actively growing planktonic culture (Figure 3.6). When this concentration was applied to biofilm growth over time, resistance developed after 4 hours. After 24 hours the gentamicin is having little effect on the biofilm as seen from the increase in CFU/ml count (Figure 3.7 B).

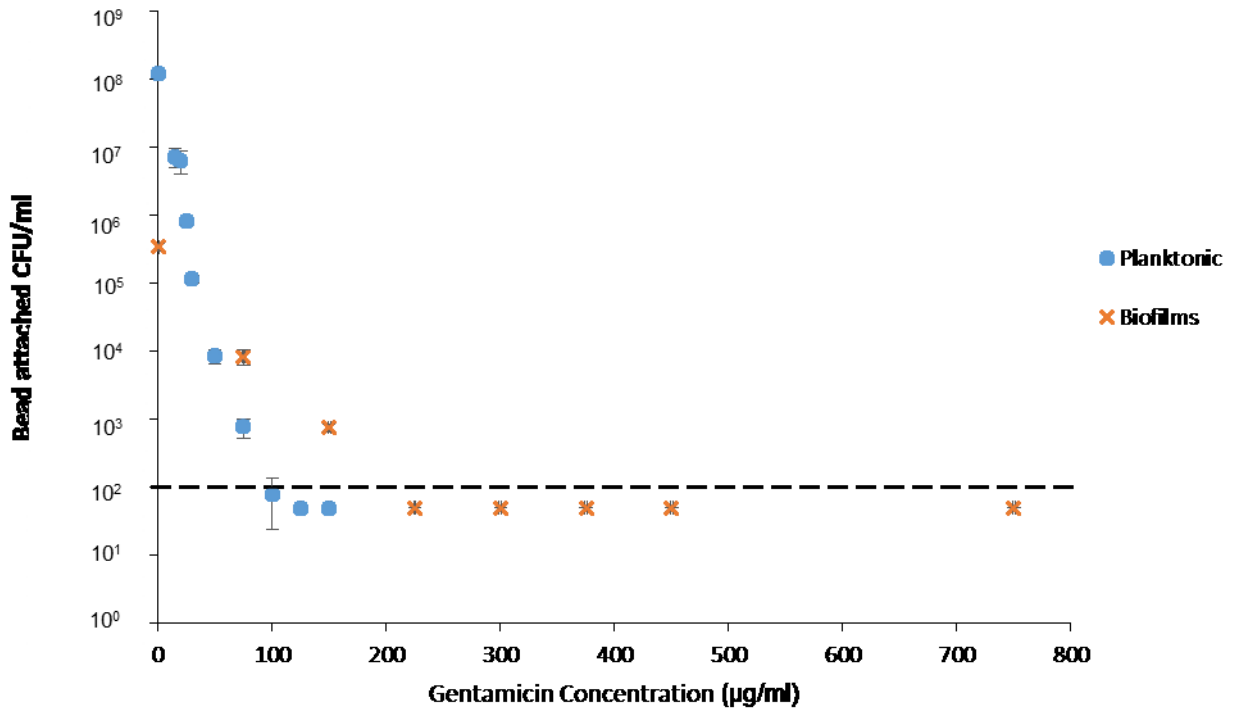


Figure 3.6: Gentamicin Concentrations Required to Inhibit 3 hour old Planktonic Cultures or 3 hour old Biofilms. Mean CFU/ml counts are plotted against antibiotic concentrations with error bars of standard deviation. A) 600 µl diluted overnight cultures were incubated for 3 hours at 37°C, 150 rpm before being challenged with different gentamicin concentrations for 2 hours. Wells were sampled, serially diluted and plated B) The biofilms were incubated for 3 hours at 37°C, 150 rpm before being challenged with different gentamicin concentrations for 2 hours. Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml)

The Development of Biofilm Resistance over 24 hours with Different Gentamicin Concentrations

In order, to explore how quickly the *Staph. aureus* biofilms developed resistance, growth curves were carried out with the biofilms challenged by gentamicin. Two different gentamicin concentrations were used (Figure 3.7).

The concentration of gentamicin required to affect and inhibit *Staph. aureus* biofilm growth was determined either by growth at 37°C overnight on agar plates (data not shown) or by the concentration required to kill an actively growing planktonic culture (Figure 3.6). This latter concentration is considered the MIC and would be the standard concentration recommended by a hospital laboratory for the treatment of a clinical infection.

Determination of the MIC is described in the Materials and Methods. The inoculum used to develop the biofilms was diluted overnight cultures and averaged $\sim 10^4$ CFU/ml.

The alginate beads were incubated at 37°C, 150 rpm for the required growth time (0.5, 1, 2, 3, 4, 6, 8 or 24 hours). The beads were removed and washed before being placed in fresh medium with the required antibiotic concentration for the 2 hour challenge. After this time the beads were washed, dissolved, serially diluted and plated. CFU/ml counts were calculated as previously described.

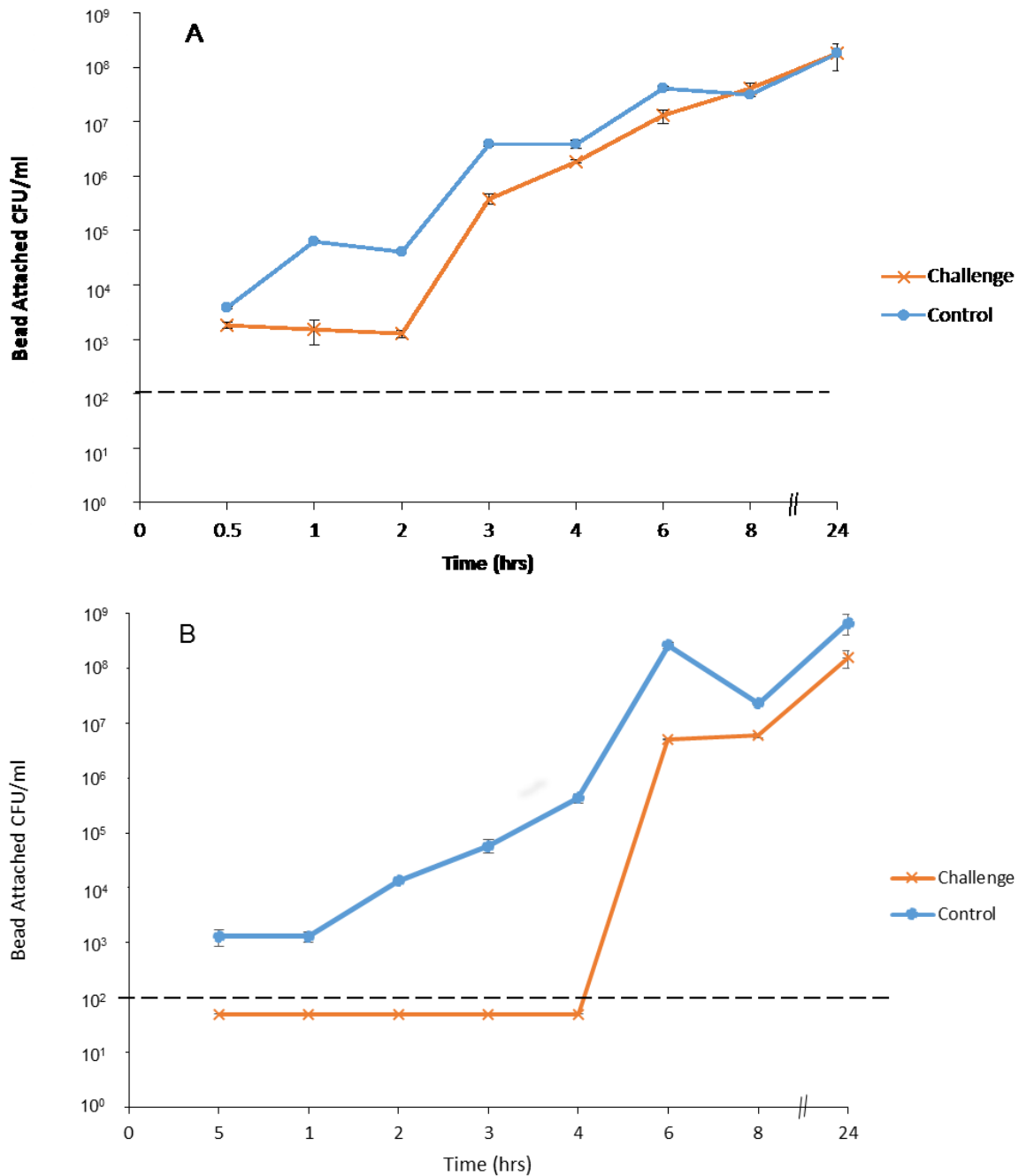


Figure 3.7: Development of Resistance in a Biofilm over 24 hours against 15 µg/ml (A) and 150 µg/ml (B) Gentamicin. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. Cultures were set up from diluted overnight cultures with triplicate beads incubated for the indicated time points as described in Materials and Methods. The beads were challenged with either 15 µg/ml (A) or 150 µg/ml (B) gentamicin for 2 hours before being washed, dissolved, serially diluted and plated. No antibiotics were added to the control beads. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

When cultivating *Staph. aureus* overnight on an LB agar plate containing 15µg/ml gentamicin, there was complete inhibition of growth. However, as can be seen from Figure 3.7 A, when the bacteria are actively growing on the alginate beads and forming the biofilms, there is low level resistance immediately and after 4 hours incubation the gentamicin concentration has a negligible effect on the bacteria.

The development of resistance with the higher gentamicin concentration of 150 µg/ml only occurs between 4 and 6 hours growth. However, resistance increases sharply and by 24 hours the CFU/ml counts between the antibiotic challenged beads and the control are similar.

As has been stated before an alginate bead has a surface area of 160.31 mm² or 160x10⁶ µm. If a *Staph. aureus* cell is ~1 µm then 1.6x10⁷ cells are needed for complete coverage. However, cells would not form evenly on the bead surface and they would naturally clump together (Figure 3.1). Thus it could be theorised that from ~10⁴/10⁵ cells biofilms would be forming and developing resistance to the antibiotics. When comparing this number with Figure 3.7 A resistance starts to develop from 10³ cells with full resistance at ~10⁷ cells supporting the argument that a biofilm has formed. In Figure 3.7 B resistance once again develops by ~10⁷ cells.

As in a clinical situation, a biofilm infection would only present after an extended period of sub-clinical symptoms; a 24 hour biofilm model is more appropriate to use to investigate the effective antibiotic concentrations required.

Increasing Gentamicin Concentrations are Required to Affect a 24 hour old Biofilm

As a biofilm develops and establishes itself there is a corresponding rise in the antibiotic concentrations required to have a significant effect. The ability to resist antibiotic, antiseptic and disinfectant treatments is considered a sign of a mature biofilm [Kiedrowski and Horswill, 2011]. In terms of experimental biofilm models this is usually deemed to be 24 hours. Therefore, determining antibiotic concentrations which affect biofilms at this time is essential.

Triplicate beads, for each gentamicin concentration, were incubated at 37°C, with shaking (150 rpm) for 24 hours. For Figure 3.8 (medium change), the beads were washed and placed into fresh medium containing the appropriate gentamicin

concentration for the 2 hour challenge. After this time the beads were washed, dissolved, serially diluted and plated. Following incubation overnight, the colonies were counted and the CFU/ml calculated as described before.

Without medium change, after 24 hours incubation the beads were not transferred into fresh medium, instead the antibiotic was added directly into the wells and the plate was incubated for the 2 hour challenge. The decision was made to not change the medium as in an infection situation the static biofilm would only have access to limited nutrients.

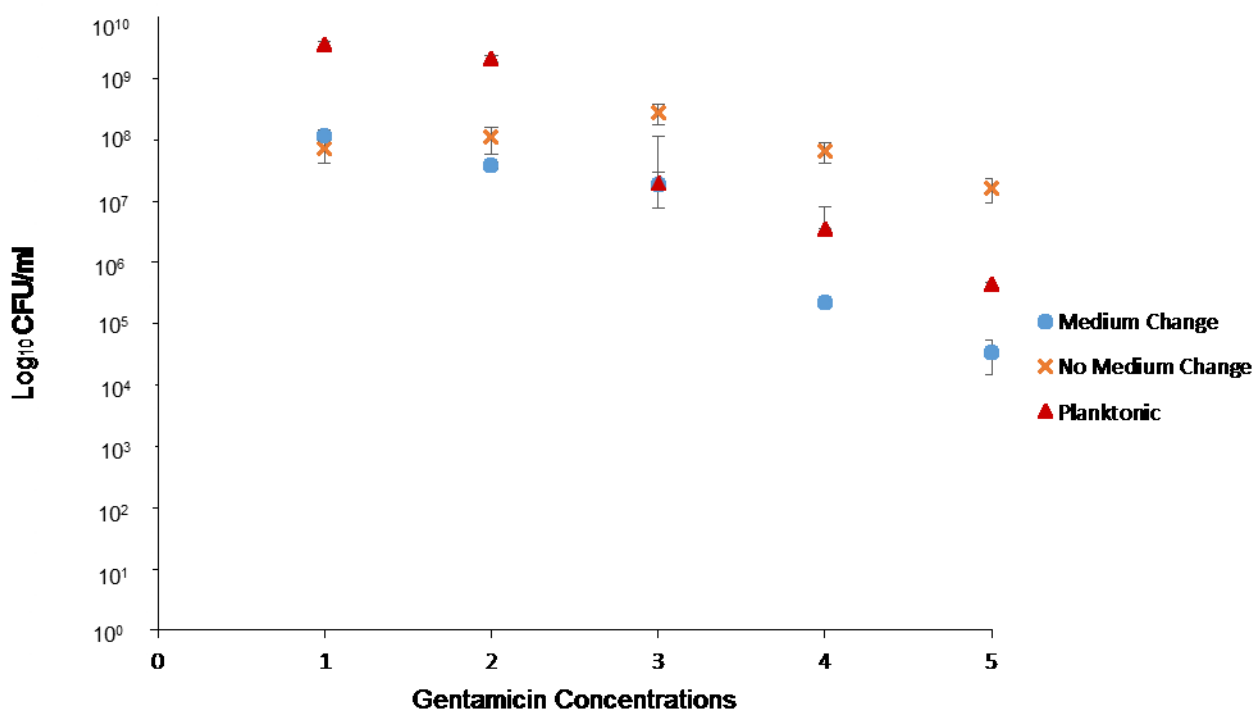


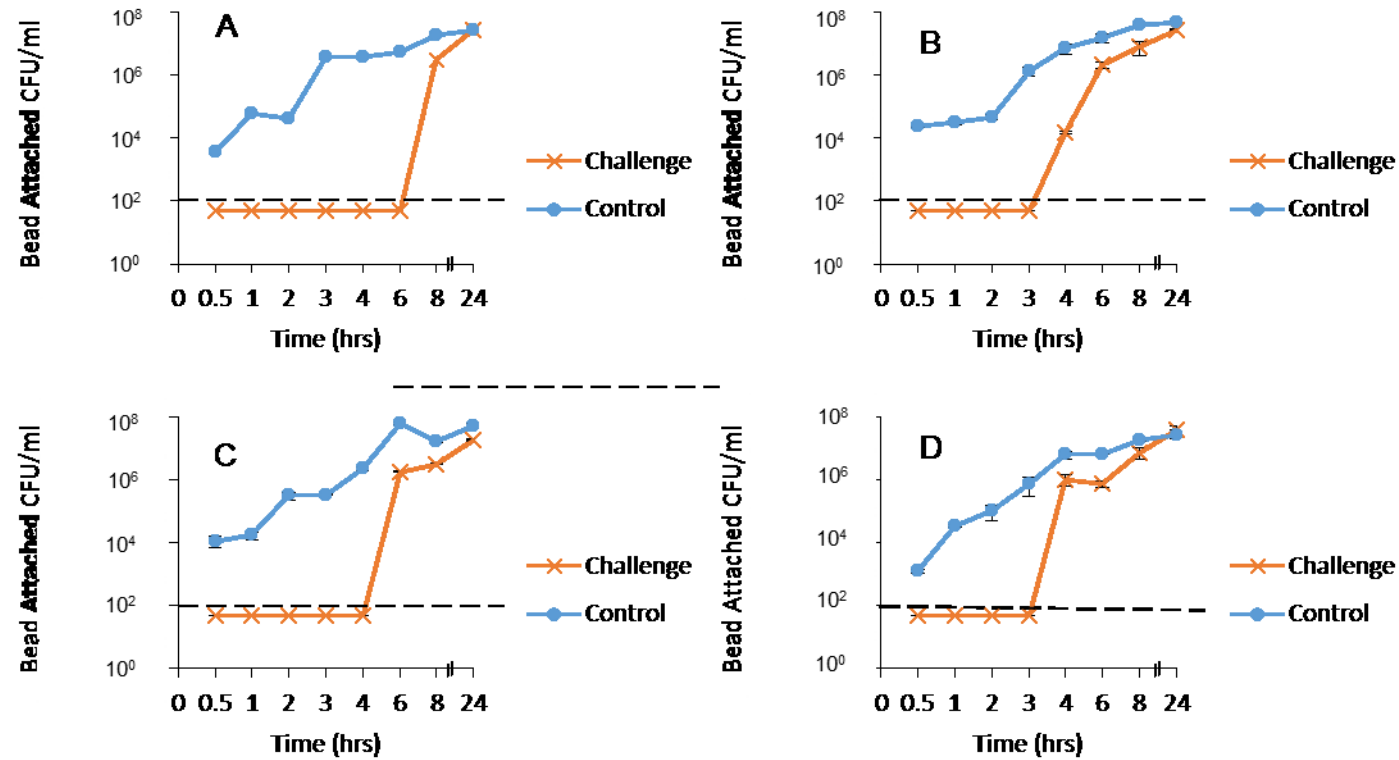
Figure 3.8: Increasing Gentamicin Concentrations are Required to affect a 24 hour old Biofilm (with/out medium change) and Planktonic Cultures. For the medium change: the beads were incubated for 24 hours before been washed and transferred into fresh LB with the appropriate gentamicin concentration. No Medium Change: The beads were incubated for 24 hours. The appropriate gentamicin concentration was added directly into the wells. Gentamicin concentrations 1: 0 µg/ml, 2: 15 µg/ml, 3: 150 µg/ml, 4: 256 µg/ml and 5: 1024 µg/ml. After the 2 hour challenge, the beads were washed, dissolved, serially diluted and plated as described in Materials and Methods. For the planktonic cultures: 600 µl diluted overnight culture was incubated for 24 hrs before been challenged with gentamicin. Wells were sampled, serially diluted and plated. CFU/ml counts are plotted against gentamicin concentrations with error bars of standard deviation.

Figure 3.8 (medium change) shows that only the higher concentrations of gentamicin had a significant effect on the biofilm load. However, even the highest concentration of 1024 µg/ml did not completely eliminate the biofilm, instead only reducing the biofilm load by 3 log orders. The CFU/ml counts detected would still cover the majority of the bead's surface suggesting that biofilms are present and are providing resistance to the antibiotics.

A reduced effect is seen in Figure 3.8 (no medium change), with even the highest concentration only decreasing the biofilm load by 1.5 logs. This is most likely due to the addition of fresh medium reactivating the cells systems and allowing the gentamicin to reach its target. The surrounding planktonic culture in Figure 3.8 (no medium change), may also provide a protective effect in that the gentamicin would first affect the free floating bacterial cells before moving onto the biofilm at a lower concentration. The planktonic cultures included on this graph were taken from the same overnight culture which was used to inoculate the beads. The resistance profile for the planktonic culture falls between the two biofilm cultures (with/out medium change), with the highest concentration reducing the bacterial load by 3 log orders. It would seem logical that after 24 hours the cells are well into stationary phase and more or less metabolically inactive and thus the antibiotic would not have a strong effect.

Development of Resistance of *Staphylococcus aureus* Biofilms to Other Antibiotics over 24 hours

Having observed that the gentamicin resistance develops quickly and by 24 hours has a negligible effect on biofilms, the next logical step would be to compare if and how antibiotic resistance develops for other antibiotic classes. The antibiotics were selected based on a previous student's work on orthopaedic infections and antibiotic loaded acrylic bone cements [Dall, 2013]. The method followed is the same as that for determining gentamicin resistance, as described for Figure 3.7.



Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The biofilms were incubated for the indicated times before being challenged with antibiotics for 2 hours. Beads were washed, dissolved, serially diluted and plated as described in the Materials and Methods. No antibiotics were added to the control. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

- A) 6 µg/ml Rifampicin,
- B) 15 µg/ml Vancomycin,
- C) 10 µg/ml Ciprofloxacin,
- D) 0.1 µg/ml Clindamycin.

Figure 3.9a: Development of Antibiotic Resistance of *Staphylococcus aureus* Biofilms over 24 hours for Rifampicin (A), Vancomycin (B), Ciprofloxacin (C) and Clindamycin (D).

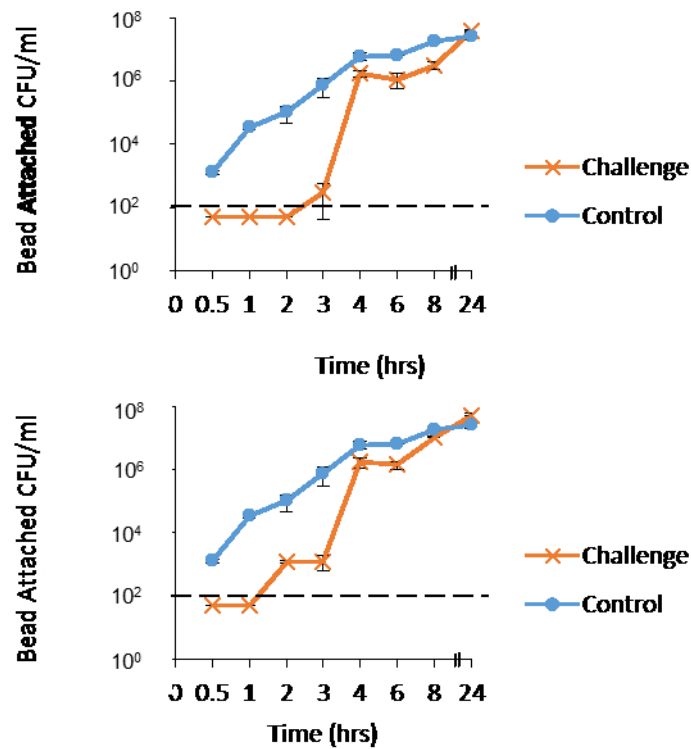


Figure 3.9: Development of Antibiotic Resistance of *Staphylococcus aureus* Biofilms over 24 hours for Linezolid (E) and Daptomycin (F). Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The biofilms were incubated for the indicated times before being challenged with antibiotics for 2 hours. Beads were washed, dissolved, serially diluted and plated as described in the Materials and Methods. No antibiotics were added for the control. E) 4 $\mu\text{g/ml}$ linezolid, F) 0.5 $\mu\text{g/ml}$ daptomycin (supplemented with 50 $\mu\text{g/ml}$ CaCl_2). The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml)

From Figure 3.9 it can be seen that resistance against the antibiotics, with the exception of daptomycin, takes at least 3 hours to develop (Figure 3.9b, F). Resistance to daptomycin developed after only 1 hour's growth. Rifampicin resistance developed between six and eight hours of growth (Figure 3.9a, A). This is the longest time observed for any of the antibiotics. However, once the resistance developed there was a steep increase in CFU/ml counts. Linezolid resistance develops by three hours with a gradual increase in CFU/ml counts. Both vancomycin and clindamycin resistance develops by four hours whereas ciprofloxacin resistance appears by six hours.

Effects of Gentamicin with other Antibiotics on *Staphylococcus aureus* Biofilms over 24 hours

As it is usual, within the clinical environment, to treat biofilm infections with a combination of antibiotics in order to improve bacterial target range and to prevent development of resistance, combination experiments were carried out. These experiments investigated synergism and antagonism between gentamicin and the above antibiotics (Figure 3.10 A-F). The antibiotic concentrations used were those which inhibited growth overnight on LB agar plates.

The beads were incubated at 37°C, 150 rpm for the required time (0.5, 1, 2, 3, 4, 6, 8 and 24 hours). The beads were then washed and transferred into fresh medium containing either gentamicin, the second antibiotic, both antibiotics, or no antibiotics (control). After 2 hours challenge, the beads were washed, dissolved, and the solution was serially diluted and plated as described in the Materials and Methods. CFU/ml counts were calculated as before.

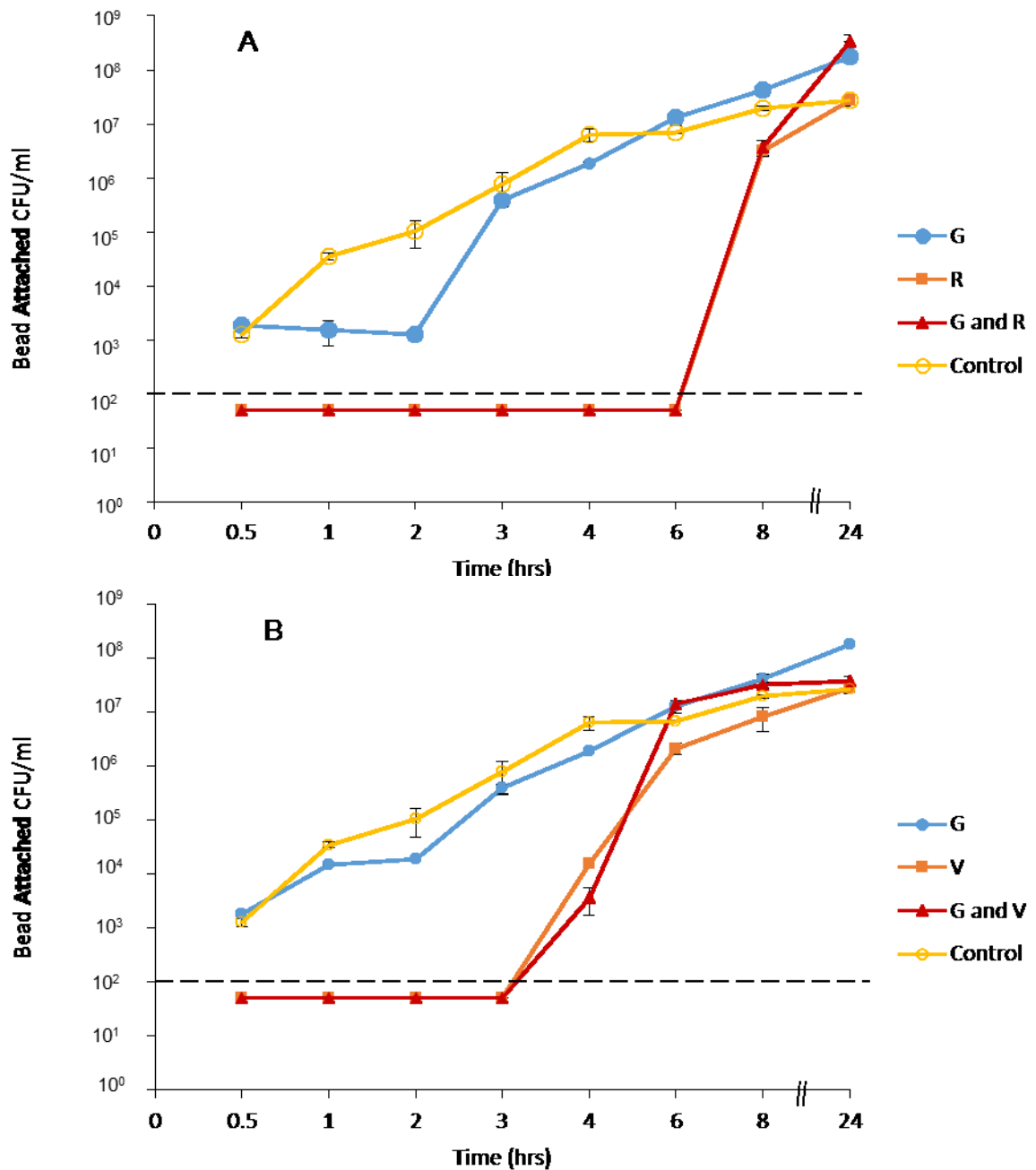


Figure 3.10a: The Effect of Gentamicin and Rifampicin (A) or Gentamicin and Vancomycin (B) on *Staphylococcus aureus* Biofilms over 24 hours. CFU/ml counts are plotted against time of growth with error bars or standard deviation. Cultures were set up from overnight cultures and grown for the indicated times as described in Materials and Methods. Beads were washed and placed into fresh medium containing either 15 µg/ml gentamicin, the second antibiotic (A: 6 µg/ml rifampicin or B: 15 µg/ml vancomycin) or both antibiotics. No antibiotics were added to the control wells. Beads were washed, dissolved, serially diluted and plated after the 2 hour long challenge. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml)

Gentamicin resistance develops immediately; however, it takes over 6 hours for the biofilm to display resistance to rifampicin as seen in Figure 3.10 A. When the antibiotics are combined the effect was the same as that of rifampicin only. Rifampicin acts by inhibiting nucleic acid synthesis, whereas gentamicin causes incorrect reading of mRNA, leading to the synthesis of non-functional proteins [Campbell *et al.*, 2001]. The process on which gentamicin acts is downstream to the process which rifampicin affects. Therefore, until rifampicin resistance develops, the gentamicin doesn't have a target and so resistance cannot develop.

For Figure 3.10 B, gentamicin and vancomycin combined, gentamicin resistance was observed by 0.5 hours and showed a steady increase, whereas vancomycin resistance developed sharply between 3 and 4 hours. When gentamicin and vancomycin are combined resistance develops in the same pattern as for vancomycin alone, beginning between 3 and 4 hours. Vancomycin acts by inhibiting cell wall synthesis and peptidoglycan formation and resistance is believed to be due to reduced permeability of the antibiotic via thickening of the cell wall [Howden *et al.*, 2010].

In Figure 3.10 C, the pattern of gentamicin resistance is the same as previously described for Figure 3.10 B. Ciprofloxacin resistance develops between 4 and 6 hours with cell numbers leaping up to 10^6 CFU/ml. When ciprofloxacin was combined with gentamicin resistance developed, as seen for ciprofloxacin alone, delaying resistance to gentamicin. Ciprofloxacin has a bactericidal action and acts by inhibiting DNA gyrase [Drlica and Zhao, 1997]. Resistance is most commonly due to mutations in DNA gyrase; the more mutations a cell produces the higher its resistance levels [Jacoby, 2005].

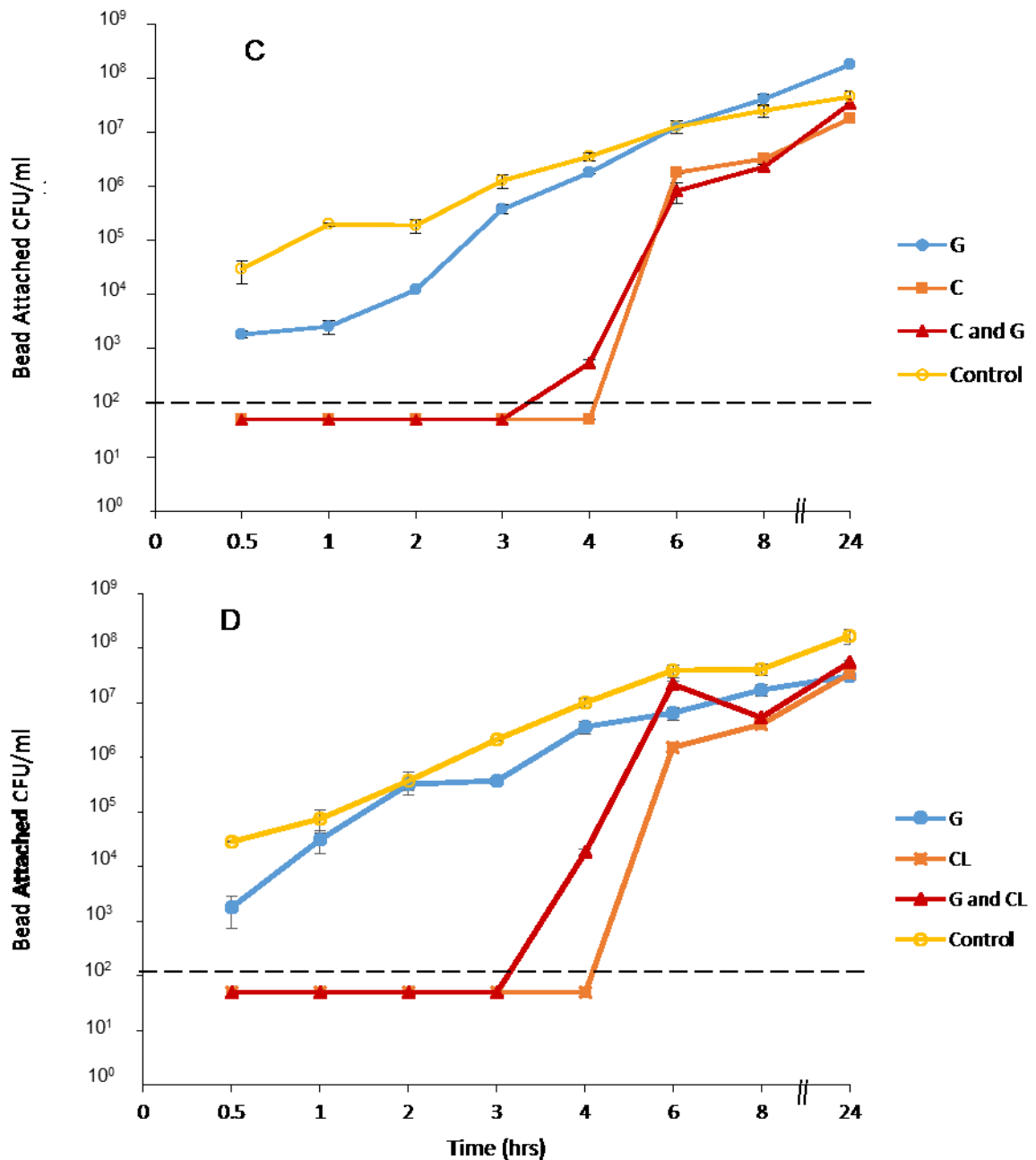


Figure 3.10b: The Effect of Gentamicin and Ciprofloxacin (C) or Gentamicin and Clindamycin (D) on *Staphylococcus aureus* Biofilms over 24 hours. CFU/ml counts are plotted against time of growth with error bars of standard deviation. Cultures were set up from overnight cultures and grown for the indicated times as described in Materials and Methods. Beads were washed and placed into fresh medium containing either 15 µg/ml gentamicin, the second antibiotic (C: 10 µg/ml ciprofloxacin or D: 0.1 µg/ml clindamycin) or both antibiotics. No antibiotics were added to the control wells. Beads were washed, dissolved, serially diluted and plated after the 2 hour long challenge. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml)

For Figure 3.10 D, gentamicin resistance developed as described previously. Clindamycin resistance developed by six hours (Figure 3.9 C). Clindamycin acts in a bacteriostatic manner through interference with protein synthesis by binding the 50S ribosomal subunit [Kohanski *et al.*, 2010]. Resistance is due to modifications of the antibiotic target site on the ribosome. This also confers resistance to other classes of antibiotics such as macrolides, azalides, lincosamides and group B streptogramins [Roberts *et al.*, 1999]. As can be seen clindamycin delayed development of resistance to gentamicin by 4 hours when the antibiotics are combined.

In Figure 3.10 E, gentamicin resistance develops as previously described whilst linezolid resistance developed by three hours as did resistance to the combined antibiotics (Figure 3.10 E). The pattern of the latter two conditions is similar suggesting that the effect of linezolid is dormant but that the combination does not enhance the overall negative effect on the cells. Linezolid has a bacteriostatic action, inhibiting protein synthesis by binding to domain V of the 23S ribosomal RNA of the 50S ribosomal subunit [Mouton and Jansz, 2001]. Resistance is caused by mutations in the antibiotic target site and should develop slowly as there are multiple copies of the 23S rRNA gene within the cells [Prystowsky *et al.*, 2001].

Examining Figure 3.10 F, gentamicin resistance develops in a similar manner as with the other antibiotics. Daptomycin resistance develops earlier than any of the other antibiotics with resistance shown after 2 hours growth. Interestingly, there is evidence for an additive effect between gentamicin and daptomycin as resistance against the combined antibiotics appeared to develop after resistance to each individual antibiotic. Daptomycin is bactericidal and requires calcium to act effectively. Its structure contains a 13 member cyclic lipopeptide with a decanoyl side chain. The antibiotic action is thought to be due to the insertion of the lipophilic tail into bacterial cell membranes which causes membrane depolarization and potassium ion efflux leading to cell death [Silverman *et al.*, 2003]. Resistance has only recently been reported and is believed to be a result of point mutations in the phospholipid biosynthesis genes [Hayden *et al.*, 2005].

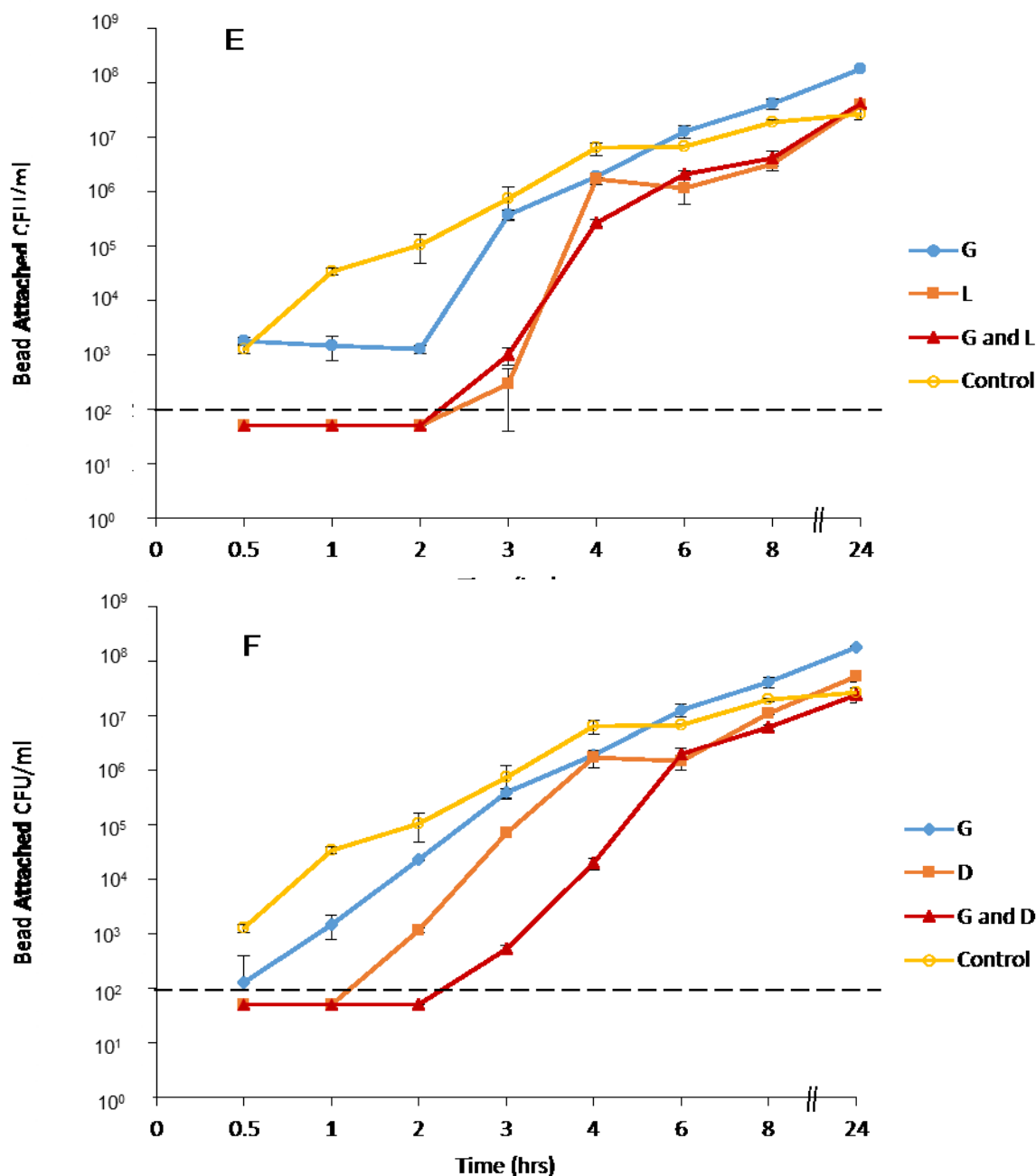


Figure 3.10c: The Effect of Gentamicin and Linezolid (E) or Gentamicin and Daptomycin (F) on *Staphylococcus aureus* Biofilms over 24 hours. CFU/ml counts are plotted against time of growth with error bars of standard deviation. Cultures were set up from overnight cultures and grown for the indicated times as described in Materials and Methods. Beads were washed and placed into fresh medium containing either 15 $\mu\text{g/ml}$ gentamicin, the second antibiotic (E: 4 $\mu\text{g/ml}$ linezolid or F: 0.5 $\mu\text{g/ml}$ daptomycin), or both antibiotics. No antibiotics were added to the control wells. Beads were washed, dissolved, serially diluted and plated after the 2 hour long challenge. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

Discussion

Stable Formation of Biofilms occurs by Four Hours' Growth

The alginate beads were found to be a suitable base on which the biofilms can form and it has been shown that the biofilms are firmly attached to the beads after just 4 hours' growth (Figure 3.2). By 24 hours the biofilms are fully attached and displaying significant antibiotic resistance, which is considered one of the hallmarks of a mature biofilm [Kiedrowski and Horswill, 2011]. This chapter focused on *Staph. aureus* and the commonly utilized clinical antibiotics used to treat staphylococcal infections. Chapter 4 covers other bacterial species and shows that other species are also able to form biofilms effectively, thus making the alginate bead method a viable alternative to the Calgary Biofilm Device.

Comparison of the Alginate bead method to the Calgary Biofilm Device

In comparison to the Calgary Biofilm Device, the alginate bead method is reliable, robust and much cheaper. The equipment and reagents required are low cost and commonly available. The one major disadvantage is that it is labour intensive and rate limiting by the number of samples which can be processed at any one time. However, with certain aspects of the method, such as the serial dilution and spot plating there is the potential for automation.

The development of biofilms on the alginate beads is supported by the previous microscopy work by Dall [2013] and the number of bacterial cells required for total surface area coverage. The paper by Ceri *et al* [1999] demonstrates that development of biofilms on the plastic pegs also occurs quickly with $\sim 10^6$ CFU/ml after 10 hours incubation (for *P. aeruginosa*, *E. coli* and *Staph. aureus*). Thus the beads can be considered a suitable base for biofilm formation.

The best recovery solutions for the biofilms seem to be a 1:1 mixture of dissolving solution and medium (Figure 3.5). Both LB and Muller-Hinton broth were used for the experiments detailed in this chapter, in the papers by Ceri *et al* [1999] and Harrison *et al* [2010] Muller-Hinton broth was used. It was found that changing the medium did

not seem to have an effect on the biofilm formation or recovery and thus could be tailored to the bacterial species required for the experiments.

Either the original method to recover the biofilms (crushing and dissolving) or low power sonication (Figures 3.4) which more closely mirrors the Calgary method could be used. The sonicating water bath (Defon F5 Minor, Ultrasonic Ltd) used is a closer match for the Aquasonic machine used by Ceri *et al* [1999] rather than the higher powered probe sonicator (Thermo Scientific) which is more commonly used to lyse cells. The probe sonicator can only process one sample at a time whereas the sonicating water bath is able to process sample batches.

The main advantages of sonication over the original method are the reduced amount of time required and that more samples can be processed at any one time. However, there are negative aspects to sonication, namely possible damage to bacterial cells. Sonication damages bacterial cells especially Gram-negative species; this is most likely due the thinner peptidoglycan cell wall cell that Gram-negative species have compared to Gram-positive species [Monsen *et al.*, 2009].

Inhibition of *Staphylococcus aureus* Biofilms by Gentamicin

It is interesting to observe the differences in gentamicin concentrations required to eradicate at different stages of bacterial growth. Figure 3.7 A, shows that biofilms display immediate resistance to 15 µg/ml gentamicin. In contrast, when this strain is plated onto solid medium (LB) and grown overnight, there is complete inhibition of growth at this concentration. Why does the agar plate not show any growth? It could be the fact, that the cells have to initiate growth to form colonies on a plate and are therefore; susceptible to the continuous gentamicin exposure. Whereas, in the biofilm situation the cells are already actively growing before the addition of gentamicin and innate cell and biofilm resistance mechanisms can react to the antibiotic.

Gentamicin acts by incorporating incorrect amino acids into peptides causing protein mistranslation, the antibiotic also increases membrane permeability allowing further antibiotic uptake into the cell through the inclusion of these incorrect proteins into the cytoplasmic membrane [Davis *et al.*, 1986].

Comparisons of the concentrations required to kill young (3 hours old) actively growing biofilms and planktonic cultures (Figure 3.6) show that nearly double the gentamicin concentration is required to kill the biofilm (225 µg/ml) compared to the planktonic culture (125 µg/ml). This is probably due to the innate resistance afforded by the structure of the biofilm matrix [Mah and O'Toole, 2001; Van Acker *et al.*, 2014].

For a 24 hour old biofilm a much higher concentration of gentamicin (1024 µg/ml) was required to reduce the biofilm load and even then only by 3 log orders (Figure 3.8). Moreover, when the same antibiotic concentration was added directly to the 24hr old biofilms (pipetted directly into the wells) the antibiotic effect decreased; the biofilm load was only reduced by 1.5 logs (Figure 3.8).

Allison *et al* [2011] have shown that aminoglycoside sensitivity can be restored by supplying persister cells and biofilms with sugar metabolites. The sugar metabolites are taken up by the bacterial cell and enter glycolysis resulting in the production of NADH. The NADH is oxidised by electron transport chain enzymes which contribute to generation of PMF. The revival of PMF enables aminoglycoside uptake into the cell leading to cell death. It is possible that some of the components within the LB also act in this way, this would account for the differences in biofilm load reductions between Figures 3.8a and 3.8b.

Further work needs to be conducted on this subject to compare the differences in antibiotic effectiveness with and without medium change with other antibiotic classes and medium types. Figure 3.8 (no medium change) is a more realistic scenario as any biofilm associated infection would be sessile without access to many fresh nutrients.

van de Belt *et al* [2001] demonstrated that *Staph. aureus* is able to form biofilms on six different gentamicin loaded polymethylmethacrylate bone cements. After 6 hours growth, when compared to the unloaded cement controls, there was no obvious difference in biofilm formation. Only after longer exposure times was there any major effect observed. Biofilm reduction occurred after 24 hours but only lasted for a further 48 hours for one of the brands (CMW3) before increasing again. It should be noted that the antibiotic effectiveness did not correlate with the kinetics for antibiotic release from the cement [van de Belt *et al.*, 2001].

There have been reports that aminoglycosides (gentamicin and tobramycin) can promote biofilm formation in both Gram-positive species (*Staph. aureus*) and Gram-negative species (*Ps. aeruginosa*/*E. coli*) which is the exact opposite of their purpose

[Hess *et al.*, 2011; Hoffman *et al.*, 2005]. This raises the question of whether gentamicin is a suitable antibiotic for use in a clinical environment especially in regards to bone cement, prosthetic joint infections and other biofilm based infections.

Development of Resistance of *Staphylococcus aureus* Biofilms to Other Antibiotics over 24 hours

The development of resistance of the other antibiotics over 24 hours all follow roughly the same pattern, although resistance does develop after different time periods (Figure 3.9). In comparison to gentamicin, the other antibiotics act at a much lower concentrations (Figures 3.7 and 3.9). This could be due to their different modes of action. However, clindamycin and linezolid also affect protein synthesis though they act on different targets within the cell. These two antibiotics are also bacteriostatic rather than bactericidal [Agar and Gould 2012; Kohanski *et al.*, 2010]. The gentamicin concentration required to have an effect on a young actively growing biofilm is 10x that which kills on an agar plate. Whereas, the same concentration which kills on a plate has an effect on the young actively growing biofilms for the other antibiotics, with resistance developing as the biofilm develops. After 24 hours growth the antibiotics have very little effect with the CFU/ml count matching that of the untreated control (Figure 3.9).

The modes of action of each of the antibiotics examined differed. The most effective antibiotic is rifampicin (Figure 3.9a, A) which is a bactericidal antibiotic that inhibits RNA synthesis by targeting RNA polymerase [Campbell *et al.*, 2001]. Resistance is due to mutations in the gene encoding the DNA-dependent RNA polymerase [Aubry-Damon *et al.*, 1998]. The resistance demonstrated in Figure 3.9a A, may be a due to a combination of the biofilm matrix's protective structure [Hogan *et al.*, 2015] and any mutations which the bacteria develop. However, it is unlikely that the majority of the bacteria present within the biofilm would have mutated to become resistant during the experimental period, so a greater weight should be placed on the matrix as the main mechanism of resistance. The reduced metabolic activity of the cells within the biofilm [Lewis, 2005] may also contribute to the resistance as it has been shown that rifampicin can penetrate biofilms without killing the cells [Zhang and Stewart, 2002].

The antibiotic for which resistance developed most rapidly was daptomycin (Figure 3.9b, F). This bactericidal antibiotic showed the least activity against biofilms which is

in contrast to the report from Smith *et al* [2009]. Daptomycin was found to eliminate 96% of biofilm-associated bacteria in 12 different *Staph. aureus* isolates, at a MIC of between 0.06-0.25 µg/ml, which was lower than the concentration used for this study (0.5 µg/ml). Mascio *et al.*, [2007] also demonstrated that daptomycin is active against log phase, stationary phase and metabolically inert Gram-positive bacteria.

Resistance to the two bacteriostatic antibiotics clindamycin and linezolid occurred by four (Figure 3.9a, D) and three hours respectively (Figure 3.9b, E). These antibiotics inhibit protein synthesis by affecting the 50S bacterial ribosomal subunit [Colca *et al.*, 2003; Kohanski *et al.*, 2010]; whether the antibiotic is bactericidal or bacteriostatic does not seem to affect when the resistance developed. There are contrasting reports about the effectiveness of linezolid against biofilms. For example, in the study by Raad *et al* [2007] results from biofilms treated with linezolid were equal to those of the negative control. However, Curtin *et al* [2003] demonstrated that linezolid was the most effective monotherapy for clearing biofilms from polyurethane coupons in an *in vitro* model system for *Staph. epidermidis* catheter related biofilm infections. Linezolid appears to be most effective when combined with rifampicin, this mixture cleared ~70% of infections from prosthetic joint infections [Gómez *et al.*, 2011].

Ciprofloxacin resistance developed between 4 and 6 hours (Figure 3.8a C). Ciprofloxacin is normally used for staphylococcal and *Pseudomonas* infections but is not effective at treating biofilms [Adire *et al.*, 2015]. Diffusion of ciprofloxacin into biofilms is dependent on its charge and that of the biofilm. At neutral pH, ciprofloxacin is positively charged and can effectively penetrate into a biofilm with enhanced activity [Adire *et al.*, 2015; Hernández-Borrell and Montero, 2003]. Resistance to ciprofloxacin may be due to mutations in the DNA gyrase or topoisomerase IV genes [Lindren *et al.*, 2003]. Increased expression of transporters which prevent the antibiotic from reaching critical concentrations within the cell is also a recognised resistance mechanism [Jacoby, 2005]. The third mechanism is plasmid mediated and produces enhanced efflux pumps [Jacoby *et al.*, 2014].

Vancomycin resistance developed between 3 and 4 hours growth (Figure 3.9a, B). Vancomycin has a slow mode of action causing bacterial death by preventing cross linking in the cell wall during synthesis, this results in a weakened wall, slow growth and death [Kiedrowski and Horswill, 2011]. There are two recognised levels of resistance to vancomycin. Intermediate resistance is due to mutations which increase the cell wall thickness [Howden *et al.*, 2010] and full level resistance is due to a mobile

genetic element which encodes for proteins that decrease the binding affinity for vancomycin [Courvalin, 2006].

Even though, all the antibiotics have specific resistance mechanisms which can be acquired or selected for under the pressure of antibiotic treatment. It is unlikely that all the resistance mechanisms were present or able to be developed from the one strain after less than 24 hours selective pressure. Thus, the more reasonable explanation for the antibiotic resistance is the biofilm itself. Biofilm resistance is based on heterogeneous gene & protein expression, reduced metabolic activity (including persisters), reduction in penetration of the antibiotics and the presence of EPS, extracellular proteins and eDNA in the biofilm matrix [Archer *et al.*, 2011; Lewis, 2005; Mann *et al.*, 2009; Xu *et al.*, 2000].

Effects of Gentamicin with other Antibiotics on *Staphylococcus aureus* Biofilms over 24 hours

The use of gentamicin is common in the clinical environment for the treatment of staphylococcal infections in prosthetic joints. Bone cements were introduced in 1970 [Buchholz and Engelbrecht, 1970, Neut *et al.*, 2005], and are used to produce a localised area of high concentration antibiotics around an implant to prevent infections. Gentamicin is used because of its broad antibacterial spectrum and stability at the high temperatures required to set the bone cement [Neut *et al.*, 2005]. Initial results were promising with lower infection rates reported despite the lack of understanding around the release mechanisms and control of the antibiotics from the cement [Wahlig and Dingeldein, 1980]. The increasing levels of gentamicin resistance, recorded at ~41%, have promoted the development of bone cements infused with gentamicin and a second antibiotic [Neut *et al.*, 2005].

Interestingly, all combinations of antibiotics resulted in delayed emergence of gentamicin resistance. At a gentamicin concentration of 15 µg/ml resistance emerged within half an hour of biofilm formation (Figure 3.7), however, when combined with another antibiotic, gentamicin resistance did not emerge until 3-8 hours after biofilm formation (Figure 3.10).

The only antibiotic to show enhanced inhibitory activity with gentamicin was daptomycin (Figure 3.10c F). The biofilm resistance was delayed by roughly one hour

when the two antibiotics were combined. Tsuji and Rybak [2005] have shown that a mixture of gentamicin and daptomycin significantly reduces bacterial cell numbers in *Staph. aureus* endocarditis. However, LaPlante and Woodmansee [2009] demonstrated that daptomycin alone proved more effective in a rabbit model of infective endocarditis than in combination with either gentamicin or rifampicin. Clinically daptomycin is often combined with rifampicin. This mixture has been reported to reduce biofilm biomass but is unable to completely clear the implant-associated infection in animal models [John *et al.*, 2009]. Further work is required to clarify the effectiveness of combinations of daptomycin with gentamicin or rifampicin.

For three of the other antibiotics, rifampicin, vancomycin and linezolid (Figure 3.10a A, 3.10a B and 3.10c E), there is no observed difference when they are combined with gentamicin compared to when they are tested as an individual substance other than a delay in gentamicin resistance. In fact, it has been shown linezolid, in combination with fusidic acid, gentamicin or rifampicin displayed no synergy but did prevent the emergence of resistant mutants [Grohs *et al.*, 2003]. This is only the case for a short period as gentamicin resistance did still emerge. Nonetheless, as previously discussed the resistance is more than likely due to the biofilm composition and should not be regarded as true genetic based resistance. Once cells are released from biofilms they revert back to an antibiotic sensitive planktonic state.

When gentamicin is combined with clindamycin (Figure 3.10b D), there seems to be a slight antagonistic effect, this contrasts with the paper published by Neut *et al* [2005]. Neut *et al* [2005] states that there is an additional antimicrobial effect when gentamicin and clindamycin are combined. However, the antibiotics were already present before the start of bacterial growth and biofilm formation, compared to Figure 3.10 D, which allowed the biofilm to form and then challenged with antibiotics. Thus, the comparison which can be made is limited by the differences in methodology. Since the difference is small, additional experiments are necessary to confirm that the observation is reproducible.

Further investigation is necessary to examine the effects of the gentamicin in combination with these antibiotics. The addition of a third antibiotic would also provide valuable information.

Chapter 4:
Biofilm Formation, Resistance and Disruption

Introduction

Many bacterial species form biofilms causing numerous HCAs and contamination in healthcare environments with significant increases in length of hospital stays & costs and decreases in patients' quality of life.

The bacterial species investigated in this chapter are *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *Escherichia coli* (2 strains), *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (2 strains). All these species are active in the healthcare environment with some causing more serious infections than others (Table 1.5).

Notable biofilms infections include catheter, prosthetic valves, artificial joints and other device related sepsis by *Staph. epidermidis*, dental caries by *Strep. mutans*, and respiratory infections (ventilator associated) by *K. pneumoniae* and *Ps. aeruginosa*. *Ps. aeruginosa* is also a significant problem in cystic fibrosis patients and on burn wards due to the compromised immunity of the patients [Goering *et al.*, 2008].

Biofilms are considerably harder to treat than planktonic cultures due to the nature of the matrix, the reduced metabolic functions of the cells, and the presence of different cells types such as persisters. Even with prolonged treatment, once the selective antibiotic pressure has been removed the infection can reactivate unless the underlying biofilm has been eradicated [Fux *et al.*, 2005].

Occasionally, in the clinical setting an infection can be caused by more than one species. A multispecies biofilm may provide protection for one or both species and antibiotic resistance levels are often higher than in biofilms formed by the individual species. Resistance mechanisms can also be transferred between bacterial species in this situation.

As treatment of biofilms is a hugely complicated issue, new and novel ways of treating and disrupting biofilms are constantly being investigated. One of these methods which has recently been published is the use of sugar metabolites to reactivate the metabolic systems of persister cells and biofilms, subsequently restoring function to the specific antibiotic targets [Allison *et al.*, 2011]. A second approach uses D-amino acids which modulate peptidoglycan synthesis and inhibit attachment of biofilm matrix proteins

thereby, preventing and disrupting biofilm formation [Hochbaum *et al.*, 2011; Kolodkin-Gal *et al.*, 2010].

Aims of this Chapter

To examine:

- How different health care associated bacterial species form biofilms using the alginate bead method.
- The development of antibiotic resistance over 24 hours in these biofilms.
- The antibiotic concentrations required to reduce the biofilm load after 3 and 24 hours growth.
- How a mixed species biofilm develops and its antibiotic resistance in comparison to the component species.
- How natural molecules, such as sugar metabolites and D-amino acids, affect biofilm formation and composition.

Development of Biofilms

As virtually all bacteria can form biofilms under the correct conditions, a selection of different bacterial species was chosen to further validate the alginate bead method and investigate antibiotic resistance. The majority of the species examined were clinical isolates (Table 2.1).

The biofilms (Figures 4.1 A and 4.2 A) were developed using overnight cultures grown in LB, at 37°C with shaking (200 rpm) which were diluted 1/10 000. This culture was used to inoculate triplicate alginate beads (prepared as described in Materials and Methods) for each time point in 48 well plates. The plates were incubated at 37°C, 150rpm for the required time. After their removal under aseptic conditions the beads were individually washed briefly in dH₂O (using a 48 well plate) and transferred to 15ml falcon tubes. The tubes contained 2ml dissolving buffer in which the beads were crushed using the base of a flamed metal loop and dissolved. The tubes were rotated until the beads were fully dissolved (~15 minutes). This solution was vortexed for 10 seconds before a sample was taken, which was serially diluted, and plated using the Miles Misra method (20 µl spots). Spots were counted if they contained between 2 and 30 colonies.

The planktonic cultures (Figures 4.1 B and 4.2 B) were set up from the same diluted overnight culture as the biofilms. 600 µl was pipetted into 48 well plates. Triplicate wells were used for each time point. After the required time, the wells were sampled. These samples were serially diluted and plated in the same way as the biofilm samples.

The CFU/ml was calculated using $N = (C.V)/D$, where C is the number of colonies. For the biofilm samples V is 250 (to adjust for the 2ml dissolving solution) whereas for the planktonic samples V is 50. D is the dilution factor. The graphs show mean CFU/ml counts against time of growth with error bars of standard deviation.

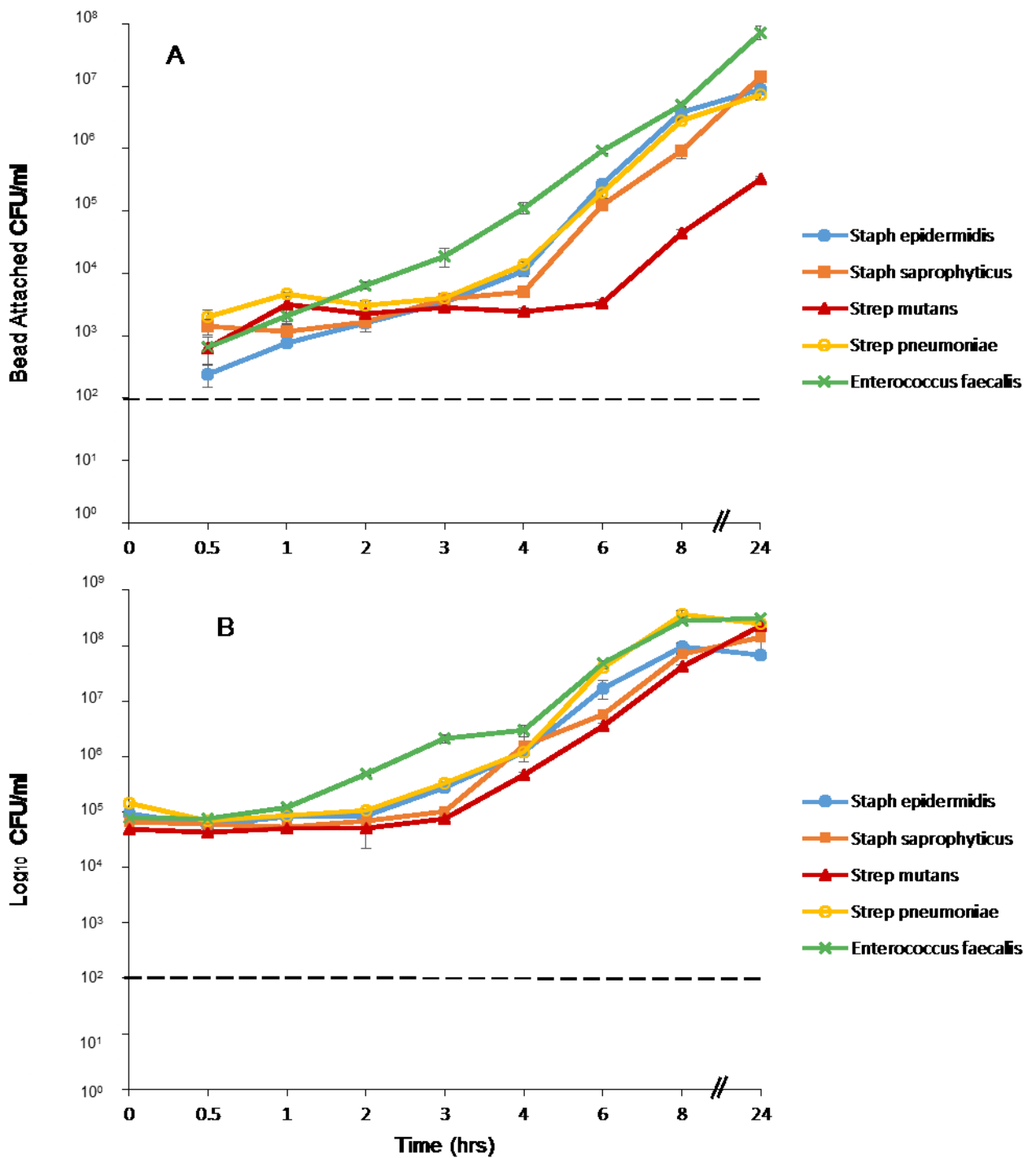


Figure 4.1: Development of Biofilms on Alginate Beads (A) and Planktonic Growth (B) by Various Gram-positive Organisms. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

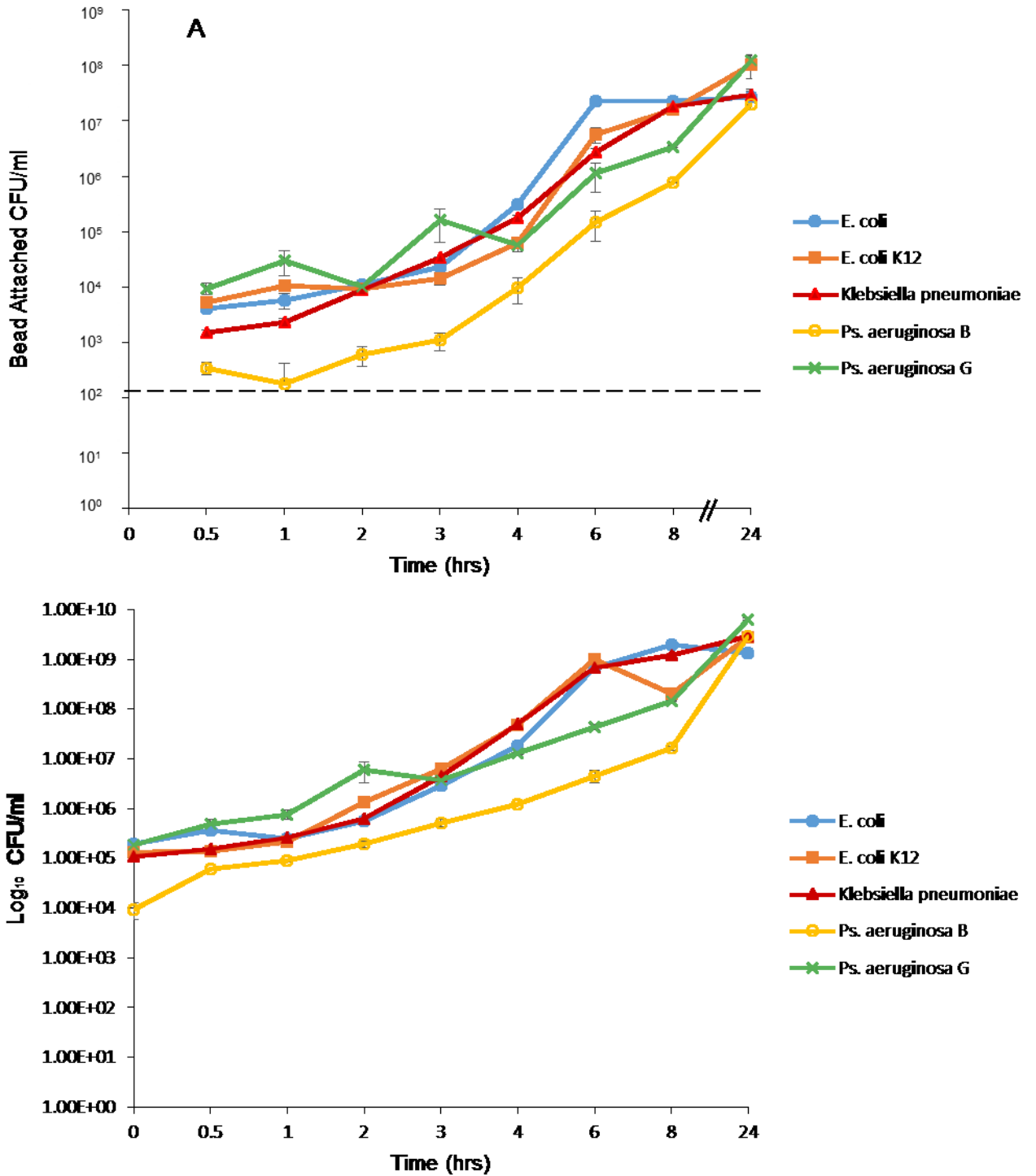


Figure 4.2: Development of Biofilms on Alginate Beads (A) and Planktonic Culture (B) by Various Gram-negative Organisms. Cultures were set up from overnight cultures as described in the Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

As can be seen from Figures 4.1 A, and 4.2 A, the development of biofilms of the different bacterial species follows the same basic growth pattern with the biofilm firmly attached by 4 hours and fully established by 24 hours. This can be confirmed by the emergence of resistance as seen in Figure 4.3. It should be noted, that the alginate composition of the beads does not appear to have an inhibitory effect on biofilm development. Moreover, the assay seemed robust even with the use of a mucoid alginate producing strain of *Ps. aeruginosa B* (Figure 4.2 A).

Some species did appear to form the biofilm more slowly than others but this perhaps could be accounted for by the differences in individual replication times for the particular species. Initial attachment to beads appeared rapid and is probably non-specific for the first couple of hours. After this time, there was a steady increase in colonisation, with both replication and further planktonic attachment contributing to the biofilm. The planktonic growth of the organisms from the same overnight culture and diluted inoculum is provided for control purposes.

As all the bacterial species selected were able to form biofilms the next step involved determining their antibiotic resistance and the profiles for how antibiotic resistance developed over 24 hours.

Development of Resistance over 24 hours

In order to determine how quickly biofilms develop resistance, growth curves were carried out with the biofilms challenged by either gentamicin or vancomycin. The inoculum used to develop the biofilms was grown overnight in LB at 37°C with shaking (200 rpm) and diluted in fresh LB to $\sim 10^4$ CFU/ml. 600 μ l of this culture was pipetted into a 48 well plate wells.

Triplicate beads for each time point were added to the well plate and incubated at 37°C, with shaking (150 rpm) for the required growth time (0.5, 1, 2, 3, 4, 6, 8 or 24 hours). The beads were removed under aseptic conditions and rinsed individually in dH₂O, before being placed in fresh medium with the required antibiotic concentration for the 2 hour long challenge. The beads were once again washed, dissolved (see

Materials and Methods) and CFU/ml counts were determined by plating. CFU/ml counts were calculated as previously stated.

The antibiotic concentration used was initially reflected by the level required to kill an actively growing planktonic culture (MIC as described in Materials and Methods). Table 4.1 summarises the antibiotic and concentration used for each species. Gentamicin was the first antibiotic choice however, some species were resistant. Vancomycin was selected if this was the case. Figure 4.3 (a, b and c) show the development of resistance for each species.

Table 4.1: Antibiotics and Planktonic MIC Concentrations Used for Each Bacterial Species.

Bacterial Strain	Antibiotic	Concentration (ug/ml)
<i>Staph. epidermidis</i>	Gentamicin	50
<i>Staph. saprophyticus</i>	Vancomycin	50
<i>Strep. mutans</i>	Vancomycin	50
<i>Strep. pneumoniae</i>	Gentamicin	75
<i>E. coli</i>	Gentamicin	90
<i>E. coli</i> K12	Gentamicin	50
<i>E. faecalis</i>	Vancomycin	75
<i>K. pneumoniae</i>	Gentamicin	75
<i>Ps. aeruginosa</i> G	Gentamicin	75

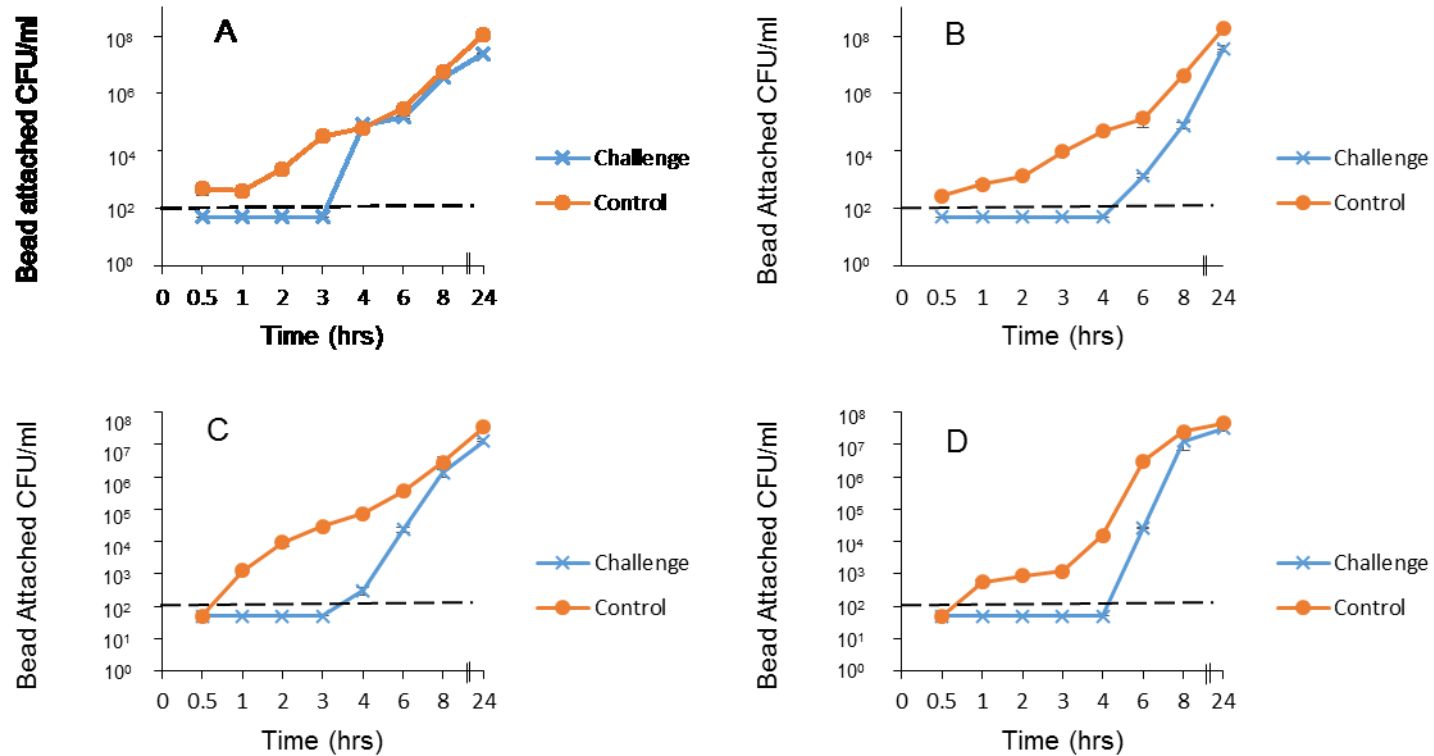


Figure 4.3a: Development of Antibiotic Resistance of Biofilms of *Staphylococcus epidermidis* (A), *Staphylococcus saprophyticus* (B), *Streptococcus mutans* (C) and *Streptococcus pneumoniae* (D) over 24 hours.

Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The biofilms were incubated for the indicated times before being challenged with antibiotics for 2 hrs. Beads were then washed, dissolved, serially diluted and plated as detailed in Materials and Methods. No antibiotics were added to the control. Triplicate beads were set up for each time point. The dashed line indicates the detection point of the assay (≤ 50 CFU/ml).
 A) *Staphylococcus epidermidis* (50 µg/ml gentamicin),
 B) *Staphylococcus saprophyticus* (50 µg/ml vancomycin),
 C) *Streptococcus mutans* (50 µg/ml vancomycin), and
 D) *Streptococcus pneumoniae* (75 µg/ml gentamicin).

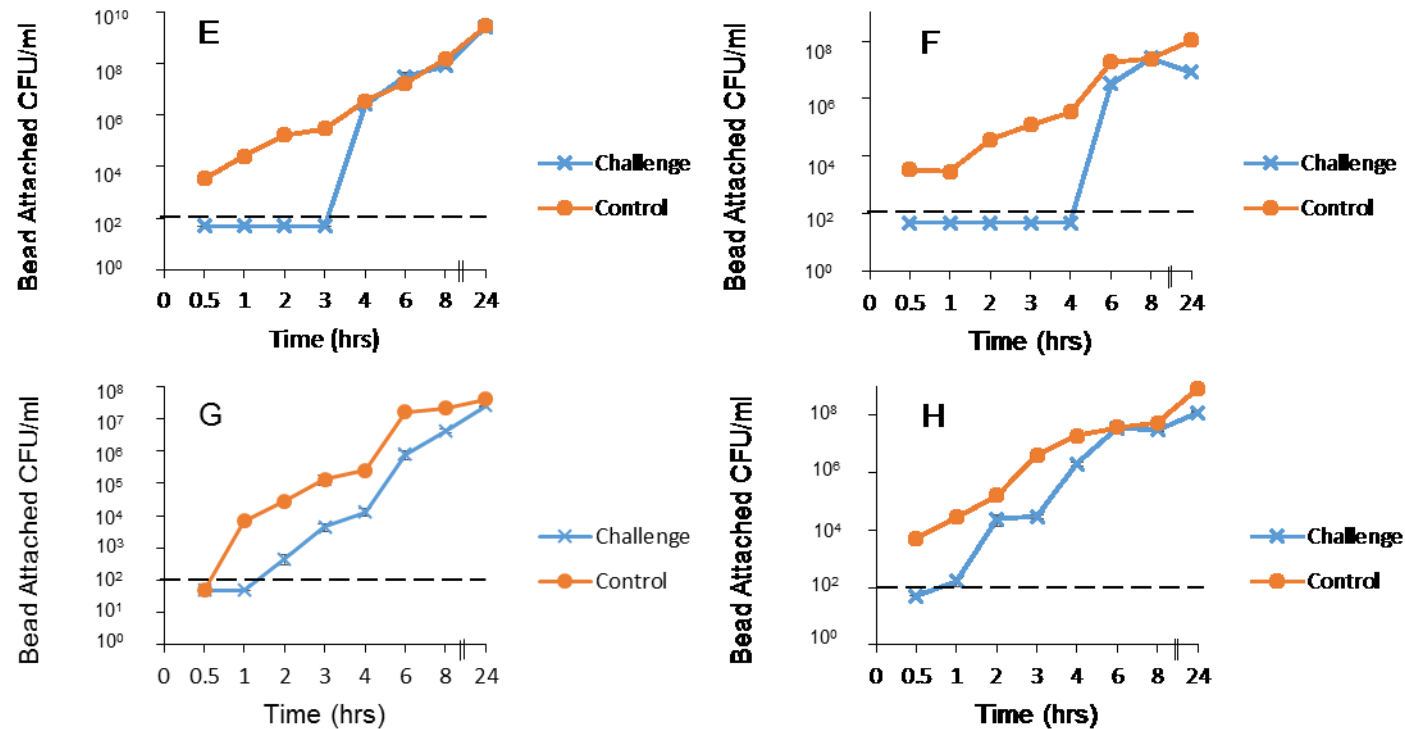


Figure 4.3b: Development of Antibiotic Resistance of Biofilms of *Escherichia coli* (E), *Escherichia coli* K12 (F), *Enterococcus faecalis* (G) and *Klebsiella pneumoniae* (H) over 24 hours.

Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The biofilms were incubated for the indicated times before being challenged with antibiotics for 2 hrs. Beads were washed, dissolved, serially diluted and plated as detailed in Materials and Methods. No antibiotics were added to the control. Triplicate beads were set up for each time point. The dashed line indicates the detection point of the assay (≤ 50 CFU/ml).
 E) *Escherichia coli* (90 µg/ml gentamicin),
 F) *Escherichia coli* K12 (50 µg/ml gentamicin),
 G) *Enterococcus faecalis* (75 µg/ml vancomycin),
 H) *Klebsiella pneumoniae* (75 µg/ml gentamicin).

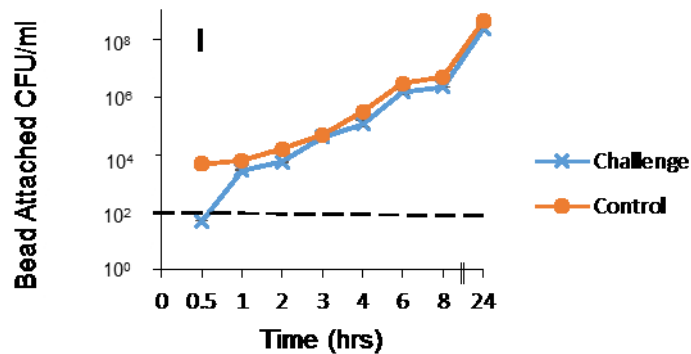


Figure 4.3c: Development of Antibiotic Resistance of Biofilms of *Pseudomonas aeruginosa* G over 24 hours. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The biofilms were incubated for the indicated times before being challenged with antibiotics for 2 hrs. Beads were washed, dissolved, serially diluted and plated as detailed in Materials and Methods. Triplicate beads were set up for each time point. No antibiotics were added to the control. *Pseudomonas aeruginosa* was challenged with 75 µg/ml gentamicin. The dashed line indicates the detection point of the assay (≤ 50 CFU/ml).

Of the Gram-positive bacterial species, resistance became evident between 4 and 6 hours, with the exception of *E. faecalis* which displayed resistance after 2 hours of biofilm formation (Figure 4.3b, G). It is interesting to note that there is a wide range of cell densities at which resistance develops. *E. faecalis* and *Strep. mutans* developed resistance at 10^2 CFU/ml, though *Strep. mutans* took longer to reach this point. *Staph. saprophyticus* resistance developed at 10^3 CFU/ml but *Staph. epidermidis* and *Strep. pneumoniae* resistance only developed once the cell density was above 10^4 CFU/ml.

K. pneumoniae and *Ps. aeruginosa* exhibited resistance after 1 hour growth, whereas, the two *E. coli* strains only developed resistance after 4 (Figure 4.3b, E) and 6 hours (Figure 4.3b, F). As with the Gram-positive organisms the cell density ranges from 10^2 CFU/ml (*K. pneumoniae*) and 10^3 CFU/ml (*Ps. aeruginosa*) to 10^6 CFU/ml (*E. coli*) before increasing resistance develops.

Differences in Levels of Resistance of 3 and 24 hour old Biofilms

In a clinical situation, the ultimate goal is to eradicate the biofilm completely. However, this may not be possible. Therefore, significantly reducing the biofilm load is considered the next logical step. In order to determine the antibiotic concentrations required for this, each bacterial species biofilm was grown for 3 or 24 hours (Figure 4.4) and challenged for 2 hours with increasing antibiotic concentrations.

Triplicate beads were incubated for each antibiotic concentration in 600µl diluted overnight culture ($\sim 10^4$ CFU/ml) for 37°C, 150 rpm for 3 or 24 hours. Beads were then removed and washed before being placed in 600 µl fresh LB with the different antibiotic concentrations for the 2 hour long challenge. The beads were once again washed, dissolved and the resulting solution sampled, diluted and plated. CFU/ml counts were calculated as before. Graphs show CFU/ml counts plotted against antibiotic concentrations with error bars of standard deviation.

Resistance Levels of 3 hour old Biofilms

As the biofilms are still developing at three hours, it is logical to assume that the antibiotics will still have a significant effect, and this is especially evident with *Staph. epidermidis* and *Strep. mutans* (Figure 4.4a, A and C). *E. faecalis* shows significant resistance with only the highest vancomycin concentration (96 µg/ml) having a detrimental effect on the biofilm. This correlates with the early development of resistance in Figure 4.3b (G), and as resistance to vancomycin was first identified in *Enterococcus* species [Cetinkaya *et al.*, 2003] therefore, it is reasonable that resistance might develop quickly.

Of the Gram-negative species, biofilm resistance was observed until the higher antibiotic concentrations, though for *K. pneumoniae* the biofilm was not completely eradicated. Gram-negative resistance to gentamicin relies mainly on active transport of the antibiotic out of the cell [Aires *et al.*, 1999] and sequestering the antibiotic [Magent *et al.*, 2003]. As the gentamicin concentration rises it can overwhelm these systems having a detrimental effect on the cells and biofilms.

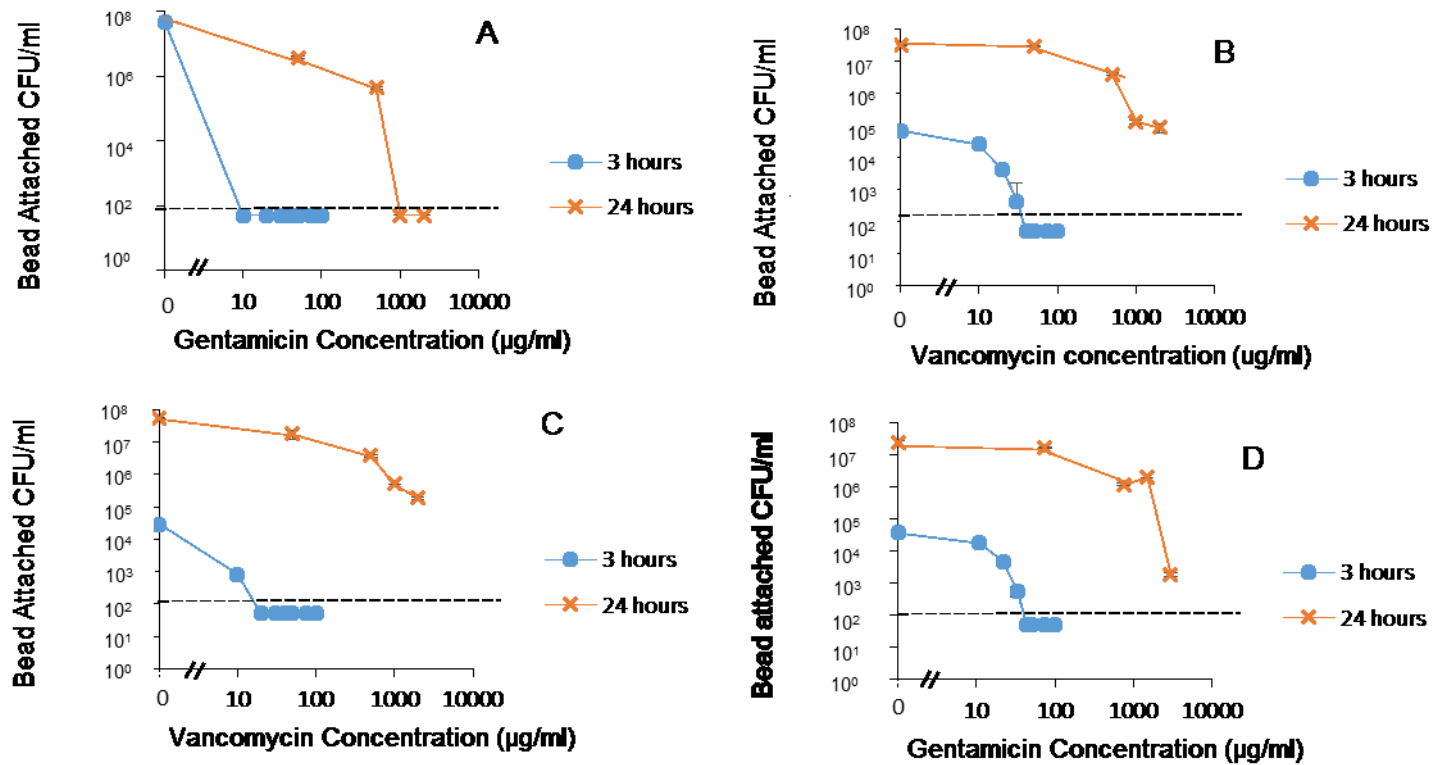


Figure 4.4a: Antibiotic Concentrations required to reduce Biofilm Load after 3 or 24 hours growth of *Staphylococcus epidermidis* (A), *Staphylococcus saprophyticus* (B), *Streptococcus mutans* (C) and *Streptococcus pneumoniae* (D).

Mean CFU/ml counts are plotted against antibiotic concentrations with error bars of standard deviation. Triplicate beads were set up for each condition. The biofilms were incubated for 3 or 24 hours before being challenged with antibiotics for 2 hrs. Beads were washed, dissolved, serially diluted and plated as detailed in Materials and Methods. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml). A) *Staphylococcus epidermidis*, B) *Staphylococcus saprophyticus*, C) *Streptococcus mutans*, and D) *Streptococcus pneumoniae*.

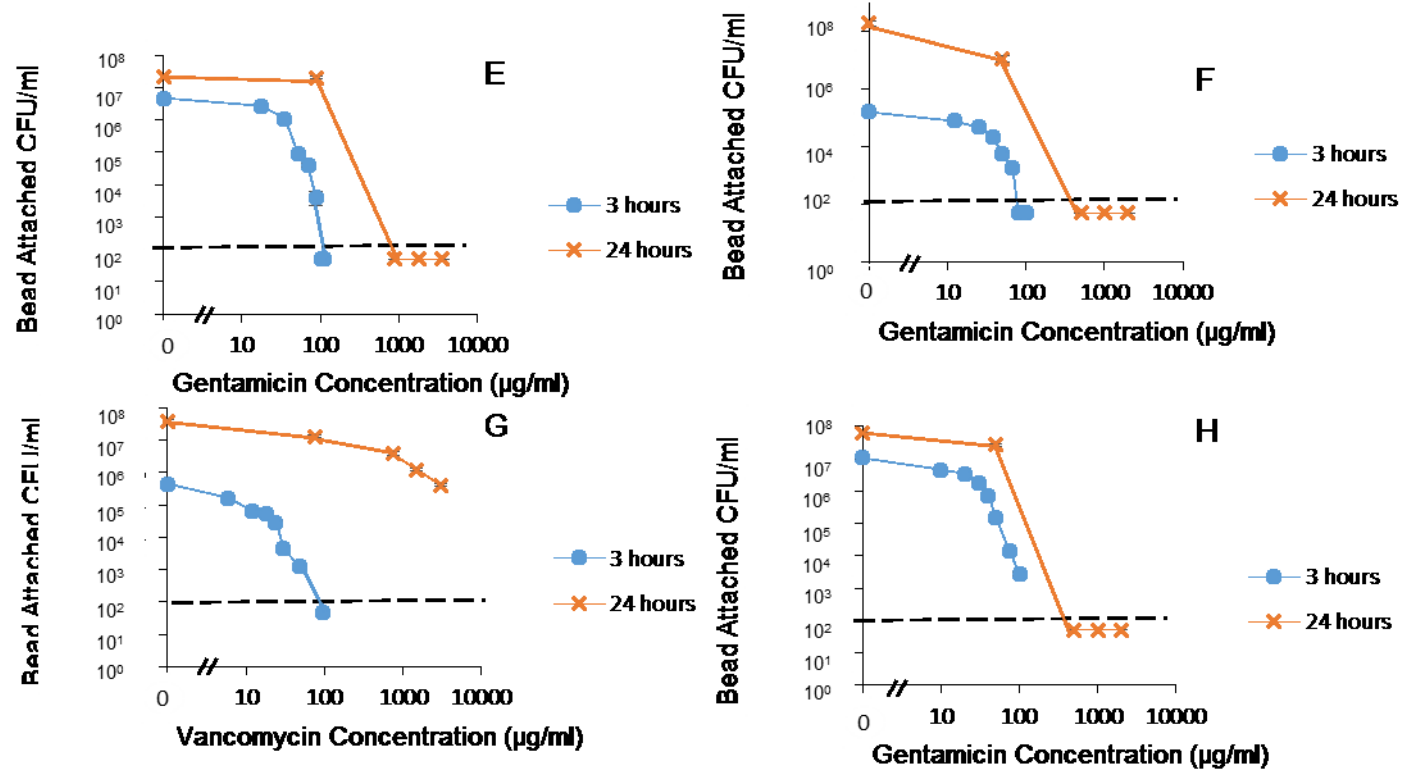


Figure 4.4b: Antibiotic Concentrations required to reduce Biofilm Load after 3 or 24 hours growth of *Escherichia coli* (E), *Escherichia coli* K12 (F), *Enterococcus faecalis* (G) and *Klebsiella pneumoniae* (H).

Mean CFU/ml counts are plotted against antibiotic concentrations with error bars of standard deviation. Triplicate beads were set up for each condition. The biofilms were incubated for 3 or 24 hours before being challenged with antibiotics for 2 hrs. Beads were washed, dissolved, serially diluted and plated as detailed in Materials and Methods. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml). E) *Escherichia coli*, F) *Escherichia coli* K12, G) *Enterococcus faecalis*, and H) *Klebsiella pneumoniae*.

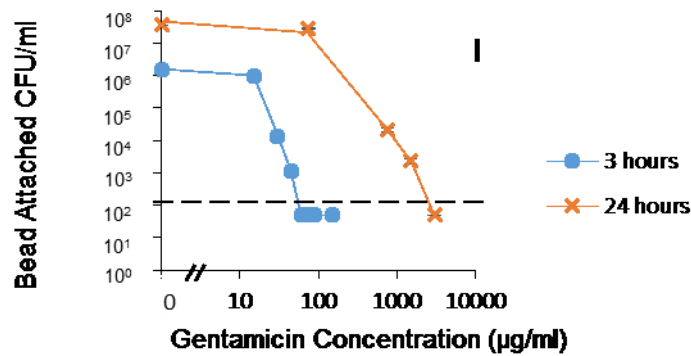


Figure 4.4c: Antibiotic Concentrations required to reduce Biofilm Load after 3 or 24 hours growth of *Pseudomonas aeruginosa* G. Mean CFU/ml counts are plotted against antibiotic concentrations with error bars of standard deviation. Triplicate beads were set up for each condition. The biofilms were incubated for 3 or 24 hours before being challenged with antibiotics for 2 hrs. Beads were washed, dissolved, serially diluted and plated as detailed in Materials and Methods. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

Resistance Levels of 24 hour old Biofilms

Within a clinical environment, a biofilm infection will be firmly established before a patient shows signs of clinical infection [Costerton *et al.*, 1999; Lindsay and von Holy, 2006]. Thus it is important that the antibiotic resistance levels of older biofilms are examined to determine what concentration of antibiotic is required to reduce the bacterial load.

As has been discussed in Chapter 3 for older *Staph. aureus* biofilms (>24 hours old) a substantial increase in antibiotic concentration is required to have any effect on the biofilms. The same result is seen with the other bacterial species, in some cases over 10x the antibiotic amount that was effective on a 3 hour old biofilm, only has a minimal effect on a 24 hr old biofilm.

The species which has the lowest tolerance for the higher antibiotic concentrations is *E. coli*, with both strains biofilms being destroyed at relatively low antibiotic concentrations compared to the other species. It could be, as mentioned above, that

the cell's defence mechanisms against gentamicin are just overwhelmed by the excessive amount of antibiotic. It can be theorised that there is a relationship between the antibiotic concentration required for destroying the biofilm and cell density.

However, the main reason behind the resistance has to be the biofilm structure itself. The different cell types within the biofilm are less susceptible because of changes in growth rates, gene expression and protein production. If the antibiotic targets are not active or less active then the antibiotic just won't have the expected activity. The biofilm matrix including the EPS would also have a protective effect.

Development and Challenge of Mixed Species Biofilms

In order to discover whether the presence of another bacterial species has any effect on biofilm development and resistance, an experiment was undertaken whereby *Staph. aureus* and *Ps. aeruginosa* G were combined in a mixed culture. These two species were chosen as in a clinical setting, they can be found in the same host sites and have been shown to cause dual infections [Hendricks *et al.*, 2001].

Development of the Biofilms

An equal starting inoculum of each species was combined and grown with beads for 0.5, 1, 2, 3, 4, 6, 8, and 24 hours, to examine the development of the mixed species biofilm (Figure 4.5). The beads were incubated at 37°C, 150 rpm for the required time before being washed and placed into fresh LB containing 150 µg/ml gentamicin. The biofilms were challenged for 2 hours after which they were washed, dissolved, serially diluted and plated as described previously.

The culture was plated on either LB-rifampicin (5 µg/ml) or LB-Linezolid (4 µg/ml) to select for *Staph. aureus* and *Ps. aeruginosa* G respectively. Previous experiments had determined the antibiotic concentration required in the agar plates to select for each species (data not shown).

Development of the mixed biofilm by each species was relatively even (Figure 4.5) and follows the same pattern as single species biofilms (Figures 3.5 B and 4.2A). The

final CFU/ml counts at 24 hrs showed that neither species has a detrimental effect on the other in terms of forming a biofilm. However, this is without any external pressures, such as an antibiotic challenge, which could change how the biofilm develops and reacts. This issue was therefore addressed.

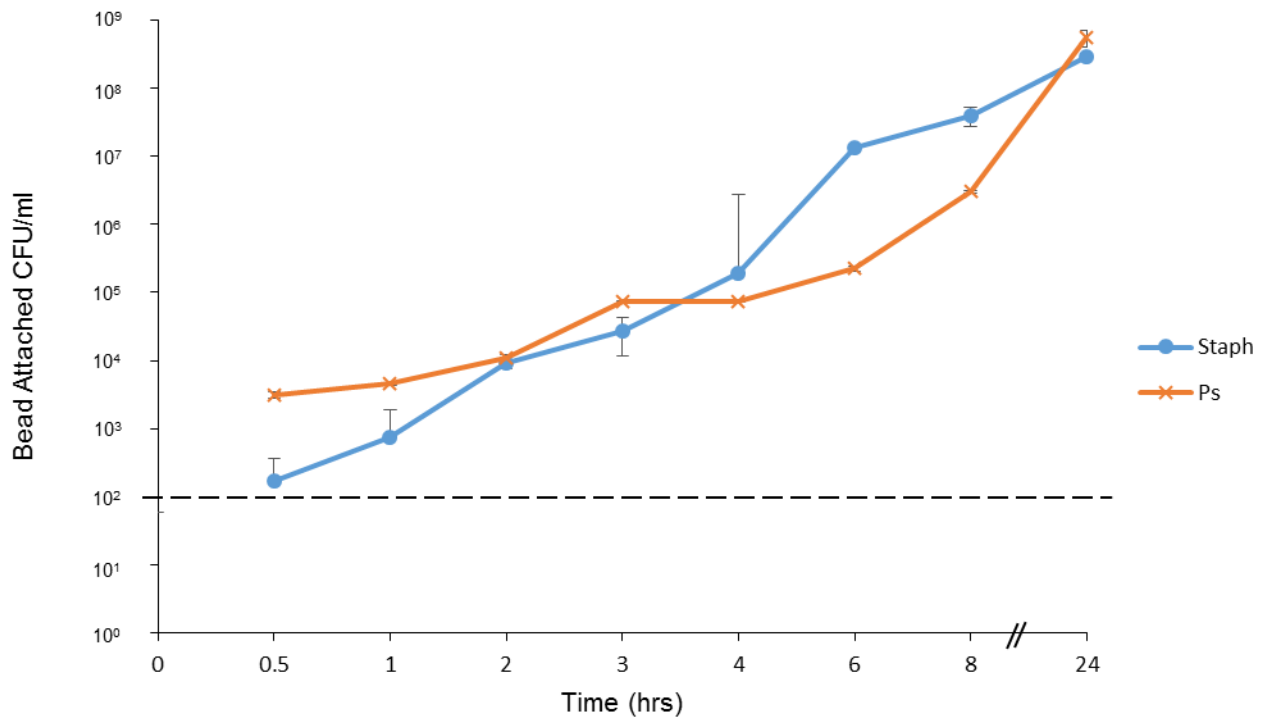


Figure 4.5: Development of a Mixed Species Biofilm Consisting of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. Cultures were set up from overnight cultures (as described in Materials and Methods) with triplicate beads sampled at the indicated time points. Starting inoculum of *Staph. aureus* was 4.08×10^3 CFU/ml and for *Ps. aeruginosa* 5.15×10^3 CFU/ml. Beads were washed, dissolved, serially diluted and plated on to selective plates for each species. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

Development of Resistance over 24 hours

Figure 4.6 shows how the addition of an antibiotic affects biofilm viability and when resistance develops. The protocol used is identical to that described for Figure 4.3.

When the mixed biofilm was challenged with 150 µg/ml gentamicin *Ps. aeruginosa* showed immediate resistance. Comparing this to *Ps. aeruginosa* in a single species biofilm which, when challenged with 75 µg/ml gentamicin (Figure 4.3c), only showed resistance after 1hr growth.

A similar effect can be seen with the *Staph. aureus* in that the gentamicin resistance develops earlier than when it is growing on its own (Figure 3.6). In a single species biofilm antibiotic resistance develops at 6hrs ($\sim 10^6$ CFU/ml) whereas, in a mixed species biofilm, resistance develops at 3hrs and at a lower cell density ($\sim 10^3$ CFU/ml).

Thus, a mixed biofilm appears to have a beneficial effect on both species in terms of antibiotic resistance in the early stages of a biofilm (Figure 4.6). However, by 24 hrs the *Ps. aeruginosa* isolated from the mixed biofilm showed a 2 log difference in CFU/ml to that of the unchallenged control. This suggests that the beneficial effect of the mixed biofilm is reducing especially as the *Ps. aeruginosa* single culture (Figure 4.3c) biofilm challenged with gentamicin matches that of the unchallenged control.

There is a difference in the survival numbers of *Staph. aureus* as well but it is not as great. There is a possibility that the gentamicin is still present within the bacterial cells however, the biofilms have been rinsed to remove any planktonic cells, as well as the surrounding gentamicin. As the culture sample was diluted, any remaining gentamicin would also be diluted. If this is the case any bactericidal effect would be minimal due to the extremely low gentamicin concentrations.

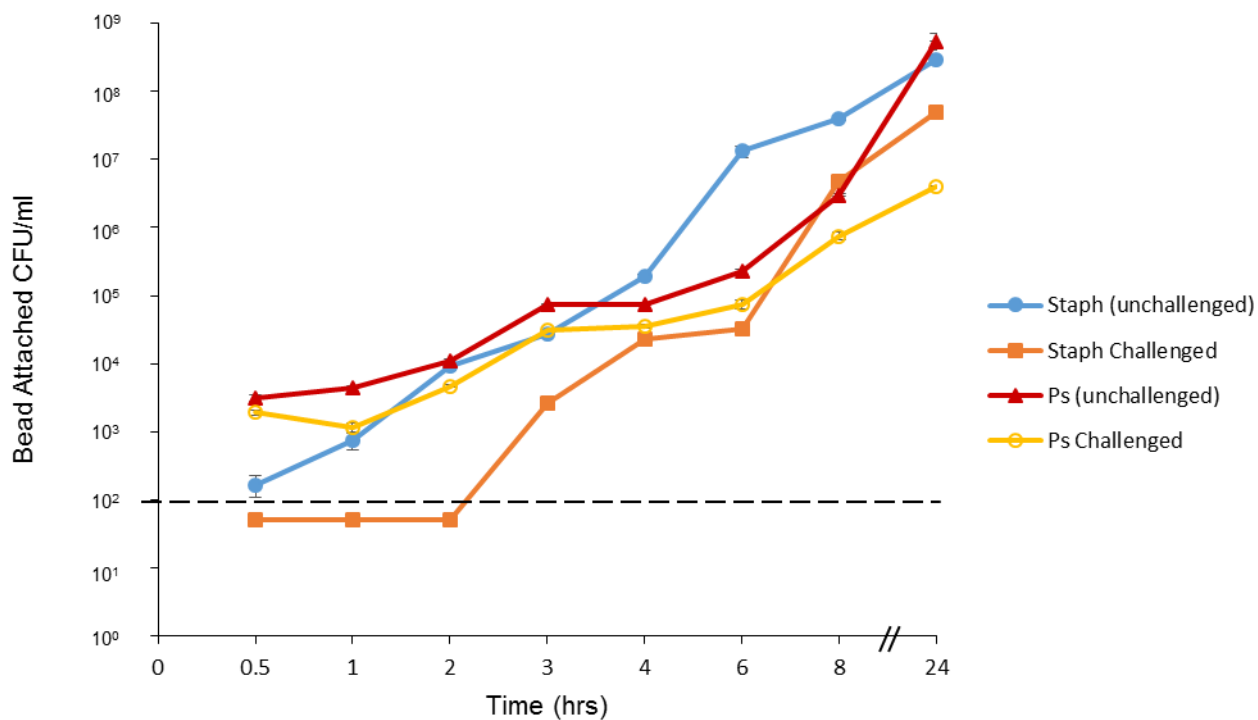


Figure 4.6: Development of Resistance of a Mixed Species Biofilm Consisting of *Staphylococcus aureus* and *Pseudomonas aeruginosa* over 24hrs. Mean CFU/ml counts are plotted against time of growth with error bars of standard deviation. The biofilms were incubated for the indicated times before being challenged with 150 ug/ml gentamicin for 2 hrs (Staph/Ps Challenged). Cultures were set up from overnight cultures with triplicate beads for each time points. Starting inoculum of *Staph. aureus* was 4.08×10^3 and for *Ps. aeruginosa* 5.15×10^3 which were mixed equally. Beads were washed, dissolved, serially diluted and plated on to selective plates for each species. No antibiotic were added to the control (Staph/Ps unchallenged). The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

Levels of Resistance of 3 hour old Mixed Species Biofilms

As with the single species biofilms the resistance levels of the mixed biofilm, after different periods of growth (3 and 24 hours), were determined and compared to the individual species biofilms to see how the mixed biofilm influences antibiotic resistance levels.

The three cultures used for the following two experiments (Figures 4.7 and 4.8) were *Staph. aureus*, *Ps. aeruginosa* and the mixed culture. The experiment was set up in the same way as described for Figure 4.4.

The mixed biofilm confers some protection from gentamicin for *Ps. aeruginosa* with this species surviving in higher antibiotic concentrations than in the individual culture which correlates with what is seen in Figure 4.6; initial faster growth and development of *Ps. aeruginosa* biofilms. There is some difference in the survival rates between the *Staph. aureus* from the mixed and individual biofilms, with 225 µg/ml gentamicin eradicating the individual *Staph. aureus* whereas, 450 µg/ml gentamicin was required to completely eradicate the *Staph. aureus* from the mixed species biofilm (Figure 4.7).

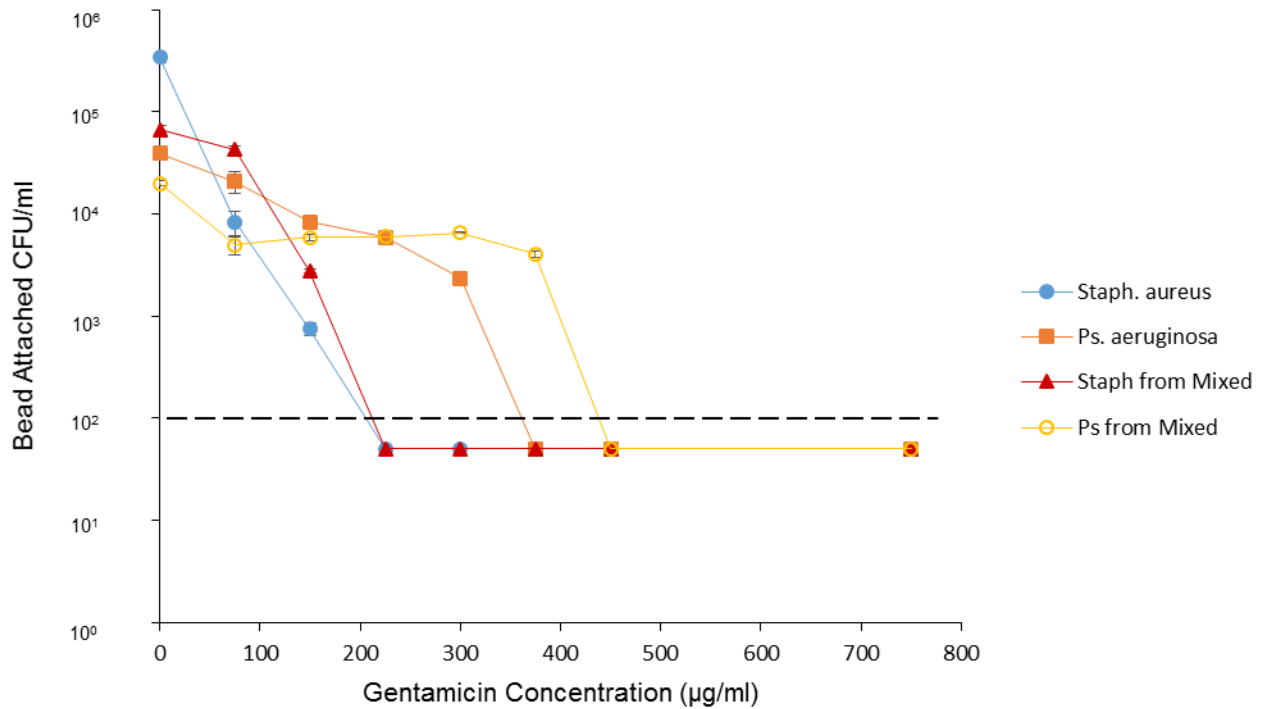


Figure 4.7: Antibiotic Concentrations required to Reduce Biofilm Load after 3 hours growth of a Mixed Species Biofilm Consisting of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Mean CFU/ml counts are plotted against antibiotic concentrations with error bars of standard deviation. The biofilms were incubated for 3 hrs before being challenged with gentamicin for 2 hrs. Cultures were set up from overnight cultures with triplicate beads for each time point as described in Materials and Methods. Beads were washed, dissolved, serially diluted and plated on to selective plates for each species from the mixed biofilms. The individual species were plated on to LB agar. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

Levels of Resistance of 24 hour old Mixed Species Biofilms

In order, to determine how a mature biofilm responds to increasing levels of antibiotic, a 24 hour old biofilm was challenged with different antibiotic concentrations. The protocol for this is identical to that described for Figure 4.4. As with the three hour old biofilms (Figure 4.7), 3 different cultures were used, an individual *Staph. aureus* culture, an individual *Ps. aeruginosa* culture, and a mixture of the two species.

It is clear that an older mixed biofilm confers protection to *Staph. aureus* as even 3000µg/ml gentamicin could not eradicate the biofilm. However, there is a 4 log reduction when challenged with gentamicin for 2 hours compared to the unchallenged control (Figure 4.8).

In direct contrast, to the effects of antibiotics on a 3 hour old biofilm (Figure 4.7), which showed that *Ps. aeruginosa* survived in the higher antibiotic concentrations, a 24 hour old biofilm seems to have a detrimental effect on *Ps. aeruginosa*. However, the reduction in the *Ps. aeruginosa* CFU/ml count does correlate with Figure 4.6 showing that after 24 hrs *Ps. aeruginosa* numbers are less in a mixed biofilm situation than in an individual culture.

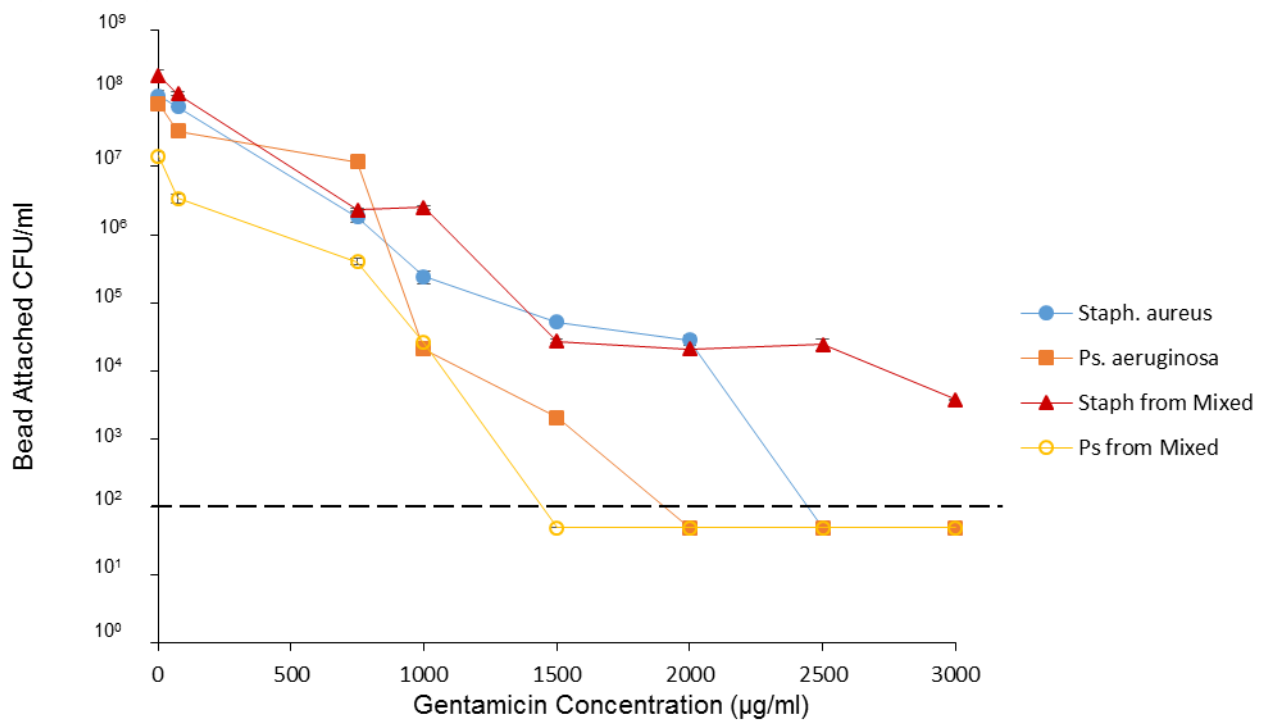


Figure 4.8: Antibiotic Concentrations required to Reduce Biofilm Load after 24 hours growth of a Mixed Species Biofilm Consisting of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Mean CFU/ml counts are plotted against antibiotic concentrations with error bars of standard deviation. The biofilms were incubated for 24 hrs before being challenged with gentamicin for 2 hrs. Cultures were set up from overnight cultures with triplicate beads for each time points. Beads were washed, dissolved, serially diluted and plated (as described in Materials and Methods) on to selective plates for each species from the mixed biofilms. The individual species were plated on to LB. The dashed line indicates the detection limit of the assay (≤ 50 CFU/ml).

Novel Methods of Disruption to Biofilms may Include D-amino acids and Sugar Metabolites

The antibiotic amounts required to kill or significantly reduce biofilm loads are extremely high. It is reasonable to assume that these levels could never be used in a clinical setting due to the difficulty in administration and toxic side effects of such antibiotic concentrations to the patient. Therefore, other approaches need to be identified which can be used in conjunction with conventional therapies to treat biofilms.

Use of D-Amino Acids to Disrupt *Staphylococcus aureus* Biofilms

In *Bacillus subtilis* cultures, D-amino acids are naturally produced during late stationary phase and have an effect on the structure of pellicles within the culture [Kolodkin-Gal *et al.*, 2010]. The same activity has been found to affect *Staph. aureus*, *Ps. aeruginosa* and *Staph. epidermidis* [Kolodkin-Gal *et al.*, 2010]. The D-amino acids which have the greatest effect on *Staph. aureus* are D-phenylalanine, D-proline and D-tyrosine [Hochbaum *et al.*, 2011].

The D-amino acids have been reported to disrupt both the formation of a biofilm when added to the culture medium and to disrupt a fully formed biofilm when added after the biofilm has become established.

In the experiments carried out by Hochbaum *et al* [2011] an older biofilm (over 24 hours old) was used to examine the effect of D-amino acids on biofilm formation. It would be interesting to examine whether the D-amino acid inhibitory effect occurs immediately after biofilm formation or whether the biofilms need to be established first. Figure 4.10 shows the effects of D-amino acids on the growth of young *Staph. aureus* biofilms.

Triplicate beads for each time point were inoculated with a 1/10 000 dilution from an overnight culture ($\sim 10^4$ CFU/ml). D-amino acids were included in the medium either singularly or in combination at a concentration of 100 μ M (as detailed in Hochbaum *et al.*, [2011]). After incubation at 37°C, 150 rpm for the required time (2, 4, 6, or 8 hours) the beads were washed, dissolved, serially diluted and plated. CFU/ml counts were calculated as before.

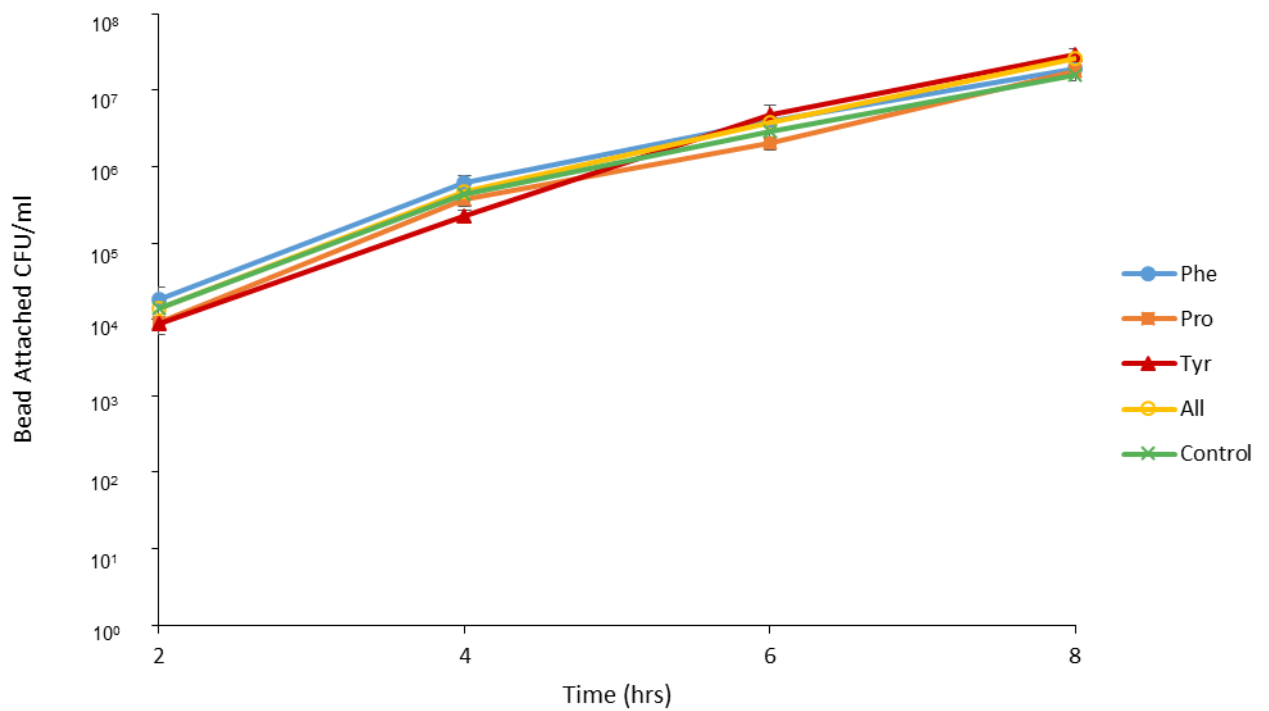


Figure 4.9: Effects of D-amino acids on Young *Staphylococcus aureus* Biofilms. Mean CFU/ml counts are plotted against growth time with error bars of standard deviation. Overnight cultures were diluted and used to incubate alginate beads for the indicated times. Triplicate beads were set up for each condition. Various D-amino acids were included in the inoculum: Phenylalanine (Phe), Proline (Pro), Tyrosine (Tyr), all of them or none (control). Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods.

Figure 4.9 illustrates that the CFU/ml counts still increased over time even with the D-amino acids in the culture. This indicates that the presence of D-amino acids has no detectable effects on biofilm formation over the first few hours of a biofilm's development, with the strain examined.

Hochbaum *et al.*, [2011] reported two different experiments. In the first, the biofilms were grown in the presence of D-amino acids at different concentrations (Figure 4.10 A and B). The second experiment involved growing the biofilm for 24 hours then placing the biofilms into fresh medium and challenging with D-amino acids to disrupt the established biofilms (Figure 4.11).

For the first experiment (Figure 4.10) biofilms were grown in the presence of D-amino acids. Triplicate beads were inoculated with a 1/10000 dilution from an overnight culture. D-amino acids were included in the medium either singularly or in combination at a concentration of 100 μ M (Figure 4.10 A) or 500 μ M (Figure 4.10 B). The different concentrations are based on the paper published by Hochbaum *et al* [2011], which reported that higher D-amino acid concentrations affected biofilm formation to a greater extent. After incubation at 37°C, 150 rpm for 24 hours the beads were washed, dissolved, serially diluted and plated. CFU/ml counts were calculated as before. CFU/ml counts are plotted against the different D-amino acids on the graphs with error bars of standard deviation.

For the second experiment (Figure 4.11), the D-amino acid challenge, the biofilms were pre-grown for 24 hours at 37°C, 150 rpm after which the beads were washed and placed into fresh medium with the appropriate D-amino acid for a further 24 hours at 37°C. After the challenge time the beads were treated as above.

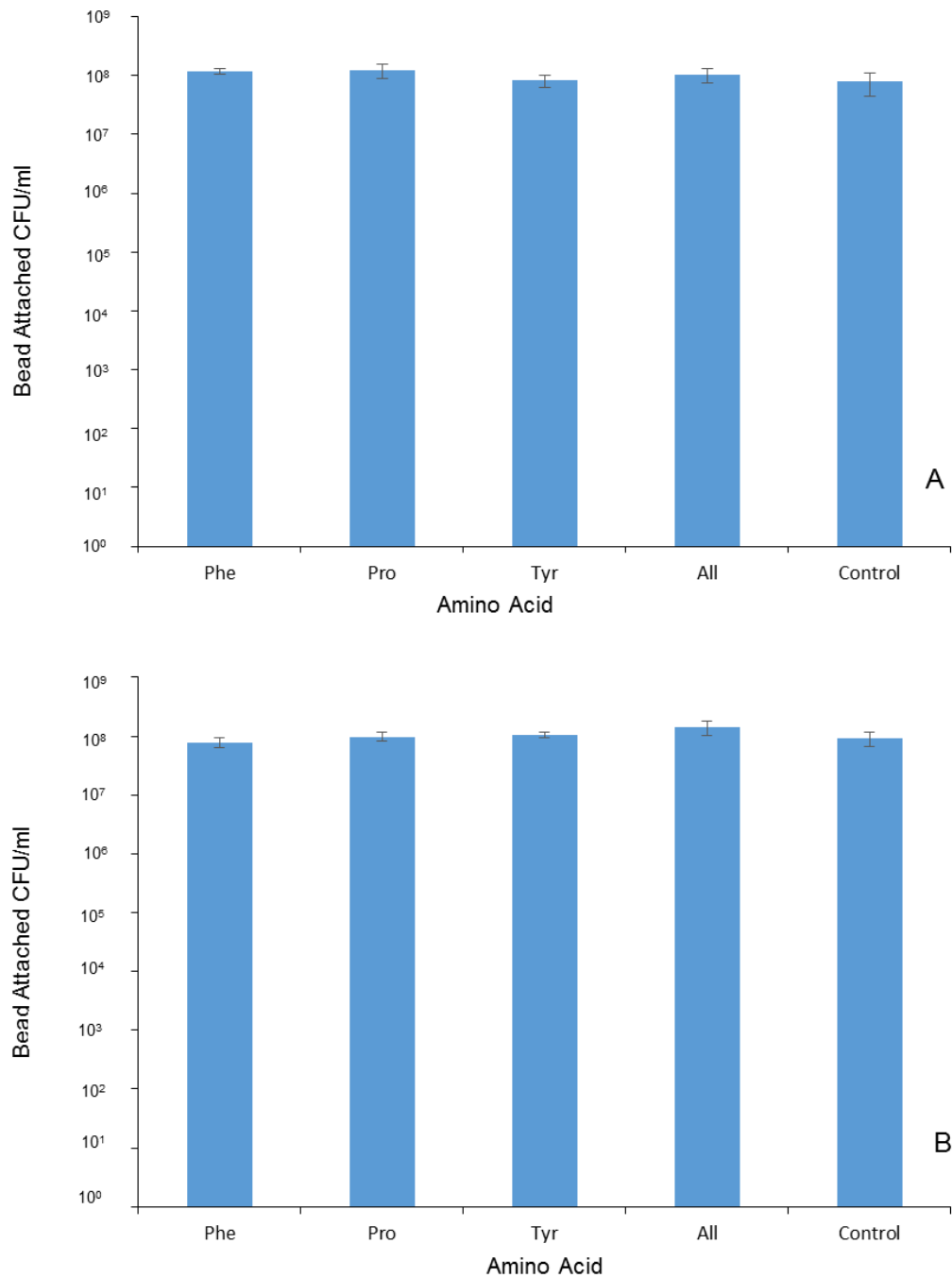


Figure 4.10: Growth of *Staphylococcus aureus* Biofilms with 100 μM (A) and 500 μM (B) D-Amino Acids. Mean CFU/ml counts are plotted against D-amino acids with error bars of standard deviation. Overnight cultures were diluted and used to inoculated alginate beads for 24 hours. 100 μM (A) or 500 μM (B) of Phenylalanine (1), Proline (2), Tyrosine (3) all of them (4) or none (5: control) were included in the inoculum. Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods. Triplicate beads were used for each condition.

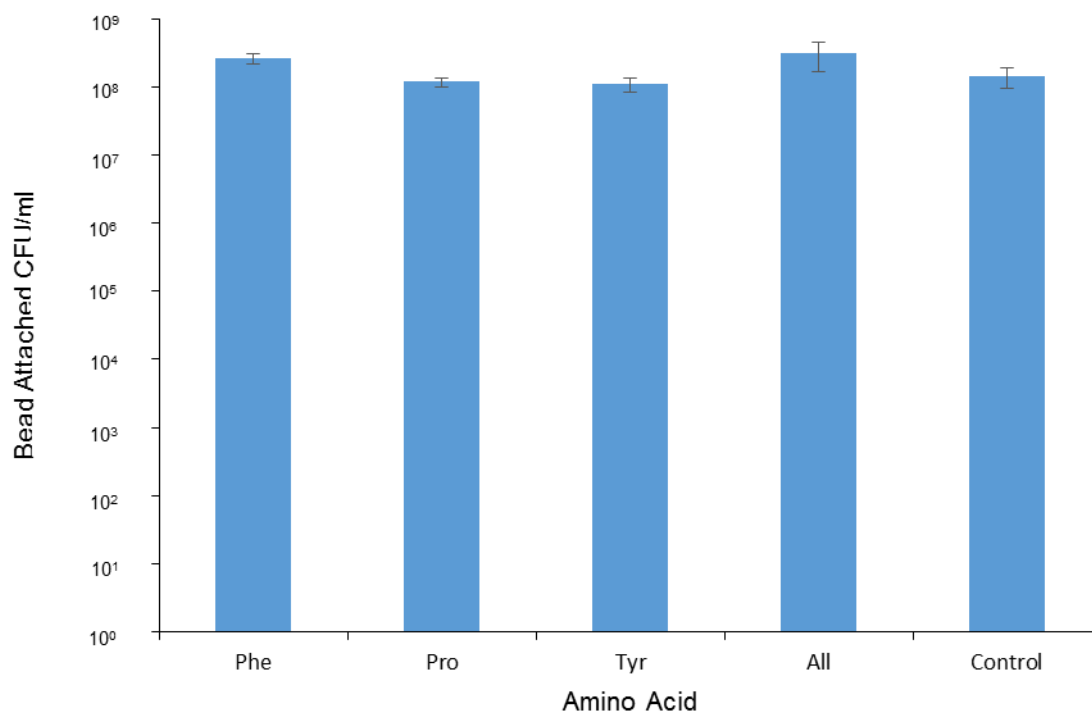


Figure 4.11: Growth and Challenge of *Staphylococcus aureus* Biofilms with 100 μM D-Amino Acids. Mean CFU/ml counts are plotted against D-amino acid with error bars of standard deviation. Overnight cultures were diluted and used to inoculate alginate beads for the 24 hours. Beads were washed and placed in fresh medium with 100 μM D-amino acids: Phenylalanine (1), Proline (2), Tyrosine (3) all of them (4) or none (5: control) for 24 hours. Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods. Triplicate beads were used for each condition.

Even after repeated experiments, with different D-amino acid concentrations added immediately or after a growth period, there was no measureable effect on the biofilm formation and cell viability. This contradicts the published papers.

It can be theorised that the D-amino acid effect is strain specific, as has been reported with *Staph. epidermidis* [Ramón-Peréz *et al.*, 2014]. A recently published paper has also been unable to replicate the results of the original *Staph. aureus* experiments [Sarkar and Pires, 2015] even using the same strain. Moreover, the D-amino acids effect observed in the original *Bacillus subtilis* study has been shown to be due to a strain specific mutation [Leiman *et al.*, 2013].

Using Sugar Metabolites to Restore Gentamicin Sensitivity to *Staphylococcus aureus* Biofilms

A previous study by Allison *et al* [2011] details how the addition of sugar metabolites can reverse aminoglycoside resistance in biofilms by activating the proton-motive force (PMF) transport system. The sugar metabolites, specifically fructose, are taken up into the cell by the PMF transport system. This then triggers the uptake of the antibiotic.

Established (24 hours old) biofilms were used for the Allison *et al* [2011] experiments, as it has previously been shown that the biofilms are firmly attached to the alginate beads by 4 hours (Figure 3.4) and are resistant to 15 µg/ml gentamicin (Figure 3.5). It would be interesting to investigate whether young biofilms have the same reaction to the addition of fructose and revert to a gentamicin sensitive phenotype (Figure 4.12).

Triplicate beads were incubated with diluted overnight culture for 2 hours before being transferred into stationary phase medium (see Materials and Methods) with either 15 µg/ml gentamicin, 10 µg/ml fructose, both additives or neither and then challenged for the required time (1, 2, 3, or 4 hours). After the challenge time, the beads were dissolved, serially diluted and plated. CFU/ml counts were calculated as previously described. Graphs show CFU/ml counts plotted against challenge times (Figure 4.12).

Figure 4.12 shows that, while there is no major alteration in antibiotic sensitivity, there is a slight dip after 2 hours' challenge however, the effect is transient. As this experiment did not yield the expected result of a reversion to gentamicin sensitivity in

the biofilms, the original experiment from Allison *et al* [2011] was replicated. The published results were not achieved (Figure 4.13).

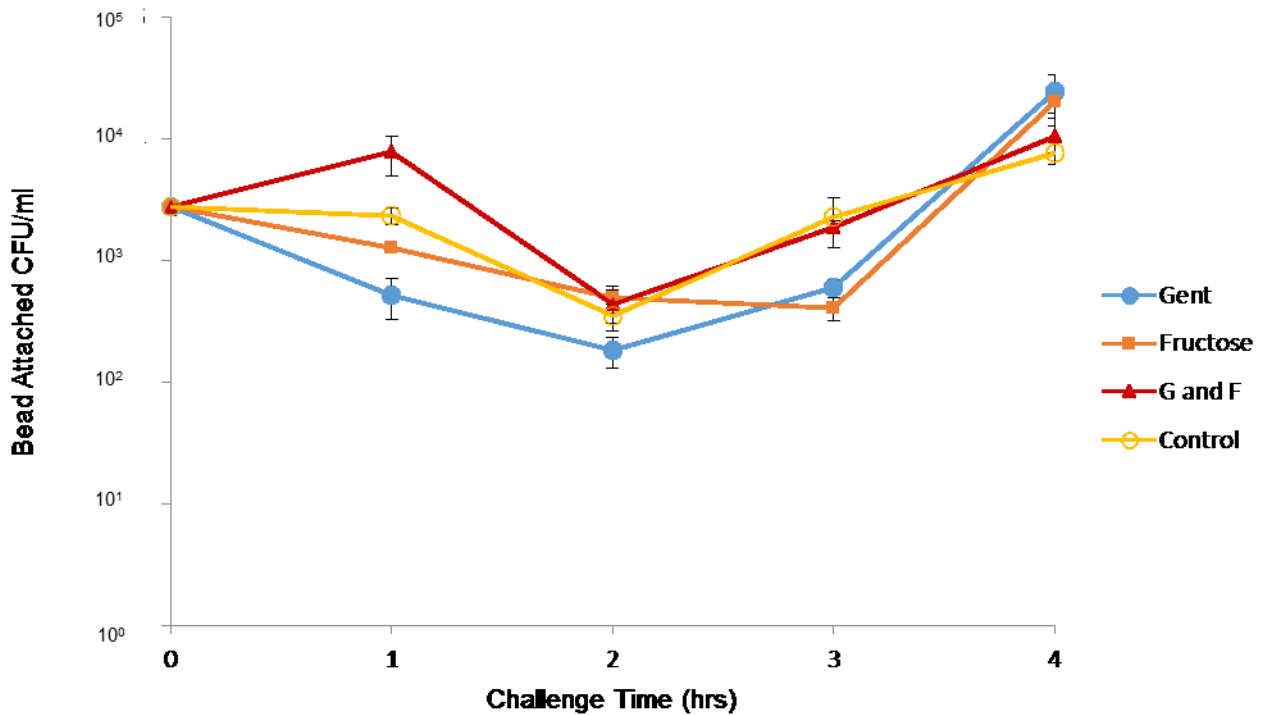


Figure 4.12: Reversal of Antibiotic Resistance is not Achieved in Young Biofilms (<4 hours) of *Staphylococcus aureus* with the Addition of Fructose. Mean CFU/ml counts are plotted against challenge time with error bars of standard deviation. The biofilms were incubated for 2 hours, washed and transferred into stationary phase medium with 15 µg/ml gentamicin (Gent), 10 µg/ml fructose (Fructose) or both (G and F). Control beads had no additions. Triplicate beads were set up for each time point. Biofilms were incubated and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods.

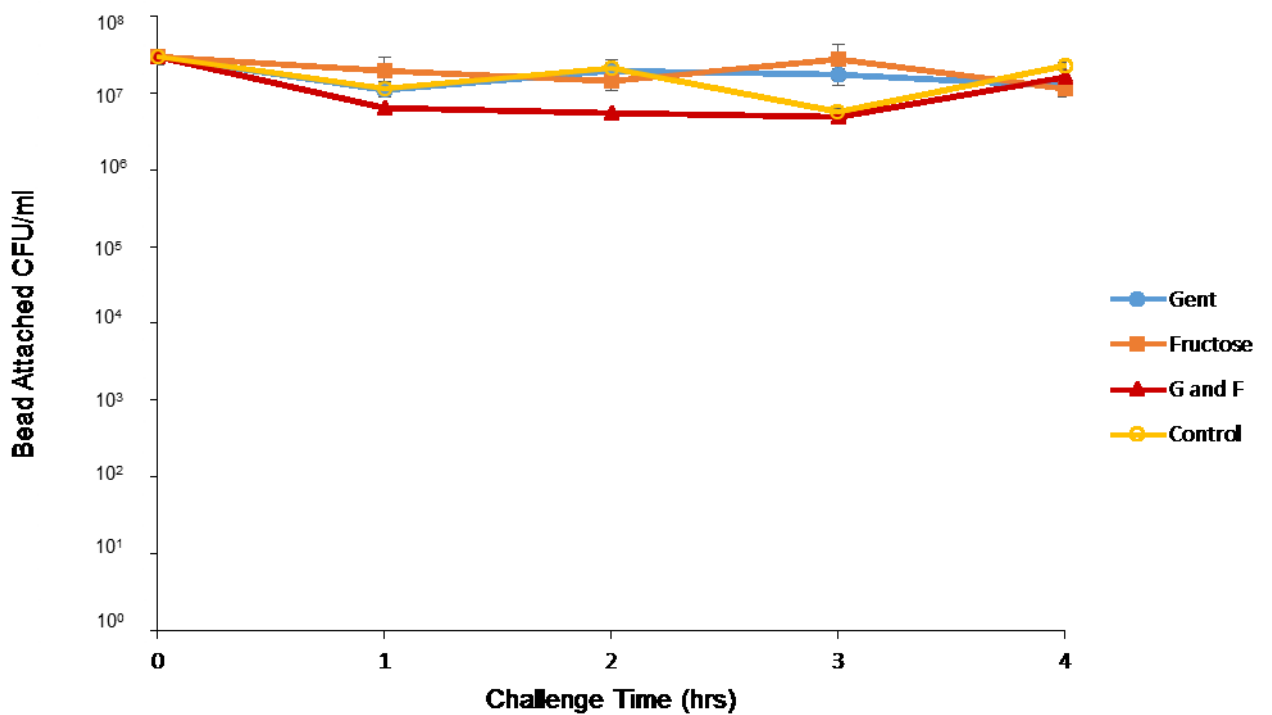


Figure 4.13: Gentamicin Resistance is not Reversed with the Addition of Fructose to a 24 hour *Staphylococcus aureus* Biofilm. Mean CFU/ml counts are plotted against challenge time with error bars of standard deviation. The biofilms were incubated for 24 hours, washed and transferred into stationary phase medium with 15 µg/ml gentamicin (Gent), 10 µg/ml fructose (Fructose) or both (G and F). Control beads had no additions. Triplicate beads were used for each condition. Biofilms were incubated and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated as described in Materials and Methods.

Discussion

Development of Biofilms

It is well documented that numerous bacteria and other microorganisms can form biofilms [Burmølle *et al.*, 2014; Costeron *et al.*, 1987]. Indeed biofilms are one of the most dominant cell states (>95%) in which bacteria are found [Whitman *et al.*, 1998].

The species used in this study were *Staph. epidermidis*, *Staph. saprophyticus*, *Strep. mutans*, *Strep. pneumoniae*, *E. coli* (2 strains), *E. faecalis*, *K. pneumoniae* and *Ps. aeruginosa*. The infections caused by these species include catheter and implant associated infections, osteomyelitis, burns and chronic wounds, otitis media and pneumonia. All of these infections have a large biofilm component [Bjarnsholt, 2013]. Other infections and diseases caused by these bacteria are detailed in Table 1.5 [Goering *et al.*, 2008].

Figures 4.1 A and 4.2 A demonstrated that like so many other bacterial species, the species utilised for this study, most of which are clinical isolates, can form biofilms. The pattern of the biofilm formation between the species is similar with rapid non-specific attachment followed by development of a mature biofilm with significant resistance to antibiotics.

Most published studies have been conducted on biofilms which have been grown for 24 hours or even longer [Ceri *et al.*, 1999; Hochbaum *et al.*, 2011]. Indeed there are very few papers detailing the development of young biofilms (under 24hrs old) and their resistance profiles. It is true, that within a clinical environment, a biofilm infection would only be diagnosed after various tests over a period of weeks [Stoodley *et al.*, 2011]. Therefore, working with older biofilms allows an appropriate comparison. However, it is interesting to note that biofilms are firmly established and exhibit substantial antibiotic resistance after such a short growth period.

Another conclusion which can be drawn from Figures 4.1 A and 4.2 A, is that the alginate bead method is further validated as a viable alternative to other biofilm models. This relates to the work detailed in Chapter 3. Dall [2013], who initially developed the alginate bead method only worked with staphylococcal species. The inclusion of these other Gram-positive and Gram-negative bacteria in the present study, demonstrates the versatility of the alginate bead method. The inclusion of a mucoid alginate producing strain of *Ps. aeruginosa* was to determine if the alginate

composition of the beads affected the formation of biofilms. As a stable biofilm was formed on the beads it shows that, even with an alginate producing species (as seen in Figure 4.2 A), the alginate of the beads can be regarded as inactive and should have no effect on biofilm formation and composition.

Development of Resistance

Biofilm resistance to antibiotics developed much earlier than expected, in some cases within an hour of colonisation (Figure 4.3). Most published biofilm papers are based on older (over 24 hours) models and the resistance which develops as the biofilms age [Pettit *et al.*, 2009; Yang *et al.*, 2015].

The protection a biofilm affords is multifactorial [Burmølle *et al.*, 2014] and may influence resistance through innate cell mechanisms, such as efflux pumps, sequestering of the antibiotic, and changes in the chemical microenvironment [Mah and O'Toole, 2001]. The biofilm matrix itself would also contribute to resistance through reduced diffusion rates of compounds [del Pozo and Patel, 2007], low or stalled growth rates [Stewart, 2002] and altered gene and protein expression patterns [Van Acker *et al.*, 2014]. The associated EPS and extracellular proteins could also play a significant role in biofilm resistance [Davis, 2003]. Increasing bacterial densities, especially in older biofilms, result in more waste products which could affect antibiotic actions inside the biofilms [del Pozo and Patel, 2007].

Transport mechanisms, such as efflux pumps, which prevent the antibiotic from reaching their target or actively transport compounds out of the cell have been extensively investigated [Webber and Piddock, 2003; Piddock, 2006].

Efflux pumps can either export a specific drug or a group of drugs out of a cell: their main function is thought to be the export of natural toxic substances out of the cell, and the transport of antibiotics and ensuing resistance is simply a by-product of this process [Hooper, 2005; Piddock, 2006]. Although increased expression of efflux pumps, especially multidrug resistance (MDR) pumps, is required for active transport of antibiotics some wild type bacteria have sufficient constitutive basal pump activity to display innate resistance to some antibiotics, for example *E. coli* and linezolid or *Ps. aeruginosa* and fluoroquinolones. When these pumps are inactivated the bacteria

demonstrate susceptibility to these antibiotics [Livermore, 2003; Lomovskaya *et al.*, 1999].

In a clinical situation gentamicin is used against Gram-negative aerobic bacilli and Gram-negative species, such as the strains used in this study [Ramirez and Tolmasky, 2010]. The known resistance mechanisms of gentamicin include modification of the antibiotic target site [Galimand *et al.*, 2005], reduced membrane permeability [Hancock, 1981], active transport out of the cell [Aires *et al.*, 1999], sequestering the antibiotic [Magnet *et al.*, 2003] and enzymatic inactivation of the antibiotic [Ramirez and Tolmasky, 2010].

Of these mechanisms, it is probably the active transport and sequestering of the antibiotic which are the major cell specific resistance mechanisms in use in Figure 4.4. For the lower concentrations of antibiotics resistance was present thus, these two mechanisms are functioning effectively. As the antibiotic concentrations rise the mechanisms could be overwhelmed thus leading to a killing effect and a reduction in biofilm load (4.4b).

Other resistance mechanisms are a consequence of the biofilm matrix itself as the negatively charged biofilm matrix inhibits penetration of positive aminoglycosides and the absence of oxygen within the biofilm reduces aminoglycoside activity [del Pozo and Patel, 2007].

Three of the strains used in the current study, were innately resistant to gentamicin (*Staph. saprophyticus*, *Strep. mutans* and *E. faecalis*), as determined by growth overnight on agar plates. Therefore, vancomycin was used to test the development of resistance. Vancomycin is the clinical treatment of choice for invasive staphylococcal infections [Kiedrowski and Horswill, 2011]. Vancomycin is a slow acting antibiotic [Chamber and DeLeo, 2009], however, in *E. faecalis* resistance was seen to develop within 1 hour of growth (Figure 4.3b, G). When the biofilm is challenged after 3 hours growth only the highest vancomycin concentration destroys the *E. faecalis* biofilm (Figure 4.4b, G). The same effect can be seen with the 24 hour old biofilm only being reduced by ~2 log growth rather than completely eliminated like the strains treated with gentamicin (Figure 4.4b, G).

Vancomycin resistance was first identified in *Enterococcus* species in the mid-1980s and though it has spread resistance remains at relatively low levels. There are two recognised forms of resistance. High-level resistance is due to a plasmid encoding

proteins which decrease the binding affinity for vancomycin [Courvalin, 2006]. The second form of resistance is termed intermediate and is due to mutations which increase cell wall thickness and teichoic acid D-alanylation resulting in reduced penetration of the antibiotic into the bacterial cell [Howden *et al.*, 2010].

The three strains treated with vancomycin all showed the same resistance pattern of incomplete biofilm clearance even with higher antibiotic concentrations (Figure 4.4). It may be that as vancomycin is slow acting [Chambers and DeLeo, 2009] the biofilm is able to establish itself and become resistant before the antibiotic can take effect. A significant limitation to the use of vancomycin for biofilm treatment is that vancomycin only effectively acts on actively growing cells which only make up a small percentage of a biofilm [Chamber and DeLeo, 2009]. Although, vancomycin has been shown to be able to penetrate and damage biofilms, cells remain viable. Thus it is rarely used as monotherapy in a clinical environment [Dunne *et al.*, 1993].

Whilst some innate cell mechanisms such as efflux pumps play a role in biofilm resistance, the main reason behind the resistance must be due to the biofilm itself. Biofilms resistance mechanisms have been discussed previously but to summarise heterogeneous gene and protein expression, reduced diffusion into the biofilm matrix, EPS, formation of persister cells, and different growth rates all contribute to biofilm resistance to antibiotics, disinfectants, antiseptics and other environmental stresses. Antibiotic sensitivity is restored when the bacteria re-enter a planktonic state, this indicates that biofilm resistance is an adaptive mechanism rather than one which requires genetic modification [Stewart, 2002].

Development and Challenge of Mixed Species Biofilms

Mixed species biofilms develop in much the same way as a single species biofilm. However, the presence of more than one species may generate a more complex structure and different functions and mechanisms need to be considered [Sanchez-Vizuet *et al.*, 2015]. Multispecies biofilms are common in nature and can be found in many geochemical cycles, human health and homeostasis, industrial and clinical settings [Davey and O'Toole, 2000].

Bacteria forming multispecies biofilms have been shown to have a protective effect on each other and to produce a higher biofilm load when compared to the individual

species [Burmølle *et al.*, 2006]. These mixed biofilms are more resistant to chemical stress, antimicrobials and host immune defence mechanisms [Bridier *et al.*, 2012; Harriott and Noverr, 2009; Stewart, 2002]. Multispecies biofilms can be composed of bacteria, yeast or a combination of these two microorganisms [Harriott and Noverr, 2009].

The mixed biofilm investigated in this study was constructed of *Staph. aureus* and *Ps. aeruginosa*. These two species are serious HCAs and cause a variety of diseases, infections, and problems within healthcare environments as detailed in Chapter 1. The combination of these two species was chosen as, in a clinical environment, they are found in the same host sites and associated with similar types of infections and diseases and thus could form biofilms together [Hendricks *et al.*, 2001].

Staph. aureus and *Ps. aeruginosa* are among the most frequently isolated organisms from HCAs [ECDC, 2011]. The ability of these two organisms to form a mixed biofilm which can delay wound healing and alter the production of virulence factors [DeLeon *et al.*, 2014]. Dual infections by *Ps. aeruginosa* and *Staph. aureus* result in worse patient outcomes than individual infections [Rosenbluth *et al.*, 2004].

Synergistic effects have been reported in mixed biofilms of *Staph. aureus* and *Ps. aeruginosa*, and Figure 4.6 correlates with the published results [DeLeon *et al.*, 2014]. The resistance of the biofilm is immediate, and was developed at a lower cell density than the biofilms formed by the individual species. Higher antibiotic concentrations were also withstood (Figure 4.7).

However, it is interesting to note that for the 24 hour old biofilm (Figure 4.8) there seems to be a reduction in the *Ps. aeruginosa* cell numbers from the mixed biofilm compared to the individually formed biofilm. It may be by the 24 hour time point that the culture medium is exhausted so the cells are becoming competitive against the second species.

The *Staph. aureus* quorum sensing system, unlike that in *Ps. aeruginosa* [O'Loughlin *et al.*, 2013] actually encourages biofilm detachment by the activation of the *agr* system and release of extracellular proteases [Kiedrowski and Horswill, 2011; Thoendel *et al.*, 2011]. It is possible that the *Staph. aureus* cells are dissociating from the biofilm and this and the resulting proteases are damaging the *Ps. aeruginosa* cells and causing their detachment allowing the antibiotics to once again penetrate the biofilm and have an effect. This could account for the difference in colony numbers

between the challenged and unchallenged cultures of Figure 4.6, and the fact that a lower antibiotic concentration is required to kill the *Ps. aeruginosa* from the mixed culture than that of the individual culture (Figure 4.8).

Use of D-Amino Acids to Disrupt *Staphylococcus aureus* Biofilms

The antibiotic concentrations required to reduce the biofilm load in both single and mixed species biofilms are immense and not necessarily practical in a clinical setting therefore, alternative approaches are needed to disrupt biofilms and act in conjunction with conventional therapies.

The ability of D-amino acids to disrupt biofilms is controversial. It was originally suggested that the D-amino acids inhibit and disrupt biofilm formation and was first observed for *B. subtilis*, although it was then reported for various other species including *Staph. aureus* and *Staph. epidermidis* [Kolodkin-Gal *et al.*, 2010; Hochbaum *et al.*, 2011; Ramón-Peréz *et al.*, 2014].

The theory behind the disruptive effect is that the D-amino acids are incorporated into the cell wall affecting peptidoglycan synthesis and preventing major biofilm matrix proteins from attaching to the cell wall. This might lead to decreased intracellular adhesion and poor formation of biofilms [Lister and Horswill, 2014].

Different D-amino acids have been reported to be effective against different species. It was reported that D-Phenylalanine, D-Proline and D-Tyrosine were most effective against *Staph. aureus*. D-methionine and D-alanine were reported to affect *Staph. epidermidis* whereas apparently D-Tryptophan, D-Tyrosine, D-leucine and D-methionine were most effectual against *B. subtilis* biofilms [Hochbaum *et al.*, 2011; Kolodkin-Gal *et al.*, 2010; Ramón-Peréz *et al.*, 2014].

The initial experiments carried out in the current study examined the effect of D-amino acids on young biofilms (Figure 4.9). When no effect was observed one of the Hochbaum *et al* [2011] experiments was replicated as closely as possible (Figure 4.10 A). Once again no effect was seen even with the higher D-amino acid concentrations. A combination of the three D-amino acids, which had been reported as being more effective than them individually, did not produce any significant disruption to the biofilms (Figure 4.10 B).

The second experiment detailed in the Hochbaum *et al* [2011] paper reported that if biofilms were allowed to develop normally for a period (24 hours), the addition of D-amino acids extensively damaged the biofilms. However, once again no reduction in biofilm load or damage to the biofilm was seen when the experiment was replicated, using this *Staph. aureus* strain (Figure 4.11).

An explanation for these results could be that the original reports of the D-amino acids effects were incorrect or exaggerated. In fact, there have been recently published papers which have found that the original experimental results were not reproducible, even when using the same *Staph. aureus* strain. Sankar and Pires [2015] demonstrated that none of the three reported effective D-amino acids inhibited biofilm formation or enhanced disruption of biofilms in *Staph. aureus*, *Staph. epidermidis* or *B. subtilis*. Sankar and Pires [2015] also screened 96 unnatural D-amino acids, none of which had any inhibitory effect on *Staph. aureus* biofilms.

The *B. subtilis* strain used in the original experiments [Kolodkin-Gal *et al.*, 2010] was found to have a strain specific mutation in the *dtd* gene [Leiman *et al.*, 2013]. This gene encodes D-tyrosyl-tRNA deacylase which prevents misincorporation of D-amino acids into proteins. When this mutation was repaired the strain was no longer susceptible to the D-amino acids effects and was able to correctly develop and maintain biofilms.

When the use of D-amino acids to inhibit biofilms was first proposed it seemed like a promising addition to the arsenal of alternative biofilm treatments. However, the recent publications and the results gathered from this study, have proven that the D-amino acids have little to no effect on biofilm formation and disruption. Thus the use of D-amino acids is not a viable adjunctive therapy to antibiotic treatment of biofilms.

Using Sugar Metabolites to Restore Gentamicin Sensitivity to *Staphylococcus aureus* Biofilms

The possible use of sugar metabolites in returning persister cells and biofilms to an aminoglycoside sensitive state was first reported by Allison *et al* [2011]. This paper reported that various sugar metabolites are transported by PMF into the cell's cytoplasm where they enter glycolysis. The catabolism of the sugars produces NADH which is oxidised by electron transport chain enzymes contributing to subsequent

generation of PMF. The increase in PMF facilitates entry of aminoglycosides into the cell where the antibiotics are able to act upon their target and induce cell death.

Both *E. coli* and *Staph. aureus* were used in the Allison *et al* [2011] experiments. Interestingly, only fructose was reported to aid gentamicin activity in *Staph. aureus* whereas in *E. coli* mannitol, glucose and pyruvate also had an effect. When the sugar metabolites were tested with other antibiotic classes, it was demonstrated that the sugar assisted eradication of biofilms was aminoglycoside specific. It was suggested that as aminoglycoside uptake into cells is based on PMF [Taber *et al.*, 1987] this is only this class of antibiotics whose transport and activity is affected by the sugar metabolites and that the cells did not revert back to a normal growing state – as evidenced by the lack of renewed activity of the β -lactams and quinolones antibiotic classes.

Based on the Allison *et al* [2011] paper, this study attempted to disrupt young (>4hrs old) *Staph. aureus* biofilms with the addition of fructose. As can be seen from Figure 4.12 a slight dip was observed in the CFU/ml count after 2 hours challenge with gentamicin and fructose. However, this decrease is present in all conditions, even the control and thus should not be considered significant. The results indicated that fructose does not disrupt biofilm formation in young biofilms

In the Allison *et al* [2011] experiment, the biofilms were incubated for 24 hours before been challenged with fructose and gentamicin. As no inhibition was detected in Figure 4.12, this experiment was replicated (Figure 4.13). However, there were no observable differences in the biofilms when challenged with gentamicin alone, fructose alone, gentamicin and fructose combined or when nothing was added to the culture.

Possible explanations for these results are that, although the experimental protocol was followed as closely as possible there is always variation in personal and laboratory techniques and equipment. A different *Staph. aureus* strain was used in this study compared to the Allison *et al* [2011] study which may suggest that the effect of sugar metabolites is strain specific. However, this seems unlikely as the PMF system is universal between bacteria [Kashket, 1985].

The use of sugar metabolites to disrupt biofilms is worth further investigation although, the effects published in Allison *et al.*, [2011] were not able to be reproduced. Other papers have demonstrated that sugar metabolites have a detrimental effect on biofilm

formation. In the study, conducted by Durmus *et al* [2012], *Staph. aureus* bacterial growth on endotracheal tubes (ETTs) was reduced when the tubes were coated with fructose.

Bucior *et al* [2013] reported a sugar mixture of mannitol, fucose and galactose inhibited bacterial adhesion to bronchial epithelial cells *in vitro* in a murine model of acute *Ps. aeruginosa* pneumonia. Synergistic activity was also demonstrated when the sugars were administered with conventional antibiotics reducing bacterial lung colonization and damage.

From these papers it appears that a mixture of sugars are required for Gram-negative bacteria whereas a single sugar (usually fructose) is able to produce an effect in Gram-positive bacteria. However, further work is required to clarify this and further examine the use of sugar metabolites in inhibiting bacterial biofilms.

Chapter 5:

The Bacterial Binding Proteins; CPGRP and SP-D, and their expression in *Pichia pastoris* and *Escherichia coli*.

Introduction

As has been described previously in Chapters 3 and 4, biofilms are a major issue in the health care environment and can form on catheters, prosthetic valves, artificial joints and other devices [Goering *et al.*, 2008]. Successful eradication of biofilms is difficult due to the nature of where the biofilms form and the antibiotic resistance that they develop. Treatment is commonly long term, high dose antibiotics combined with removal and replacement of the affected device. However, in some cases, such as prosthetic joints, replacement is not an option. Therefore confirmation of existing biofilm presence and/or the successful eradication of biofilms would be a huge advantage.

This chapter details experiments in which a fusion protein consisting of a bacterial binding protein and a fluorescent detector molecule were explored. Potential uses of such a fusion protein, in a clinical setting could include the imaging of prosthetic devices such as catheters which are commonly colonised but difficult to image and treat [Leevy *et al.*, 2008; White *et al.*, 2010].

If bacterial biofilms were found at a site treatment could be tailored specifically thereby reducing the investigation cost and time, the antibiotic usage and improving the patient's quality of life.

The two potential bacterial binding proteins examined are Camel Peptidoglycan Recognition Protein (CPGRP) and Surfactant Protein-D (SP-D) from both yeast expression systems and bacterial expression systems.

Camel Peptidoglycan Recognition Protein (CPGRP)

Discovery of Peptidoglycan Recognition Proteins (PGRPs)

The term Peptidoglycan Recognition Protein was first used by Yoshida, Kinoshita and Ashida [1996] in reference to the protein purified from the hemolymph of the silkworm *Bombyx mori*. The discovery of this protein was related to the hemolymph prophenol oxidase cascade which is one of the silkworm's defence pathways against infection. PGRP is triggered by recognition of non-self molecules including lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), flagella of Gram-negative and Gram-positive bacteria, as well as elements of the fungal cell wall [Yoshida *et al.*, 1996; Swaminathan *et al.*, 2006].

Since then, PGRPs have been identified in many species including cows, camels, humans, pigs, moths and mosquitoes [Dziarski, 2004], showing that this group of proteins is highly conserved between insects and mammals [Kang *et al.*, 1998; Guan and Mariuzza, 2007]. There have been nearly 100 PGRPs identified to date [Guan and Mariuzza, 2007]. Cysteine residues in mammalian PGRPs are considered to be especially important as they form disulphide bonds (Cys6-Cys130, Cys22-Cys67 and Cys43-Cys49, see Figure 5.1) [Sharma *et al.*, 2011a] which assist in stabilising the protein in the extracellular or leukocyte granule environments [Dziarski, 2004].

Within insects, PGRPs and other similar molecules provide the main defence against infecting pathogens as invertebrates lack an adaptive immune system, instead relying on their innate immunity [Kang *et al.*, 1998]. The basic function of a PGRP is the recognition of conserved molecular patterns found on the surfaces of bacterial cells, which are not native to the host. [Swaminathan *et al.*, 2006; Medzhitov and Janeway, 1998]. This recognition function triggers various innate immune system cascades and in vertebrates, activates the adaptive immune system.

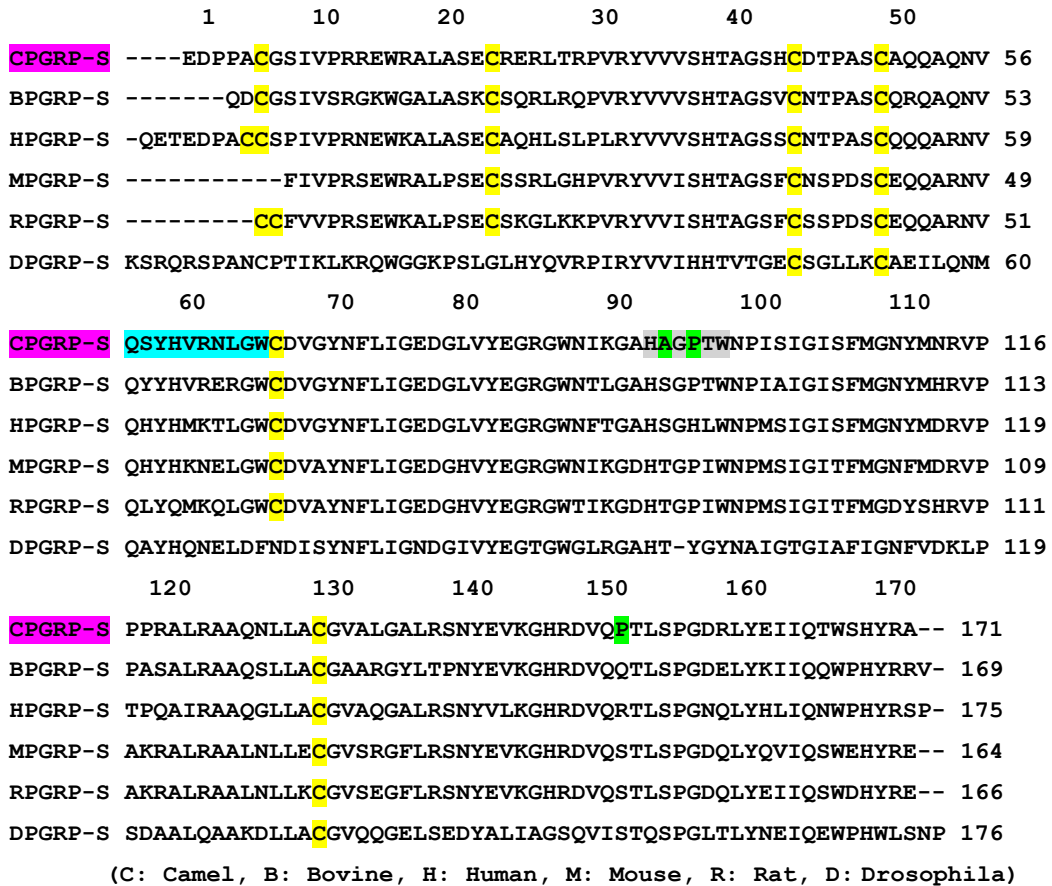


Figure 5.1: Alignment Diagram showing the Conserved Amino Acid Sequences from Various Species [Adapted from Sharma *et al*, 2011b]. CPGRP-S is the molecule of interest for this study (pink). Yellow highlighted portions denote cysteine residues. The important amino acids which favour dimerisation in CPGRP are shown in green. Subsite I consists of residues 59-66 (turquoise) and Subsite II is highlighted in grey (residues 93-98).

Peptidoglycan is the most common component which is recognised as part of a Pathogen Associated Molecular Pattern (PAMP), with the components N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) making up this polymeric molecule in a series of repeating units. [Swaminathan *et al.*, 2006; Sharma *et al.*, 2012b].

Peptidoglycan activates the mammalian immune system through two pattern recognition receptors; Toll like receptor 2 (TLR-2) and CD14 [Weidemann *et al.*, 1994; Weidemann *et al.*, 1997]. These receptors are found on the surface of macrophages and induce cytokine and chemokine production in response to invasion by foreign and unrecognised molecules. If the cytokine and chemokine reactions are too excessive, symptoms of infection such as inflammation, fever, hypotension and in severe cases, circulatory shock and multiple organ failure may be caused [Swaminathan *et al.*, 2006; Lie *et al.*, 2000].

Classifications of PGRPs Types

Approximately 100 different PGRPs have been identified, all of which contain a peptidoglycan binding site of ~165 amino acids in length [Guan and Mariuzza, 2007]. Those found in insects can be divided into two groups; short PGRPs (PGRP-S) which are no more than 20kDa and long PGRPs (PGRP-L) which are no more than 90kDa [Dziarski 2004]. These are extracellular (PGRP-S only), intracellular or transmembrane proteins, found in the insect's immune organs (fat body, gut and haemocytes), some of whose expression may be up-regulated upon exposure to either bacteria or peptidoglycan [Dziarski, 2004; Guan and Mariuzza, 2007]. There are two possible roles within the immune system which these proteins can fulfil, either activating proteolytic cascades and signalling pathways (non-catalytic PGRPs), or hydrolysing peptidoglycan (catalytic PGRPs) [Dziarski, 2004].

Four classes of mammalian PGRPs: short (PGRP-S), intermediate, of which there are two sub-groups (PGRP-I α and PGRP-I β), and long (PGRP-L) have been identified [Sharma, 2008]. All are soluble intracellular or secreted proteins [Guan and Mariuzza, 2007] with immune defence roles [Liu *et al.*, 2000]. One of the effects that a mammalian PGRP has within a system is the reduction of the immune cascade activation, in effect reducing the consequences of recognition of peptidoglycan and other PAMPs, within the immune system [Sharma, *et al.*, 2008; Liu, *et al.*, 2000].

Camel Peptidoglycan Recognition Protein

CPGRP-S is a soluble conserved pattern recognition molecule of the innate immune systems of camels [Kappeler *et al.*, 2004; Medzhitov and Janeway, 1998; Sharma *et al.*, 2008; Sharma *et al.*, 2011a; Sharma *et al.*, 2011b]. CPGRP has a more pronounced role than in other mammals because of the camel's unusual immune system [Sharma *et al.*, 2008; Sharma *et al.*, 2011b]. Molecules which are common in other mammalian immune systems including lysozyme C, lactoferrin and lactoperoxidase are only found at low concentrations within the camel. This may explain the prominence of CPGRP [Sharma *et al.*, 2008; Sharma *et al.*, 2011a] as it provides a first line of defence against infecting pathogens. When the molecule was originally discovered it was isolated from camels' milk, [Kappeler *et al.*, 2004] with the highest concentration produced during mastitis. Mastitis is rarely reported in camels in comparison to cattle and other mammals used for milking due to the presence of CPGRP [Sharma *et al.*, 2011b].

CPGRP has a similar specificity, but higher affinity, for peptidoglycan than another molecule of the immune system, lysozyme C. Lysozyme C antimicrobial properties include hydrolysing peptidoglycan, as well as immunomodulation and anti-inflammatory functions [Kappeler *et al.*, 2004]. CPGRP acts by inhibiting bacterial infections through interaction with peptidoglycan and other PAMP components, including LTA, LPS and their moieties [Sharma *et al.*, 2011b; Sharma *et al.*, 2012a]. The binding of CPGRP to these molecules occurs with varying affinities, specificities and potencies for each one [Sharma *et al.*, 2011a; Sharma *et al.*, 2012a].

A subsequent effect of this interaction is the reduction in LTA/LPS induced expression of the pro-inflammatory cytokines (Tumour Necrosis Factor- α and Interleukin-6). This results from the blocking of available LTA/LPS to the PAMP receptors (CD14, Toll and CD6) expressed on T-cells. Studies have shown that a reduction of these potentially excessive immune responses may prevent severe inflammatory reactions and sepsis [de Kimpe *et al.*, 1995].

CPGRP is made up of four polypeptide chains (Figure 5.2) each consisting of 171 amino acids [Sharma *et al.*, 2011b]. Of all the known PGRPs, CPGRP is the only tetrameric complex structure which has been identified, that gives rise to a versatile binding site [Sharma *et al.*, 2011a].

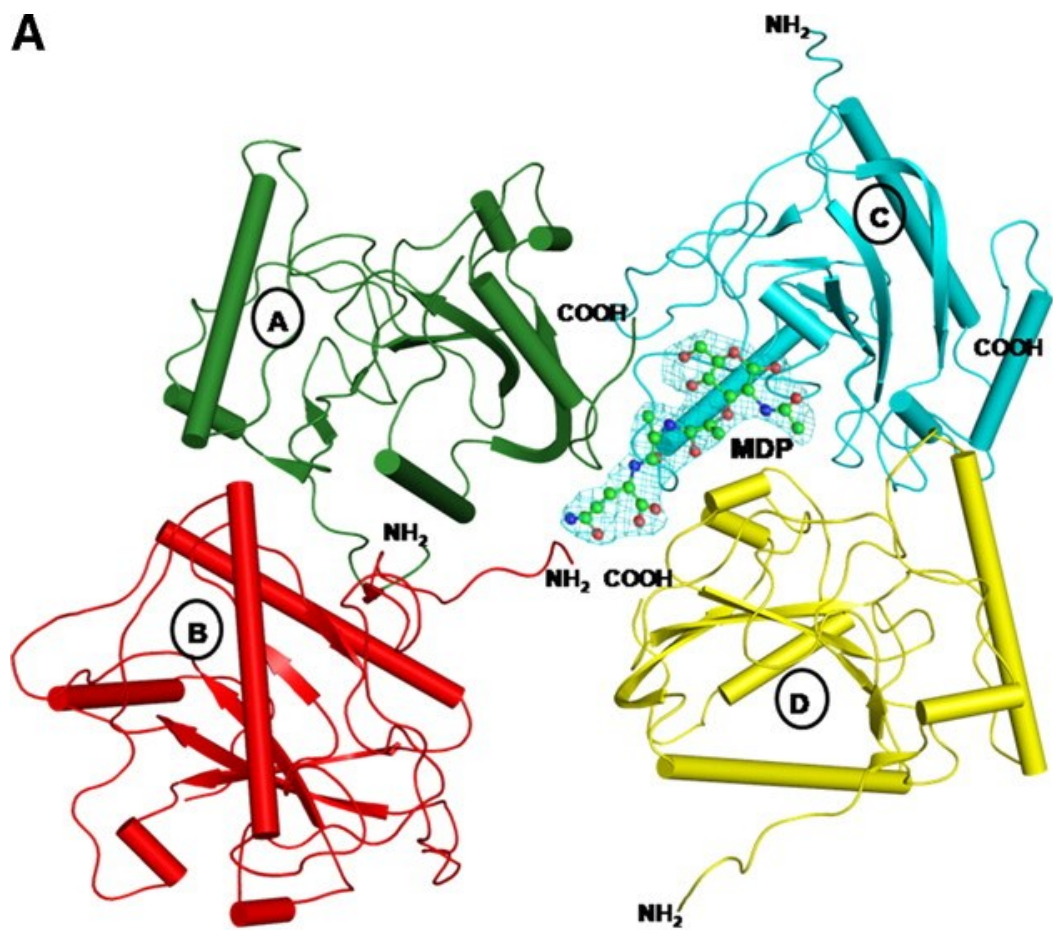


Figure 5.2: Representation of Camel Peptidoglycan Recognition Protein Showing the Channel and Binding Region [Sharma *et al.*, 2011b]. Major α -helices are represented by the long cylinders, the central β -sheet by the arrows, the remaining cylinders show minor α -helices. MDP: muramyl dipeptide is a moiety of peptidoglycan. Subsite I is located along the boundary of molecular C and D with some extension into the interface of molecular A and C. The tetramer is made up four identical monomers.

Comparisons with PGRPs from other species e.g. human PGRP binding sites, show that their amino acid residues do not favour dimerisation as in CPGRP. The amino acids which impart this multimerisation property onto CPGRP include an Alanine residue at position 94 and Proline moieties at positions 96 and 151 (highlighted in green on Figure 5.1) [Sharma *et al.*, 2011a].

The basic architecture of a CPGRP monomer is a central β sheet surrounded by 3 major α helices, stabilized by 3 disulphide linkages [Sharma *et al.*, 2011a]. A CPGRP monomer is ~20kDa [Sharma *et al.*, 2008] and, therefore, is considered a short PGRP. The tetramer itself is constructed of non-covalent associations between the four molecules leading to the formation of a binding site which is located inside the asymmetric homotetramer [Sharma, 2008]. The four monomers naturally assemble into a multimer when expressed [Sharma *et al.*, 2011b].

The CPGRP binding channel is formed from the lack of intermolecular interactions between monomers B and D, with the final binding region residing at the interface of monomers C and D with some extension into the boundary between monomers A and C. Binding of ligands does not disrupt the structural formation of the binding site or molecule, however, the orientations of some of the side chains are slightly altered [Sharma *et al.*, 2011b].

The shape of the channel is similar to a funnel in that it has a wider opening at the exterior of the molecule than the internal end in the middle of the molecule; this may help in capturing PAMPs more effectively. Another factor which may influence PAMP recognition is the flexible N termini of monomers B and D which may filter out non-PAMP molecules lacking stereo-chemical complementarity [Sharma *et al.*, 2011b].

The binding channel contains multiple subsites therefore, it is able to detect different PAMPs with specificity through direct interaction [Sharma *et al.*, 2012b]. Different compounds bind to different subregions of the binding site. Two different subsites have been identified (residues highlighted in Figure 5.1). Subsite I (S I) recognises the glycan moieties of peptidoglycan more readily than Subsite II (S II) [Sharma *et al.*, 2012a; Sharma *et al.*, 2012b].

Sharma *et al.*, [2011a] compared the binding of LPS (from *E. coli* serotype O55:B5) and LTA (from *Staph. aureus*) and found that there are common amino acid residues but also some which are polymer specific. LPS connects with the amino acids Lys-C90, Ala-C92, Asn-C99 and Arg-A170 (mainly from monomer C) whereas LTA interacts with Thr-D27, Asn-C99, Gln-D150 and Asn-A140 residues (primarily from monomer D) [Sharma *et al.*, 2011a].

CPGRP targets cell wall molecular patterns rather than cell membranes and as such could possibly be developed as a protein antibiotic, with a bacteriostatic action [Kappeler *et al.*, 2004], which would be unlikely to encounter bacterial resistance [Sharma *et al.*, 2011a]. Whole bacterial cells (*Staph. aureus*) were used to determine the effect of CPGRP on growth inhibition [Sharma *et al.*, 2011b]. The binding channel interacts directly with the molecules of the bacterial cell wall, thereby, inhibiting further cell growth. Activation of immune cells is also reduced, thus there is less amplification of immune system reactions [Sharma *et al.*, 2011b].

CPGRP is capable of detecting a wide range of microorganisms including many Gram-positive and some Gram-negative bacteria [Sharma *et al.*, 2011b]. *Enterobacter agglomerans*, *E. coli*, *Staph. aureus*, *Micrococcus* species and lactic acid bacteria including *Lactobacillus delbrueckii* spp. *bulgaricus* (whole cells) have been used in studies examining the binding of CPGRP to bacteria [Kappeler *et al.*, 2004]. Binding was found to be strong against the Gram-positive bacteria with weak binding against the Gram-negative bacteria [Kappeler *et al.*, 2004]. However, subsequent studies have found that CPGRP does in fact bind to Gram-negative bacteria mainly through the LPS component of the bacterial cell membrane [Sharma *et al.*, 2008; Sharma *et al.*, 2011a].

When the 3D structure of CPGRP is examined closely (Figure 5.2) it can be seen that the N termini are more accessible than the C termini, with slight 'tails' reaching out from the main structure of the protein. Consequently the addition of a detector molecule, such as a fluorescent protein, onto these ends should not interfere with protein folding and function. This would prove advantageous in the potential construction of a fusion protein comprising of CPGRP and a fluorescent detector molecule.

Surfactant Protein D (SP-D)

Discovery and History

Pulmonary surfactant is composed of a mixture of phospholipids (90%) and proteins and is found at the interface of alveolar gas and liquid hypophase in the airways and alveoli [Wright, 1997], maintaining the surface tension in the lungs to prevent the lungs from collapsing during respiration [Head *et al.*, 2003; Kishore *et al.*, 2006]. Surfactant deficiency can lead to a range of problems such as laboured breathing and inadequate oxygenation, this is most commonly seen in premature infants unable to produce enough surfactant [Wright, 1997].

There are four surfactant proteins which are synthesised by the alveolar type II cells and secreted into the airspaces of the lung [Crouch *et al.*, 1994]. They were first detected in amniotic fluid in 1975; production begins between 30 and 37 weeks gestation [Lu *et al.*, 1992]. The surfactant protein's role in this environment is thought to be the clearance of pathogens from the foetal membranes and amniotic fluid via amnion cells or decidual membranes [Malhotra *et al.*, 1994].

The four surfactant proteins SP-A, SP-B, SP-C and SP-D are present at different distributions within the lungs, found at 5.3%, 0.7%, 0.4%, and 0.6% of the pulmonary surfactant respectively [Weaver and Whitsett, 1991]. SP-B (14kDa) and SP-C (6kDa) are small, hydrophobic proteins whereas SP-A and SP-D are large hydrophilic proteins. The functions of SP-B and SP-C include organisation of the surfactant, adsorption to the air-liquid interface, phospholipid packaging, and stabilisation of the phospholipid monolayer ensuring the stability of the surfactant layer [Kishore *et al.*, 2006; Lu, *et al.*, 1992].

It is interesting to note that even though SP-A and SP-D have similar structures they have very different binding patterns and modes of action. SP-D binds to the heptoses on the inner oligosaccharide core or the mannose rich O-polysaccharide of LPS while SP-A prefers the lipid A domain of LPS [Head *et al.*, 2003; Seaton *et al.*, 2010]. These differences in binding potentials could be attributed to the structural variances of the binding region's shape and orientation [Seaton *et al.*, 2010].

The placement of these two proteins within the alveolar compartment will enable them to immediately intercept any inhaled microbes coming into the lungs. Estimations of SP-A and SP-D concentrations in the lungs are 300-1800µg/ml and 36-216µg/ml respectively [Wu *et al.*, 2003]. Even though SP-D is mainly associated with the lung it is also found in other tissues including the intestinal mucosa, thymus, prostate gland and paranasal sinuses [Wang *et al.*, 2008b].

SP-A and SP-D, along with serum mannose-binding protein (MBP), are members of the collectins, a family of innate immune defence proteins named for the collagen-like and lectin domains within their structure [Gupta and Surolia, 2007]. Collectins are known for enhancing clearance of microorganisms through agglutination, opsonisation and reduction of epithelial adherence via binding to glycoconjugates on a microorganism's surface [Head *et al.*, 2003].

The main roles of SP-A and SP-D are controlling infection by having a bacteriostatic effect on microbial growth, inducing phagocytosis through activating neutrophils and macrophages, and influencing cytokine and chemokine pathways during inflammation brought about by infection, necrotic/apoptotic cells and allergen challenges [Gupta and Surolia, 2007; Kishore *et al.*, 2006].

SP-D, and SP-A, can act as immunomodulators which may assist in regulating the inflammatory response [Wu *et al.*, 2003]. This is done primarily by enhancing production of cytokines such as TNF- α , IL-1 α , IL-1 β [Wright, 2005] and regulation of LPS induced inflammation by endotoxin clearance [Quintero *et al.*, 2002].

There is ~70% identity in the amino acid sequences of human SP-D to rat and bovine SP-D [Lu *et al.*, 1992]. However, binding affinities of the proteins differ, for example rat and mouse SP-D bind more efficiently to Influenza A virus than human SP-D. Human SP-D interactions with viruses are based on binding to asparagine-linked glycans which are expressed on viral envelope proteins [Hartshorn *et al.*, 2000; Leth-Larsen *et al.*, 2007]. Human SP-D also demonstrates a different saccharide inhibition profile compared to rat and mouse SP-D [Crouch *et al.*, 2006].

Structure

Protein sequence analysis has shown that each SP-D chain is made up of a short N-terminal domain, a triple-helical collagen like sequence, a short α -helical neck domain and a non-collagenous COOH-terminal domain, [Crouch *et al.*, 1994; Seaton *et al.*, 2010] which is known as the Carbohydrate Recognition Domain (CRD), as can be seen in Figure 5.3 [Kishore *et al.*, 2006].

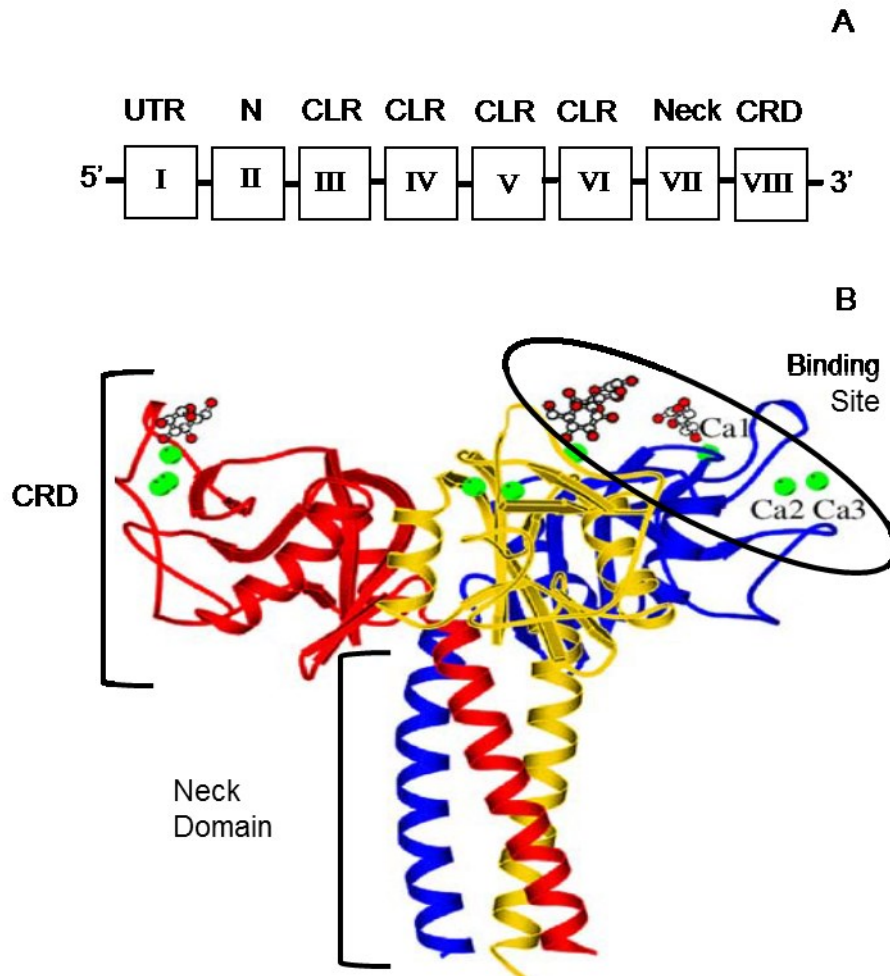


Figure 5.3: Linear Representation (A) and Crystal Structure (B) of Surfactant Protein-D bound with Maltose [Adapted from Kishore *et al.*, 2006]. A) Linear Representation of the SP-D structure. UTR: Untranslated region, N: N-terminal region, CLR: Collagen like region, Neck: Neck domain, and CRD: Carbohydrate recognition domain. B) Neck-CRD trimeric recombinant fragments of SP-D viewed perpendicular to the molecular three-fold axis. Maltose and three calcium ions are bound. Each of the three monomers is a different colour. The CRD, neck domain and binding site are indicated.

The protein is secreted as monomers of trimeric subunits [Crouch, 2000] which self-assemble to form trimers [Crouch *et al.*, 1994]. Each monomer is ~43 kDa. These monomers form the collagen-like triple helix (Figure 5.3) [Lu *et al.*, 1992]. The shape of the trimer presents a broad surface which connects with the microbial or membrane surface of interest to assist in recognition and binding [Seaton *et al.*, 2010].

Binding characteristics

Peptidoglycan has been suggested to be the generic ligand for SP-D recognition of microorganisms as it has a conserved structure between species [Gupta and Surolia, 2007]. It is the CRD of SP-D which is responsible for the binding of the molecule to LPS of bacteria specially the core oligosaccharide and/or polysaccharide chains. There seems to be a preference for 'rough' LPS though 'smooth' LPS can also be bound [Wang *et al.*, 2008a].

Ligand recognition occurs at three structural levels; the CRD, the trimeric CRD and multimers of trimeric subunits [Seaton *et al.*, 2010]. The minimal recognition unit is the CRD; fragments of this molecule which include the neck-CRD show the same binding capabilities as the entire molecule [Wang *et al.*, 2008b]. However, multiple trimers bound together may amplify the strength of ligand binding [Gupta and Surolia, 2007]. The shape of the SP-D molecule with a bound ligand is slightly different from that of the molecule without a ligand bound, in that the tilt angle of the neck region is reduced and the perpendicular orientation of the CRD region is absent [Wang *et al.*, 2008b].

As suggested by the name CRD, carbohydrate ligands such as glucose, fucose, GlcNAc and ManNAc can be bound. However, interactions with hydrophobic molecules, such as fatty acids have also been reported [Seaton *et al.*, 2010].

Bacterial species to which SP-D binding has been recorded include *P. aeruginosa*, acapsular *K. pneumoniae*, *E. coli*, *Bordetella pertussis*, *Haemophilus influenzae*, *Mycobacterium tuberculosis* and *Helicobacter pylori* [Wang *et al.*, 2008a]. Fungal and viral species include *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus fumigatus*, rotavirus, Influenza A Virus (IAV) and Respiratory Syncytial Virus, SARS

coronavirus and HIV [Crouch *et al.*, 2009]. It is the polysaccharides and highly glycosylated proteins found on fungal cell surfaces which act as ligands for SP-D and SP-A [Gupta and Surolia, 2007]. SP-D activity against viruses is facilitated by interactions with viral envelope proteins. For example IAV, consists of recognition of the oligomannose sugars on the viral hemagglutinin [Crouch *et al.*, 2005; Crouch *et al.*, 2009]. Binding and opsonising pathogens augment their uptake by macrophages and neutrophils [Crouch *et al.*, 2009].

The activity of SP-D can be inhibited by supplying the experimental system with excess free LPS vesicles, which compete with bacterial surfaces for binding leading to a reduction in aggregation and growth inhibition [Wu *et al.*, 2003].

Both Gram-positive and Gram-negative species are bound to and aggregated by SP-D in a calcium dependent manner enhancing microbial phagocytosis by alveolar macrophages. Agglutination of bacterial species would also have a negative impact upon the microbe's physiology and access to nutrients thereby further inhibiting its growth [Crouch *et al.*, 1994; Wu *et al.*, 2003]. SP-D also enhances neutrophil uptake of bacteria including *E. coli*, *Strep. pneumoniae* and *Staph. aureus* [LeVine and Whitsett, 2001].

***In vivo* Imaging**

Approaches to investigating diseases and cellular processes used to be limited to broad spectrum techniques such as *ex vivo* methods & assays, imaging procedures including magnetic resonance imaging (MRI) and computerised tomography (CT) scans and monitoring phenotypical changes of cell cultures and animal models [Dorthager *et al.*, 2009]. However, animal studies are often destructive requiring defined end point analysis involving host sacrifice and enumeration of infected organs [Dorthager *et al.*, 2009]. Such approaches only provide information for specific, pre-designed criteria and as the animal has been destroyed, a sequence showing unforeseen disease effects and possible immune reactions generally cannot be constructed [Pribaz *et al.*, 2011]. Therefore, when *in vivo* imaging technologies, such as fluorescent proteins, were developed their value quickly became apparent. The

ability to image cell and disease processes vastly improved and is now a promising and powerful method of detection [Leevy *et al.*, 2008].

Near-Infrared Fluorescent Protein (NirFP)

Currently available fluorescent proteins include a red-GFP like fluorescent protein, [Matz *et al.*, 1999] which has been derived from a GFP isolated from *Aequorea victoria* [Tsien, 1998]. Included in this near-infrared fluorescent protein group are mCherry, Katushka, mKate, Katushka-9-5 and one of the variants recently developed from Katushka-9-5; eqFP670 [Shcherbo *et al.*, 2007; Shcherbo *et al.*, 2010; Shu *et al.*, 1999].

The eqFP670 variant has since been developed by Evrogen, renamed NirFP, and is available on a mammalian expression vector [Evrogen, 2012]. eqFP670/ NirFP has been proposed to be the first GFP-like fluorescent protein which has such a long wavelength emission, half of which is in the infrared range of the spectrum [Shcherbo *et al.*, 2010].

NIR light can penetrate through two or more centimetres of most tissues; this is the area of the spectrum in which light scattering and absorbance by lipids, water and biological molecules is minimal [Shu *et al.*, 2009]. The autofluorescence issues of biological molecules such as haemoglobin does not affect NIR imaging, as the NIR region has the lowest absorption coefficient for these molecules [Weissleder and Ntziachristos, 2003].

The properties of NirFP include an excitation peak of 605nm with an emission peak of 670nm leading to a strong bathochromic shift [Shcherbo *et al.*, 2010]. The extremely high photostability and high pH stability of the protein may be the consequence of two specific amino acid mutations (Asn148 and Asn165). These two mutations are thought to result in tight packaging of the chromophore [Shcherbo *et al.*, 2010]; and consequently the proteins do not show the wavelength fluorescence of alternative chromophore forms [Evrogen, 2012].

Comparing the NirFP to its original form, Katushka, there is a fourfold increase in infrared brightness, and no observed cell toxicity or protein aggregation when utilized for imaging purposes, especially in animal models [Evrogen, 2012]. The NirFP fluorescence signal is easy to differentiate from background fluorescence (which should be minimal) and studies have shown that it can be detected for up to 48 hours

after transfection into mammalian cells [Evrogen, 2012]. The use of this protein, as the detector molecule in a fusion protein, would thus be advantageous.

Protein Expression Systems

***Pichia pastoris* Expression System**

In order to utilise the fusion proteins they must first be overexpressed and purified. The expression system which has been selected for use is the yeast *P. pastoris*. This was first developed in the 1970s as a means to produce high protein animal feed [Cereghino and Cregg, 1999]. However, this was not a viable option economically so the yeast's process of expression was developed as a system for heterogeneous protein production [Celik and Celik, 2011; Wegner, 1990]. Since that time *P. pastoris* has become an important host organism and is a well-accepted and standard tool for the production of proteins from many species [Cereghino *et al.*, 1999; Cregg *et al.*, 2000]. In the present study *P. pastoris* is the ideal choice for protein expression because of the following key factors:

The fusion protein produced is a bacterial binding protein. Thus, there is a strong possibility that a bacterial expression system would not be able to produce the protein without inflicting toxicity on itself.

P. pastoris plasmids contain a bacterial origin of replication for maintenance and propagation in *E. coli*. This is vital for ease of cloning and for the extraction of DNA for sequencing purposes. However, no bacterial promoter or Shine-Dalgarno sequence is present upstream of the gene encoding the fusion protein, essentially excluding protein transcription and translation in *E. coli* [Invitrogen, 2010c].

The *P. pastoris* expression system is capable of performing functions such as eukaryotic protein processing, protein folding and disulphide-bond formation [Celik and Calik, 2011; Cereghino and Cregg, 2000]. Many polypeptides which require post-translational modifications are expressed as functional active proteins in the *P. pastoris* system. In bacterial expression systems, such proteins may not be correctly formed or contain the glycosylation necessary to make them functional [Cereghino and Cregg, 1999].

P. pastoris is a methylotrophic yeast capable of metabolising methanol as its sole carbon source, though it is also capable of growing on glucose, glycerol or a combination of the aforementioned. This metabolic process involves the oxidation of methanol to formaldehyde, using molecular oxygen, by the enzyme alcohol oxidase (encoded by genes *AOX1* and *AOX2*). As this reaction generates formaldehyde and hydrogen peroxide it is carried out within a peroxisome, sequestering the toxic by-products away from the rest of the cell. The main promoter for expression within *P. pastoris* is the *AOX1* gene, therefore induction only occurs within the cells in the presence of methanol [Cereghino and Cregg, 1999; Invitrogen 2010b; Weider *et al.*, 2010]. Of the eukaryotic promoters used in expression systems, the *AOX1* promoter is regarded as especially strong and tightly regulated [Celik and Calik, 2011; Ellis *et al.*, 1987].

A second control mechanism regulating *AOX1* transcription is a repression/derepression system concerning growth on glucose and glycerol. Growth on glucose represses transcription even in the presence of methanol. Only when the yeast is grown on glycerol (and methanol) will there be optimal induction of the *AOX1* promoter and the best possible expression levels of the fusion protein [Cereghino and Cregg, 2000; Cereghino *et al.*, 2002].

P. pastoris can express high levels of both extracellular and intracellular proteins [Celik and Calik, 2000]. For the purposes of this project a vector (pPICZ α B) was selected that contains a α -factor signal sequence (derived from *Saccharomyces cerevisiae*), which triggers extracellular secretion. Thus the fusion protein should be expressed into the surrounding medium [Cereghino and Cregg, 2000]. As *P. pastoris* has very few native proteins which are secreted via the extracellular route [Celik and Calik, 2011], the majority of protein found in the medium should be the fusion protein. This can be considered as a first step in the purification process. Figure 5.4 shows these and other features of the plasmid [Invitrogen, 2010c].

The expression vector will be integrated into *P. pastoris* genome at the *AOX1* locus upon transformation and only those transformants which have incorporated the plasmid DNA into their genome will be cultivatable [Invitrogen, 2010c].

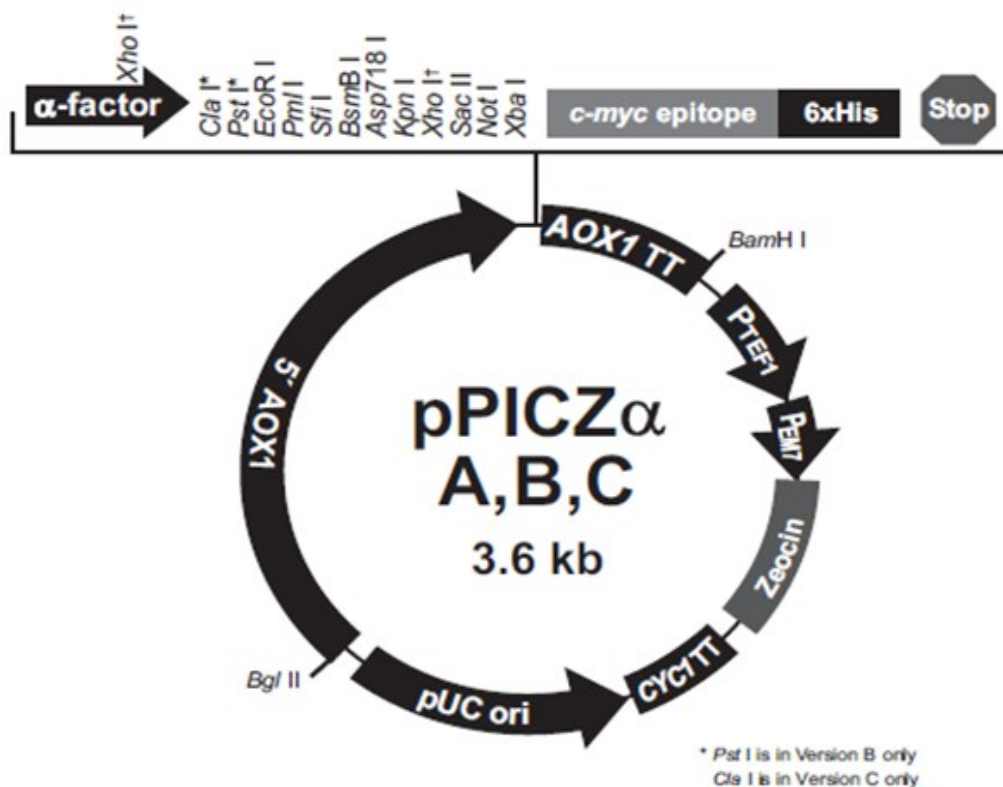


Figure 5.4: pPICZ α Vector Map [Invitrogen, 2010c]. Features of the vector include the α -factor signal sequence which triggers secretion into the surrounding medium. Zeocin resistance with promoters for use in *P. pastoris* (TEF1) and *E. coli* (EM7), bacterial origin of replication (pUC ori), AOX1 promoter which tightly regulates protein expression via methanol induction, native transcription termination and polyadenylation signal (AOX1 TT) from AOX1 gene that permits efficient 3' mRNA processing and multiple cloning site. The vector is produced in three different reading frames (A, B, and C).

Bacterial Expression Systems

A common bacterial expression system that is widely used is based around the pBAD vector. This vector, when transformed into *E. coli* and induced with arabinose, generally gives reliable and consistent protein production [Guzman *et al.*, 1995].

The pBAD/His vector (Figure 5.5) used for this study includes a polyhistidine tag which will fuse to the inserted protein, for easier detection and purification. The vector also includes a multiple cloning site, ampicillin resistance gene, *araBAD* promoter (AraC) and three different reading frames (A, B and C) [Invitrogen 2010a].

The induction of proteins is triggered in the presence of arabinose. Varying the concentration of this sugar will produce tight dose-dependent gene expression [Miyada *et al.*, 1984]. An advantage of the tight regulation by the AraC protein is allowing bacterial growth to an optimal density before protein production by not inducing the gene. This would prove beneficial in expression experiments producing potential toxic proteins [Invitrogen, 2010a].

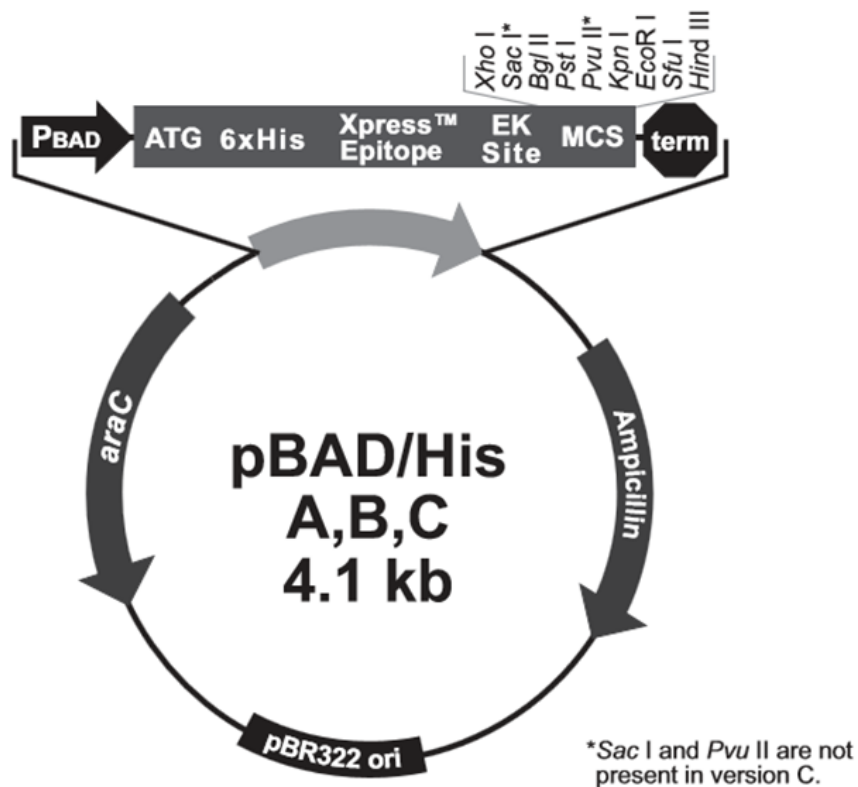


Figure 5.5: pBAD/His Vector Map [Invitrogen, 2010a]. Features of the vector include a polyhistidine tag (6x His), arabinose induced promoter (pBAD) for tight, dose dependent protein expression, ampicillin resistance for selection and maintenance, and multiple cloning site (MCS). The vector is produced in three different reading frames (A, B, and C).

Aims of this Chapter

- To construct a CPGRP-NirFP fusion hybrid gene encoding a fusion protein.
- To insert the bacterial binding gene encoding CPGRP-NirFP into the *P. pastoris* chromosome for extracellular secretable expression.
- To express and purify secreted CPGRP-NirFP from *P. pastoris*.
- To investigate CPGRP-NirFP binding specificity and characteristics.
- To construct two plasmids containing the genes encoding CPGRP and a second bacterial binding protein SP-D for expression in *E. coli*.
- To express and purify CPGRP and SP-D from *E. coli*.
- To characterise CPGRP and SP-D binding specificity and characteristics.

Results

CPGRP Gene Synthesis for Insertion into *Pichia pastoris*

This was a two stage process which involved inserting the gene encoding CPGRP into a *P. pastoris* plasmid and then constructing a hybrid between this and the gene encoding the NirFP.

As the first stage, the bacterial binding gene was synthesised by GeneArt (Invitrogen). CPGRP was codon optimised for *P. pastoris* and cloned into pPICZ α B to give the following arrangement:

EcoR1 – [His₈ – Kpn1 – Xho1 – SacII – Not1 – CPGRP] – Xba1

Codon optimisation is necessary to ensure good translation rates and accuracy for ideal protein expression. The signal sequence which allows for secretable protein production is already present within the pPICZ α B plasmid. The Kozak sequence is located before the α -factor signal sequence.

This plasmid does not contain a yeast origin of replication thus transformants can only be isolated if recombination occurs between the plasmid and the *P. pastoris* chromosome.

The plasmid was digested with SacII to verify the plasmid size of 4172bp by gel electrophoresis (Figure 5.6). DNA Sequencing was also carried out to validate the insert (data not shown).

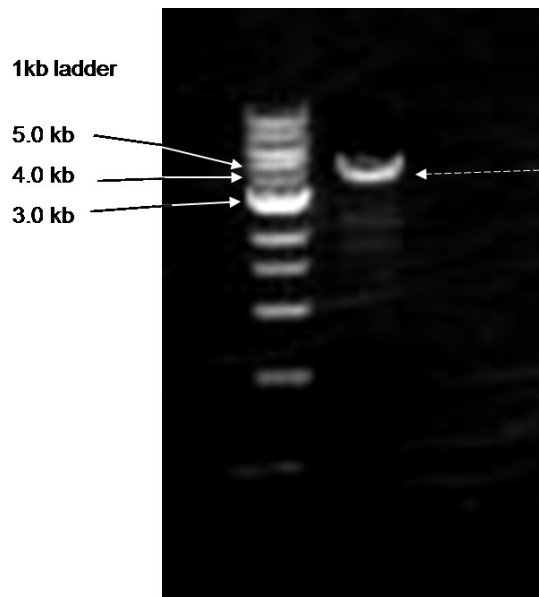


Figure 5.6: Verification of pCPGRP size. Samples of plasmid CPGRP digested with *Sac*II separated by agarose gel (0.8 w/v) electrophoresis in TAE buffer at 90 V for approximately 1 hour. Lanes (from LHS) are: 1 kb DNA ladder and pCPGRP. The linearised 4172 bp band is indicated (dashed line with arrowhead).

Construction of a Sequence Encoding a His₈-CPGRP-NirFP Fusion Protein

In order to discover and image bacterial infections the bacterial binding protein has to be detectable. The best way in which this can be done is through the use of a fluorescent protein. A Near Infrared Fluorescent Protein (NirFP) was selected because of its long wavelength emission range, high photostability and high pH stability. After extraction from its parent plasmid the NirFP gene sequence was inserted into the pCPGRP plasmid to create pCPGRP-NirFP which when inserted into *P. pastoris* KM71wt and expressed should yield a fluorescently tagged bacterial binding protein. Figure 5.7 details the order of experiments carried out.

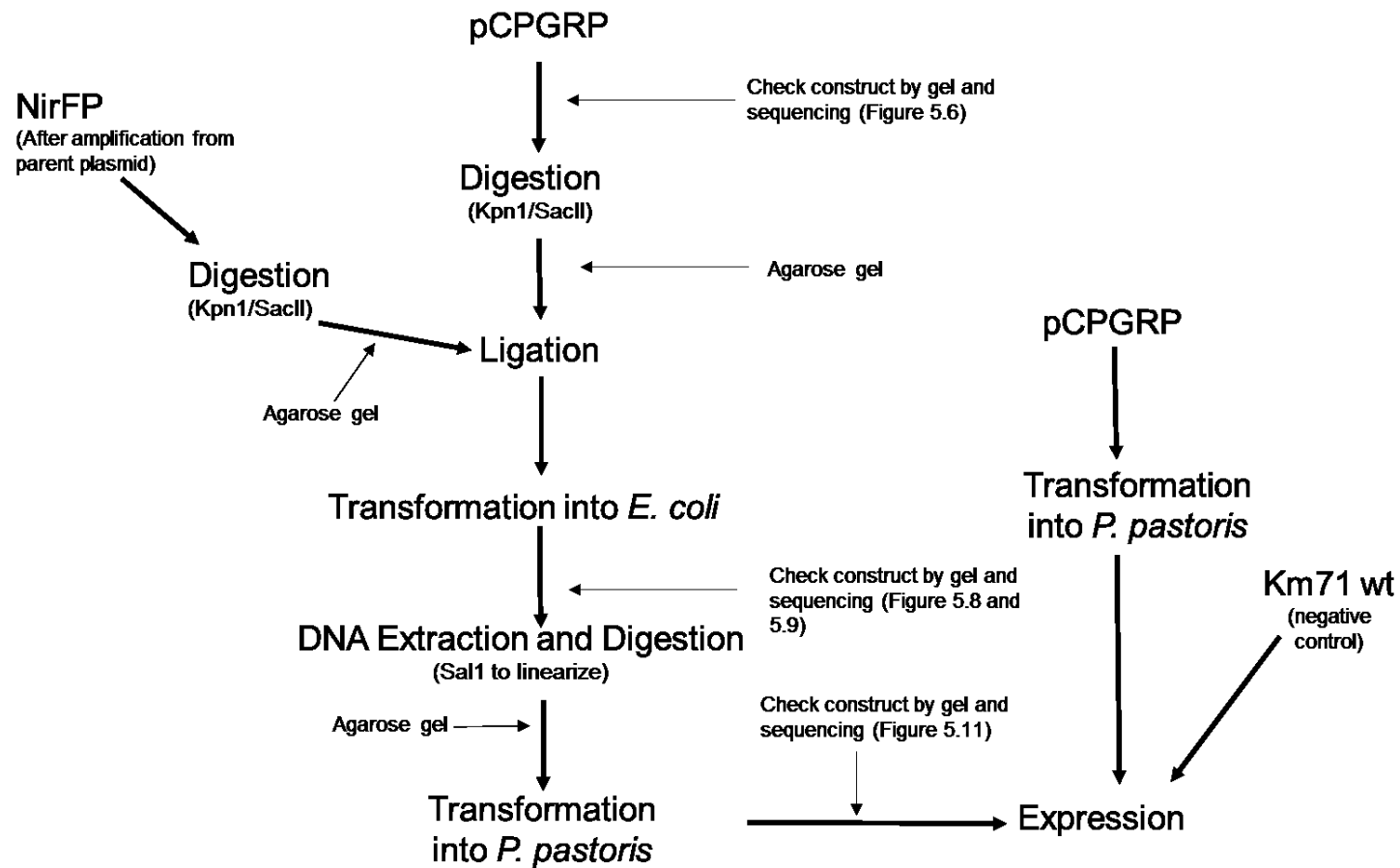


Figure 5.7: Order of Experiments from pCPGRP Gene Synthesis to Protein Expression.

PCR, with primers J and K, (Table 2.3) was used to amplify the NirFP gene sequence from its parent plasmid and incorporate KpnI and SacII sites onto the 5' and 3' ends respectively. Colony PCR, using primers J and K (see Table 2.3) was used to screen for transformants (Figure 5.8). Once possible pCPGRP-NirFP clones had been identified, they were grown overnight and DNA was extracted via Qiagen Miniprep Kits according to the manufacturer's instructions.

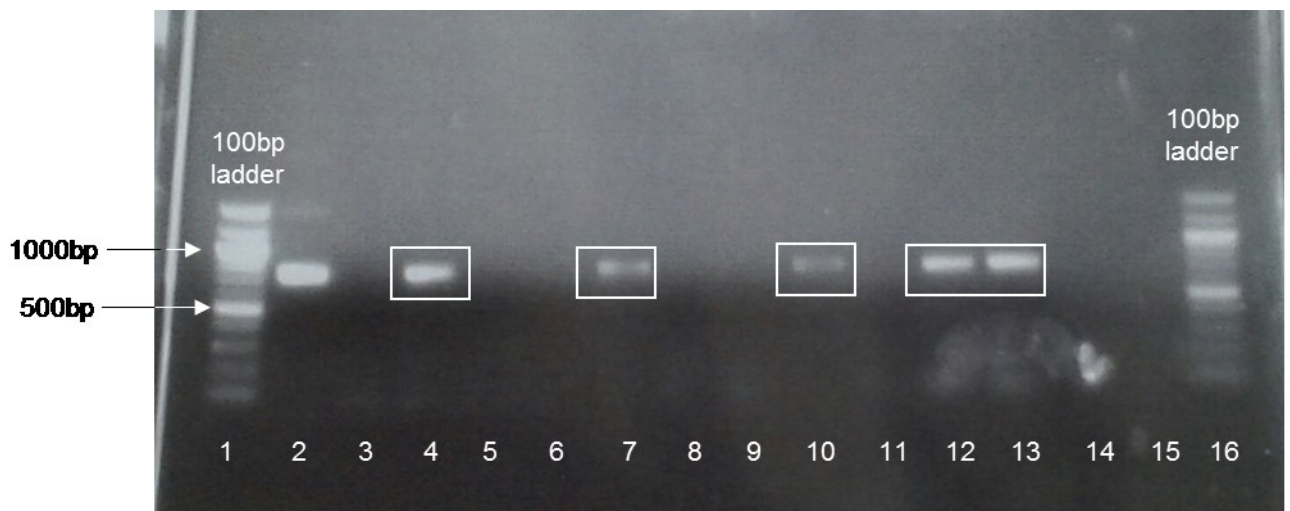


Figure 5.8: Results of Colony PCR of pCPGRP-NirFP transformants. Samples of possible NirFP clones from patch plates were separated by agarose gel (0.8 w/v) electrophoresis in TAE buffer at 120 V for approximately 1½ hours. Lanes 1 to 16 (from LHS) are as follows. Lanes 1 and 16: 100 bp Ladder. Lane 2 contains a NirFP positive control (PCR template was parent plasmid) Lanes 4, 7, 10, 12 and 13 (as indicated by the boxes) contain the correct NirFP gene sequence size at 788 bp.

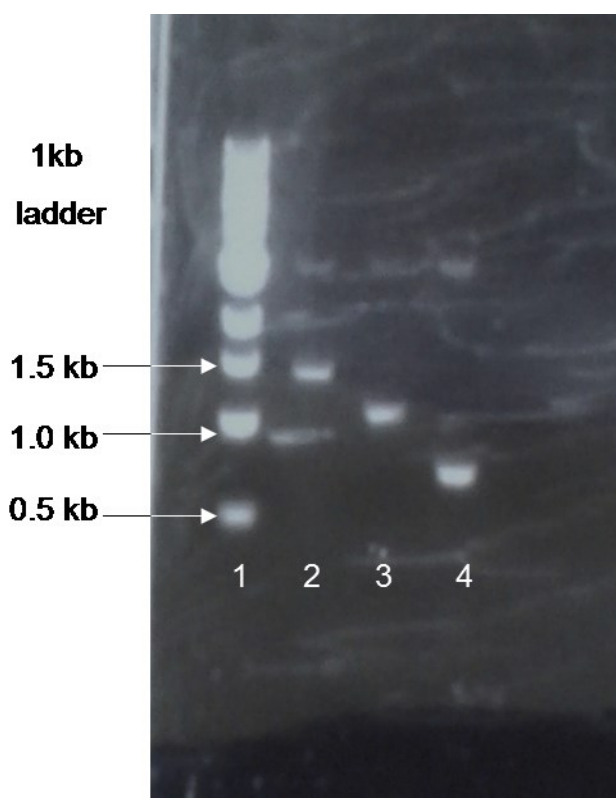


Figure 5.9: Results of PCR of Possible pCPGRP-NirFP Clones with Different Primer Combinations. Possible pCPGRP-NirFP clone DNA was extracted with Qiagen miniprep kits used according to the manufacturer's instructions. PCR was carried out with different primer combinations and was then separated by agarose gel (0.8 w/v) electrophoresis in TAE at 90V for approximately 1 hour. Lanes 1 to 4 (from LHS) are as follows. Lane 1: 1 kb Ladder. Lane 2: NirFP Forward (primer J) and CPGRP Reverse (primer H). Lane 3: NirFP Forward (Primer J) and CPGRP sequence middle reverse (primer G). Lane 4: NirFP Forward (Primer J) and NirFP Reverse (Primer K).

PCR was carried out with various primer combinations, the different sized bands which can be seen in Figure 5.9 show that both the CPGRP and NirFP gene sequences are present within the plasmid. The largest band size (lane 2) is the complete fusion protein gene sequence (1420 bp) and the smallest (lane 4) is the NirFP gene sequence (788 bp). Sequencing was used to confirm the presence of these gene sequences (data not shown). Figure 5.10 shows the final vector construct. Correct clones were stored as freezer permanents at -80°C (glycerol stocks).

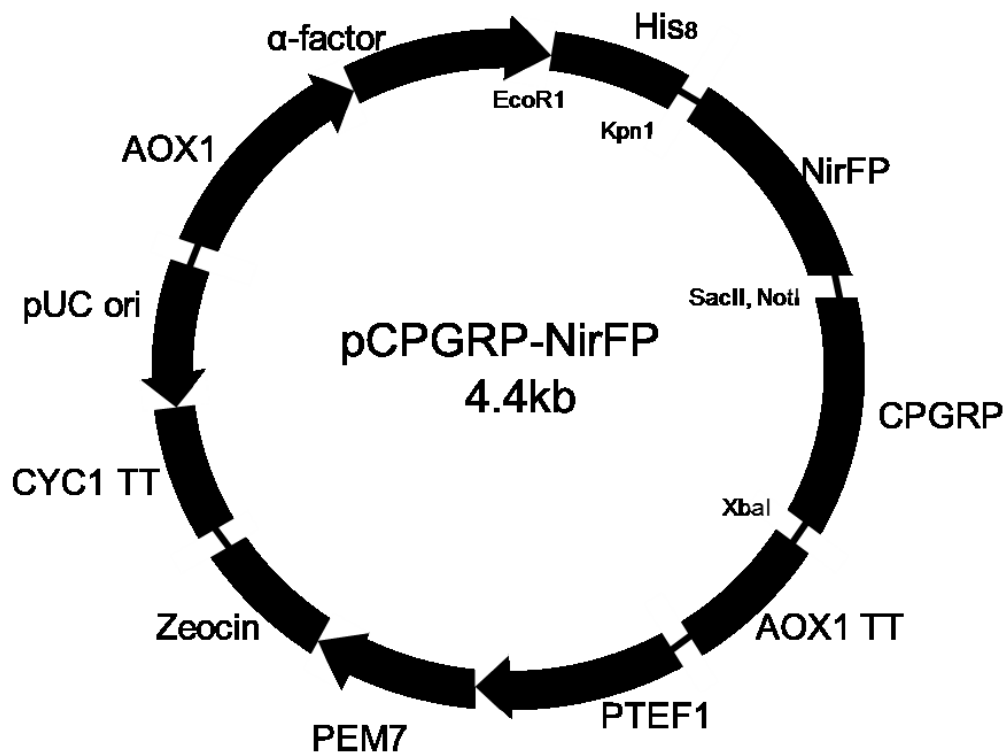


Figure 5.10: Final Vector Construct of pCPGRP-NirFP. Vector features which should be noted are α -factor secretion signal, AOX1 which permits methanol inducible, high level gene expression and the His₈ tag which allows easier protein purification. Zeocin is used for selection and maintenance in *E. coli* (25 μ g/ml in low salt LB) and selection in *P. pastoris* (100 μ g/ml in YPD).

Once the correct construct had been confirmed it was transformed into *P. pastoris*, as was pCPGRP (Figure 5.7). Once *P. pastoris* transformants were obtained, DNA was extracted using the Promega Wizard Genomic DNA Purification Kit from overnight cultures grown at 30°C, 200rpm, and sequenced (Figure 5.11).


```

TTC GAA ACG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC
-F - E - T - M - R - F - P - S - I - F - T - A - V - L - F - A - A - S - S
GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT
-A - L - A - A - P - V - N - T - T - T - E - D - E - T - A - Q - I - P - A
GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA
-E - A - V - I - G - Y - S - D - L - E - G - D - F - D - V - A - V - L - P
TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT
-F - S - N - S - T - N - N - G - L - L - F - I - N - T - T - I - A - S - I
GCT GCT AAA GAA GAA GGG GTA TCT CTC GAG AAA AGA GAG GCT GAA GCT GCA GGA ATT
-A - A - K - E - E - G - V - S - L - E - K - R - E - A - E - A - A - G - I
CAA CAT CAC CAT CAC CAC CAT CAT CAT GGT ACC ATG GGA GAG GAT AGC GAG CTG ATC
-Q - H - H - H - H - H - H - H - H - G - T - M - G - E - D - S - E - L - I
TCC GAG AAC ATG CAC ACG AAA CTG TAC ATG GAG GGC ACC GTG AAC GGC CAC CAC TTC
-S - E - N - M - H - T - K - L - Y - M - E - G - T - V - N - G - H - H - F
AAG TGC ACA TCC GAG GGC GAA GGC AAG CCC TAC GAG GGC ACC CAG ACC TGT AAG ATC
-K - C - T - S - E - G - E - G - K - P - Y - E - G - T - Q - T - C - K - I
AAG GTG GTC GAG GGC GGC CCT CTC CCC TTC GCC TTC GAC ATC CTG GCT ACC AGC TTC
-K - V - V - E - G - G - P - L - P - F - A - F - D - I - L - A - T - V - L - P
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-M - Y - G - S - K - T - F - I - N - H - T - Q - G - I - P - D - F - F - K
CAG TCC TTC CCT GAG GGC TTC ACA TGG GAG AGG ATC ACC ACA TAC GAA GAC GGG GGC
-Q - S - F - P - E - G - F - T - W - E - R - I - T - Y - E - D - G - G
GTG CTG ACC GCT ACC CAG GAC ACC AGC CTC CAG AAC GGC TGC CTC ATC TAC AAC GTC
-V - L - T - A - T - Q - D - T - S - L - Q - N - G - C - L - I - Y - N - V
AAG ATC AAC GGG GTG AAC TTC CCA TCC AAC GGC CCT GTG ATG CAG AAG AAA ACA CTC
-K - I - N - G - V - N - F - P - S - N - G - P - V - M - Q - K - T - L
GGC TGG GAG GCC AAC ACC GAG ATG CTG TAC CCC GCT GAC AGC GGT CTG AGA GGC CAT
-G - W - E - A - N - T - E - M - L - Y - P - A - D - S - G - L - R - G - H
AAT CAG ATG GCC CTG AAG CTC GTG GGC GGC GGC TAC CTG CAC TGC TCC CTC AAG ACC
-N - Q - M - A - L - K - L - V - G - G - Y - L - H - C - S - L - K - T
ACA TAC AGA TCC AAG AAA CCC GCT AAG AAC CTC AAG ATG CCC GGC TCC TAC TTC GTG
-T - Y - R - S - K - K - P - A - K - N - L - K - M - P - G - F - Y - F - V
GAC CGT AAA CTG GAA AGA ATC AAG GAG GCC GAC AAA GAG ACC TAC GTC GAG CAG CAC
-D - R - K - L - E - R - I - K - E - A - D - K - E - T - Y - V - E - Q - H
GAG ATG GCT GTG GCC AGG TAC TGC CCT AGC AAA CTG GGC GAC CAC AGC GGC CGC
-E - M - A - V - A - R - Y - C - D - L - P - S - K - L - G - H - S - G - R
GGC GGC CGC ATG ACT AGA CAT TGT GTT TTG TTG GTT TGG GCT TTG TTG GCT TTG TTG
-G - G - R - M - T - R - H - C - V - L - L - V - W - A - L - L - A - L - L
TCC TTG GGT GCT GCT AGA GAA GAT CCA CCA GCT TGT GGT TCT ATC GTT CCT AGA AGA
-S - L - G - A - A - R - E - D - P - P - A - C - G - S - I - V - P - R - R
GAG TGG AGA GCT TTG GCT TCT GAG TGT AGA GAG AGA TTG ACT AGA CCA GTT AGA TAC
-E - W - R - A - L - A - S - E - C - R - E - R - L - T - R - P - V - R - Y
GTT GTT TTT TCC CAC ACT GGT TCC CAC TGT GAT ACT CCA GCT TCA TGT GCT CAA
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CAG GCT CAA AAC GTT CAG TCC TAC CAC GTT AGA AAC TTG GGT TGG TGT GAC GTT GGT
-Q - A - Q - N - V - Q - S - Y - H - V - R - N - L - G - W - C - D - V - G
TAC AAC TTC TTG ATT GGT GAG GGC GGT TTG GTT TAC GAG GGT AGA GGT TGG AAC ATT
-Y - N - F - L - I - G - E - D - G - L - V - Y - E - G - R - G - W - N - I
AAG GGT GCT CAT GCT GGT CCA ACT TGG AAC CCA ATT TCC ATC GGT ATT TCT TTC ATG
-K - G - A - H - A - G - P - T - W - N - P - I - S - I - G - I - S - F - M
GGT AAC TAC ATG AAC AGA GTT CCA CCA CCA GCT TTG AGA GCT GCT CAG AAC TTG
-G - N - Y - M - N - R - V - P - P - P - R - A - L - R - A - A - Q - N - L
TTG GCT TGT GGT GTT GCT TTG GGT GCT TTG AGA TCC AAC TAC GAG GTT AAG GGT CAC
-L - A - C - G - V - A - L - G - A - L - R - S - N - Y - E - V - K - G - H
AGA GAT GTT CAG CCA ACT TTG TCT CCA GGT GAC AGA TTG TAC GAG ATC CAA ACT
-R - D - V - Q - P - T - L - S - P - G - D - R - L - Y - E - I - I - Q - T
TGG TCC CAC TAC AGA GCT TAA TCT AGA
-W - S - H - Y - R - A -STOP

```

Figure 5.11: Gene Sequence of CPGRP-NirFP. CPGRP was inserted into pPICZαB using EcoRI and XbaI (indicated in yellow). KpnI and SacII (indicated in pink) were used to clone the NirFP gene into pCPGRP. The region encoding the poly-His tag is shown in blue. The start and stop codons are indicated in green. The purple text represents the α-factor secretion signal, the grey highlighted region directly before is the Kozak sequence. The corresponding protein sequence (which contains no stop codons) is presented in single letter code.

Expression and Detection of CPGRP-NirFP Fusion Protein in *Pichia pastoris*

The first *Pichia pastoris* expression protocol used consisted of inoculating 25 ml BMGY (See Chapter 2) with a single colony and growing at 30°C until OD₆₀₀ was between 2 and 6. Once the correct OD has been achieved the cells were harvested by spinning at 1500 xg for 5 minutes and then re-suspended in 100ml BMMY (see Chapter 2) medium to induce expression. 100% methanol was added to a final concentration of 0.5% (v/v) every 24 hours to maintain expression. A more detailed protocol can be found in the manual [Invitrogen, 2010c].

Samples were taken at 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 hours, spun down (14000 rpm, 2 minutes) to separate the supernatant and pellet and frozen at -80°C until required. Three strains, (KM71, and the two cultures containing the CPGRP and CPGRP-NirFP genes) were set up to run concurrently. KM71 is the wild type strain which was included as a negative control. CPGRP was included to investigate whether the inclusion of the NirFP would affect the expression and characteristics of the binding protein.

The growth, expression and sample times of the cultures were altered to attempt to produce and detect the bacterial binding proteins. Expression was induced between a wide range of culture density (OD₆₀₀ 0.5 to 6), and the cultures were sampled more regularly (every hour for the first 12, then every 6 hours for 96). Both the cell pellet (after lysis) and the supernatant were tested for the bacterial binding protein though the nature of expression should have been extracellular (data not shown).

When this did not work the method of the Barlow Lab (who supplied the original strains and plasmids) was adopted. This involved taking a single colony, inoculating 5ml BMGY, and growing the culture at 30°C, 150 rpm for 24 hrs. 95 ml BMGY was added and the culture was grown for a further 60 hours. The cells were harvested by spinning at 1500xg for 5 minutes and resuspended in 25 ml BMMY. The culture was incubated at a reduced temperature of 18°C for 5 days. 125 ml methanol was added once on the second and third days, then twice on the fourth day (in the morning and evening). The culture was harvested on the fifth day by spinning down at 1500xg for 5 minutes, both the supernatant and pellets were stored separately at -80°C until required.

A positive control G1107C, supplied by the Barlow lab, was incorporated into the experiment. When testing for the binding protein through SDS-PAGE gels there was no detectable protein. Cell pellets and supernatants were boiled for 10 minutes and

visualised by SDS-PAGE gels as described in Chapter 2. In other experiments 125 ml sorbitol was added to the culture alongside the methanol. This approach also did not prove to be successful (Figure 5.12).

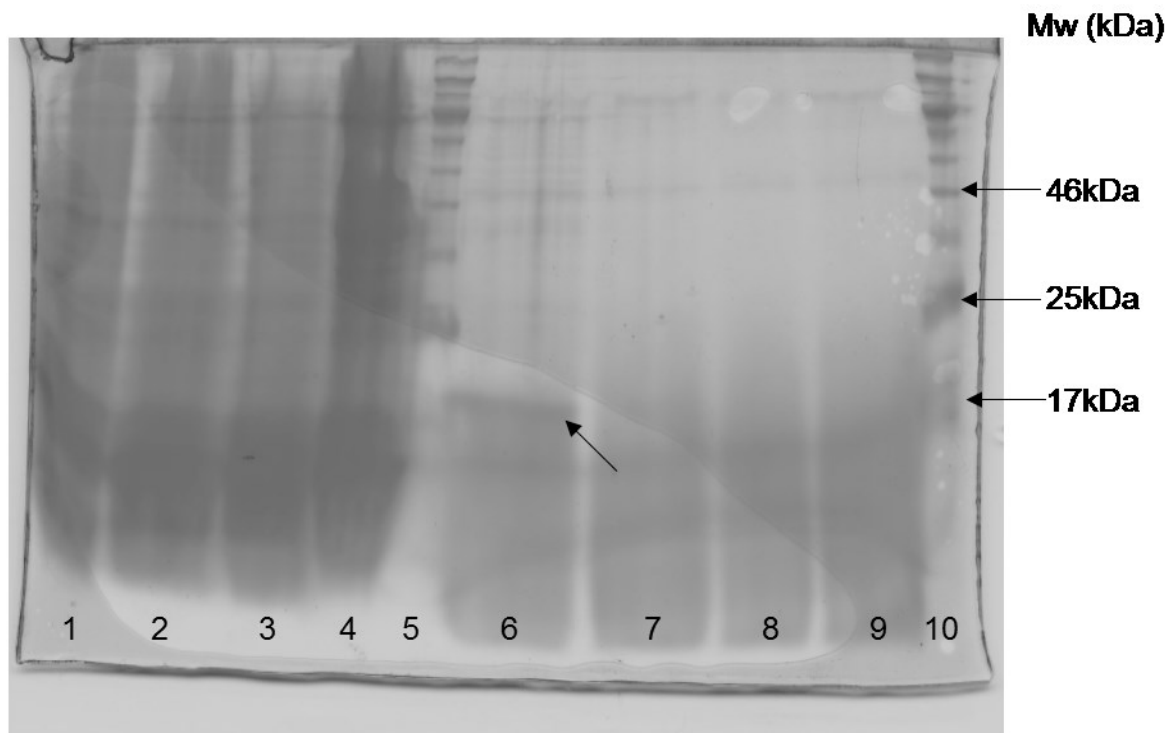


Figure 5.12: SDS-PAGE Gel showing *Pichia pastoris* Expression of Proteins in both Cell Pellets and Supernatants. Expression was carried out according to the Barlow method. After lysis the cell pellet and supernatant were run on SDS-PAGE gels. Lanes 1-4 contain G1107C, KM71 (wt), CPGRP and CPGRP-NirFP whole cell pellets. Lanes 5 and 10 contain the prestained broad range protein marker (7-175kDa) and lanes 6-9 contain G1107C, KM71 (wt), CPGRP and CPGRP-NirFP supernatant samples. The arrow indicates the positive control band from G1107C supernatant.

In further approaches, similar samples of 50ml supernatant were concentrated through Centricon Centrifugal Filter Devices Plus-70 (10K) to test if the protein was produced at a low level. The Barlow protocol was used twice and the harvested supernatant was combined to give the required amount for the filter devices. The 50 ml supernatant was concentrated down to 400 μ l through the filters in a swinging bucket centrifuge at 3500xg for 30 minutes. However, still no secreted protein was detected (data not shown).

As an additional tactic, the expression supernatant was purified on Ni-NTA Magnetic Agarose Beads using the poly-His tag on the plasmid. This process did not return any results (Figure 5.13).

After harvesting the expression supernatant on the fifth day, 5ml was incubated at 4°C with 50 µl bead resin for 4 hours with rotation. A magnetic rack was used to bind the beads allowing the supernatant to be removed. The beads were resuspended and washed in buffers (See Chapter 2) containing NaCl which reduces non-specific binding, as well as Tween and β-mercaptoethanol which prevents the beads and proteins sticking. 50 µl of elution buffer containing high levels of imidazole was used to displace any His-bound fusion protein.

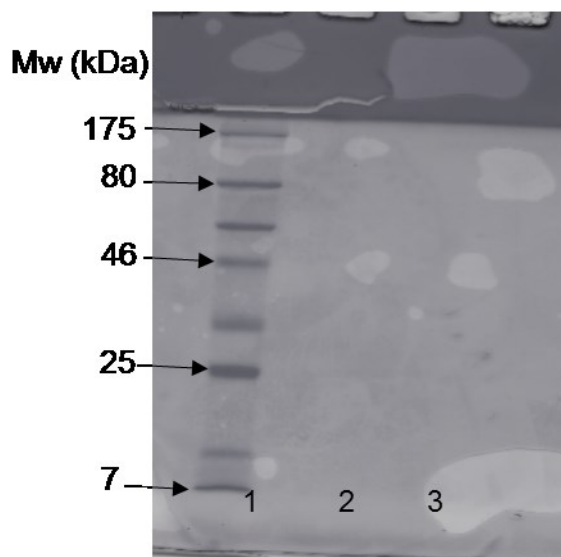


Figure 5.13: *Pichia pastoris* CPGRP and CPGRP-NirFP Supernatant Purified on Ni-NTA Magnetic Beads. CPGRP and CPGRP-NirFP expression was carried out according to the Barlow method, and purified on Ni-NTA beads before been run on a SDS-PAGE gel. Lane 1: Protein Marker, Lane 2: CPGRP and Lane 3: CPGRP-NirFP. 10 µl of each sample was loaded.

Inclusion into the Study of a Second Bacterial Binding Protein: Surfactant Protein D

With no detectable expression of either CPGRP or CPGRP-NirFP using the *P. pastoris* system the decision was taken to explore whether the CPGRP protein could be produced using a bacterial expression system.

The gene encoding a bacterial binding second protein, SP-D, was also synthesised for expression, to provide a comparison with CPGRP. SP-D was selected for use as there is a lot of available knowledge surrounding SP-D and its structure and function. The shape and orientation of the molecule is known, the amino acid sequence is available and it has been expressed previously by another group [Crouch *et al.*, 2005; Crouch *et al.*, 2006; Crouch *et al.*, 2009].

pBAD/His A [Invitrogen, 2010a] was used for the vector in both cases. This is an arabinose inducible vector with a built in His-tag (indicated in blue in Figure 5.14A and B). SacI and EcoRI (Figure 5.14A and B, yellow) were used to insert the gene sequences into the vector. A ribosome binding site (highlighted in grey in Figure 5.14A and B) is present in the vector. Both CPGRP and SP-D were codon optimised for expression in *E.coli* and synthesised by GeneArt (Invitrogen) The NirFP was not included in these experiments. After transformation into *E. coli* the genes were sequenced (Figure 5.14A and B).

```

CAG GAG GAA TTA ACC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC
-Q - E - E - L - T - M - G - G - S - H - H - H - H - H - H - G - M - A - S
ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CGA
-M - T - G - G - Q - Q - M - G - R - D - L - Y - D - D - D - D - K - D - R
TGG GGA TCC GAG CTC ATG ACC CGG CAC TGC GTG CTT CTC GTC TGG GCT CTC CTC GCC
-W - G - S - E - L - M - T - R - H - C - V - L - L - V - W - A - L - L - A
CTC CTC AGC CTC GGA GCG GCT CGA GAA GAC CCG CCG GCC TGC GGC TCC ATC GTG CCC
-L - L - S - L - G - A - A - R - E - D - P - P - A - C - G - S - I - V - P
CGC CGA GAG TGG AGG GCC CTG GCG TCC GAG TGC AGA GAA AGG CTA ACA CGG CCG GTG
-R - R - E - W - R - A - L - A - S - E - C - R - E - R - L - T - R - P - V
CGC TAC GTG GTG GTG TCG CAC ACT GCG GGC AGC CAC TGC GAC ACC CCG GCT TCG TGC
-R - Y - V - V - V - S - H - T - A - G - S - H - C - D - T - P - A - S - C
GCG CAG CAG GCC CAG AAC GTG CAA AGC TAC CAT GTG CGG AAC CTG GGC TGG TGC GAC
-A - Q - Q - A - Q - N - V - Q - S - Y - H - V - R - N - L - G - W - C - D
GTG GGC TAC AAC TTC CTG ATC GGA GAA GAT GGG CTC GTG TAC GAA GGC CGA GGC TGG
-V - G - Y - N - F - L - I - G - E - D - G - L - V - Y - E - G - R - G - W
AAT ATC AAG GGC GCC CAC GCA GGT CCC ACC TGG AAC CCC ATA TCC ATA GGC ATC TCC
-N - I - K - G - A - H - A - G - P - T - W - N - P - I - S - I - G - I - S
TTC ATG GGC AAC TAT ATG AAT CGA GTG CCC CCG CCC CGC GCC CTC CGG GCA GCC CAG
-F - M - G - N - Y - M - N - R - V - P - P - P - R - A - L - R - A - A - Q
AAT CTG CTG GCT TGT GGT GTG GCT CTG GGA GCC CTG AGA TCC AAC TAC GAG GTC AAA
-N - L - L - A - C - G - V - A - L - G - A - L - R - S - N - Y - E - V - K
GGA CAC CGG GAT GTG CAG CCG ACC CTC TCT CCA GGT GAC CGG CTC TAC GAA ATC ATC
-G - H - R - D - V - Q - P - T - L - S - P - G - D - R - L - Y - E - I - I
CAG ACT TGG TCA CAC TAC CGC GCA TGA GAATTC
-Q - T - W - S - H - Y - R - A -STOP

```

Figure 5.14a: CPGRP Gene Sequence Optimised for Expression in *Escherichia coli*. The codon optimised gene encoding CPGRP was synthesised by GeneArt (Invitrogen) and then sequenced. The poly-His tag, is shown in blue. The restriction sites, SacI and EcoRI, used for insertion are indicated in yellow. The start and stop codons are indicated in green. The grey highlighted region represents the ribosome binding site. The corresponding protein sequence (which contains no stop codons) is presented in single letter code.

```

CAG GAG GAA TTA ACC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC
-Q - E - E - L - T - M - G - G - S - H - H - H - H - H - H - G - M - A - S
ATG ACT GGT GGA CAG CAA ATG GGT CCG GAT CTG TAC GAC GAT GAC GAT AAG GAT CGA
-M - T - G - G - Q - Q - M - G - R - D - L - Y - D - D - D - D - K - D - R
TGG GGA TCC GAG CTC ATG CTG CTG TTT CTG CTG AGC GCA CTG GTT CTG CTG ACC CAG
-W - G - S - E - L - M - L - L - F - L - L - S - A - L - V - L - L - T - Q
CCG CTG GGC TAT CTG GAA GCA GAA ATG AAA ACC TAT AGC CAT CGT ACC ATG CCG AGC
-P - L - G - Y - L - E - A - E - M - K - T - Y - S - H - R - T - M - P - S
GCA TGT ACC CTG GTT ATG TGT AGC AGC GTT GAA AGC GGT CTG CCT GGT CGT GAT GGC
-A - C - T - L - V - M - C - S - S - V - E - S - G - L - P - G - R - D - G
CGT GAT GGT CGC GAA GGT CCG CGT GGT GAA AAA GGT GAT CCG GGT CTG CCA GGT GCA
-R - D - G - R - E - G - P - R - G - E - K - G - D - P - G - L - P - G - A
GCA GGT CAG GCA GGT ATG CCT GGA CAG GCA GGT CCG GTT GGT CCG AAA GGT GAT AAT
-A - G - Q - A - G - M - P - G - Q - A - G - P - V - G - P - K - G - D - N
GGT AGC GTT GGT GAA CCG GGT CCT AAA GGC GAT ACC GGT CCG AGC GGT CCT CCG GGT
-G - S - V - G - E - P - G - P - K - G - D - T - G - P - S - G - P - P - G
CCT CCT GGT GTT CCT GGT CCG GCA GGT CGT GAA GGA CCG CTG GGT AAA CAG GGT AAT
-P - P - G - V - P - G - P - A - G - R - E - G - P - L - G - K - Q - G - N
ATT GGT CCG CAG GGT AAA CCG GGA CCA AAA GGT GAA GCC GGA CCG AAA GCA GGT
-I - G - P - Q - G - K - P - G - P - K - G - E - A - G - P - K - G - E - V
GGT GCA CCG GGT ATG CAG GGT AGT GCC GGT GCA CGT GGT CTG GCA GGC CCT AAA GGT
-G - A - P - G - M - Q - G - S - A - G - A - R - G - L - A - G - P - K - G
GAA GGT GGC GTT CCG GGT GAA CGC GGT GTT CCA GGT AAT ACC GGT GCA GCC GGT TCA
-E - R - G - V - P - G - E - R - G - V - P - G - N - T - G - A - A - G - S
GCG GGT GCA ATG GGT CCT CAG GGC TCA CCG GGT GCT CGT GGT CCG CCT GGT CTG AAA
-A - G - A - M - G - P - Q - G - S - P - G - A - R - G - P - P - G - L - K
GGC GAC AAA GGT ATT CCT GGT GAT AAA GGC GCA AAA GGC GAA TCA GGT CTG CCG GAT
-G - D - K - G - I - P - G - D - K - G - A - K - G - E - S - G - L - P - D
GTT GCA AGC CTG CGT CAG CAG GTT GAA GCA CTG CAG GGT CAG GTT CAG CAT CTG CAG
-V - A - S - L - R - Q - Q - V - E - A - L - Q - G - Q - V - Q - H - L - Q
GCA GCA TTT AGC CAG TAC AAA AAA GTT GAA CTG TTT CCG AAT GGT CAG AGC GTT GGC
-A - A - F - S - Q - Y - K - K - V - E - L - F - P - N - G - Q - S - V - G
GAA AAA ATC TTT AAA ACC GCA GGT TTT GTG AAA CCG TTT ACC GAA GCA CAG CTG CTG
-E - K - I - F - K - T - A - G - F - V - K - P - F - T - E - A - Q - L - L
TGT ACC CAG GCA GGC GGT CAG CTG GCA AGT CCG CGT AGC GCA GAA AAT GCA GCC
-C - T - Q - A - G - G - Q - L - A - S - P - R - S - A - A - E - N - A - A
CTG CAG CAG CTG GTT GTT GCA AAA AAT GAA GCA GCA TTT CTG AGC ATG ACC GAT AGC
-L - Q - Q - L - V - V - A - K - N - E - A - A - F - L - S - M - T - D - S
AAA ACC GAA GGC AAA TTT ACC TAT CCG ACC GGT GAA AGC CTG GTT TAT AGC AAT TGG
-K - T - E - G - K - F - T - Y - P - T - G - E - S - L - V - Y - S - N - W
GCA CCA GGT GAA CCG AAT GAT GAT GGT GGT AGC GAA GAT TGT GTT GAA ATC TTT ACC
-A - P - G - E - P - N - D - D - G - G - S - E - D - C - V - E - I - F - T
AAT GGC AAA TGG AAT GAT CGT GCA TGC GGT GAA AAA CGT CTG GTT GTT TGT GAA TTC
-N - G - K - W - N - D - R - A - C - G - E - K - R - L - V - V - C - E - F
TAA GAA TTC
-STOP

```

Figure 5.14b: SP-D Gene Sequence Optimised for Expression in *Escherichia coli*. The codon optimised gene encoding SP-D was synthesised by GeneArt (Invitrogen) and then sequenced. The poly-His tag, is shown in blue. The restriction sites used for insertion, SacI and EcoRI, are indicated in yellow. The start and stop codons are indicated in green. The grey highlighted region represents the ribosome binding site. The corresponding protein sequence (which contains no stop codons) is presented in single letter code.

Protein Expression in *Escherichia coli*

Four bacterial strains were used for the expression of the bacterial binding proteins, DH5 α , Top10, BL21 and Rosetta-gami 2. Genotypes are detailed in Chapter 2. This was done to optimise the chances of protein expression. EHPT1, which is a His-tagged protein was used as a positive control. This is an arabinose induced protein which was constructed by a previous Gallagher lab Ph.D. student.

For expression, a single colony was used to inoculate 5 ml LB supplemented with 50 $\mu\text{g/ml}$ ampicillin, and grown overnight at 37°C until OD₆₀₀ was between 1 and 2. 10 ml LB-amp was then inoculated with 0.1ml of the overnight culture and grown at 37°C, 200rpm, until OD₆₀₀ =0.5. Arabinose was added to the cultures to induce expression. Samples were taken at 0 and 4 hours. Samples were centrifuged at top speed in a table top centrifuge for 1 minute to remove the supernatant, and the pellets were stored at -20°C until required. The bacterial binding proteins were tested for using SDS-PAGE gels and Western Blots. Cell pellets and supernatants were boiled for 10 minutes and visualised by SDS-PAGE gels. Separated proteins were transferred onto nitrocellulose membranes for the Western Blot through a semi-wet transfer (BioRad). The antibody used was an anti-His-HRP at a 1:500 dilution in blocking solution.

Initial arabinose concentrations suggested by Invitrogen [2010a] for induction are 0.2% (v/v), 0.02% (v/v), 0.002% (v/v), 0.0002% (v/v) and 0.00002% (v/v). After preliminary experiments arabinose concentrations for induction and sampling times were optimised through testing various concentrations and sampling more often (Figure 5.15). The final arabinose concentration used was 0.4% with induction at OD₆₀₀ 0.3. Experiments revealed that DH5 α , BL21 and Top10 did not produce detectable protein. Representative examples of SDS-PAGE gels are illustrated (Figure 5.15). Samples were also transferred to nitrocellulose membrane and except for the positive control the corresponding western blots did not shown any fluorescence indicating that the proteins were not produced (data not shown). Thus efforts were concentrated on the Rosetta-gami 2 strain (Figure 5.16).

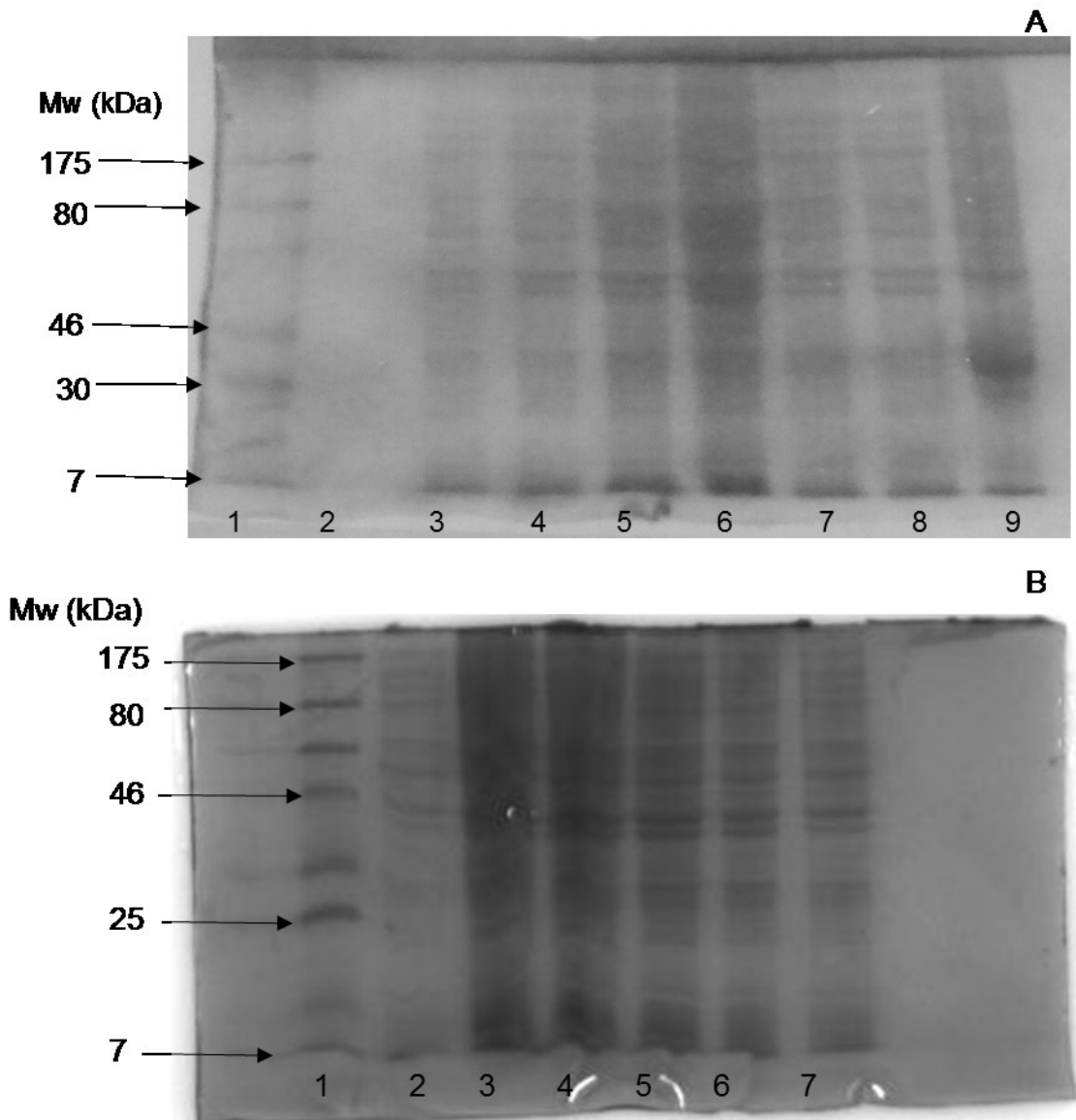


Figure 5.15: A) CPGRP Expression in TOP10 with Different Arabinose Concentrations. The culture containing pCPGRP in TOP10 was grown to an OD_{600} of 0.4 (37°C, 200 rpm) before being induced at different arabinose concentrations. Samples were taken 4 hours after induction and run on a SDS-PAGE gel. Lane 1 contains the protein marker. Lane 2 the 0 hour sample (before arabinose addition). Lanes 3-7 are samples induced at 0.0002% (v/v), 0.0002% (v/v), 0.002% (v/v), 0.02% (v/v) and 0.2% (v/v) arabinose.

B) CPGRP expression in DH5α. The culture containing pCPGRP in DH5α was grown to an OD_{600} of 0.4 at 37°C, 200 rpm, before being induced to a final concentration of 0.2% (v/v) arabinose. Samples were then taken at different time points and run on a SDS-PAGE gel. Lane 1 contains the protein marker, lane 2 is empty. Lane 3 is 0hrs (before arabinose addition). Lanes 4-8 are samples taken at 1, 2, 3, 4, and 6 hours. The positive control of EHPT1 is shown in lane 9.

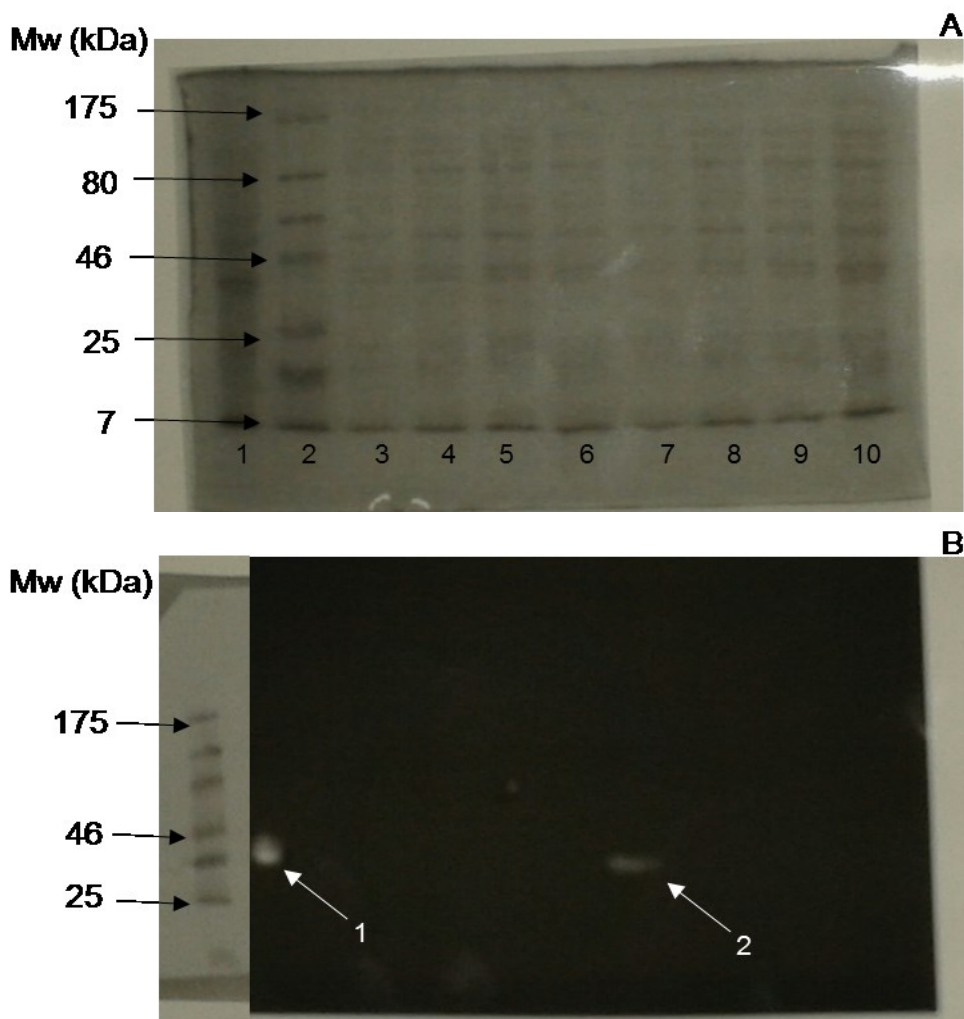


Figure 5.16: SDS-PAGE gel (A) and Western Blot (B) of Expression of CPGRP and SP-D from Rosetta-gami 2. The cultures were grown to an OD_{600} of 0.4 (37°C, 200 rpm) before being induced to a final concentration of 0.2% (v/v) arabinose. Samples were taken at specific time points and run on a SDS-PAGE gel. The positive control of EHPT1 is shown in lane 1. Lane 2 contains the protein marker. Lanes 3-6 contain CPGRP cultures, sampled at 0, 1, 2, and 4 hours. Lanes 7-10 contain SP-D samples taken at 0, 1, 2, and 4hrs after induction. For the Western Blot (B) the His-tagged positive control is indicated (1) and slight expression of SP-D can be seen (2). The proteins were transferred to a nitrocellulose membrane through a semi-wet transfer system (Biorad). A peroxidase-conjugated anti-His₆ antibody, (1:500 dilution in blocking buffer) was used and images were recorded with an Epi Chem II Darkroom under no-light conditions for 5 minutes.

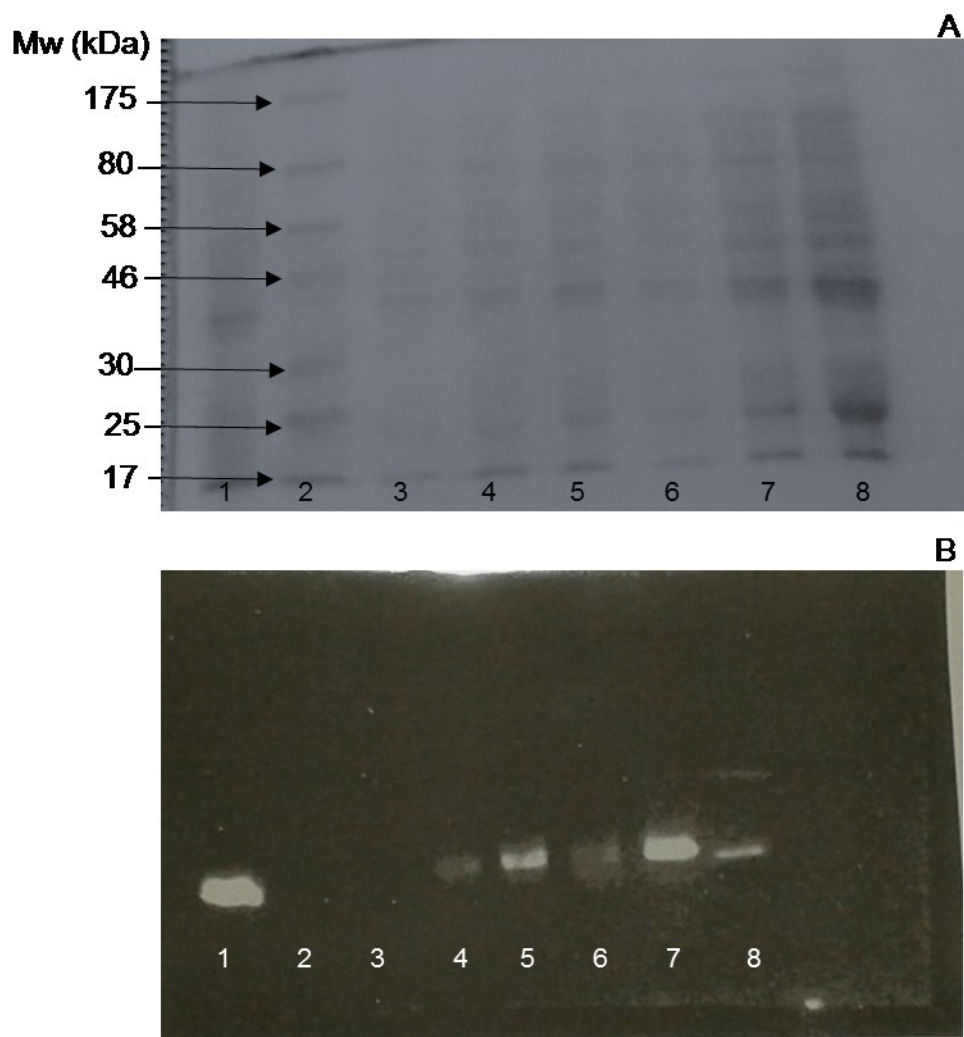


Figure 5.17: SDS-PAGE gel (A) and Western blot (B) of SP-D expression in Rosetta-Gami 2. The culture containing SP-D was grown to an OD_{600} of 0.3 (37°C, 200 rpm) before being induced to a final concentration of 0.4% (v/v) arabinose. Samples were then taken at specific time points and run on a SDS-PAGE gel (A) as well as being transferred onto a nitrocellulose membrane (B). The His-tagged positive control of EHPT1 is shown in Lane 1. Lane 2 contains the protein marker and lane 3 the 0hr sample. Lanes 4-8 are samples taken 2, 4, 6, 8, 24 hrs after induction. A peroxidase-conjugated anti-His₆ antibody, (1:500 dilution) was used for the Western Blot and images were recorded with an Epi Chem II Darkroom under no-light conditions for 5 minutes. The Display range of the image was adjusted to visualise the bands.

At no point during the experiments was CPGRP visible or detected. SP-D was observed to be expressed by the Rosetta-Gami 2 strain. However, the amount produced was extremely low, all the bands were only visible when the display range was adjusted after the photograph was captured. Thus the Western Blot (Figure 5.17) can be considered to be overexposed in order to detect the bands. It was decided that enough protein could not be produced to be characterised further.

Discussion

The yeast *P. pastoris* is well documented for use as a yeast expression system since its development in the 1970s [Cereghino and Cregg, 1999]. A wide variety of proteins have been expressed including *C. difficile* neurotoxin [Byrne *et al.*, 1998], human interleukin-17 [Eldin *et al.*, 1998], equine lactoferrin [Paramasivam *et al.*, 2002], large T antigen from Polyomavirus [Peng and Acheson, 1997] and glucoamylase from *Aspergillus awamori* [Fierobe *et al.*, 1997].

Advantages of using *P. pastoris* as an expression system include the ability to perform eukaryotic protein modifications including proteolytic processing, glycosylation, protein folding and disulphide bond formation [Macauley-Patrick *et al.*, 2005]. The system is also tightly regulated by the promoter *AOX1* which only induces protein production in the presence of methanol [Celik and Calik, 2011]. A secondary control mechanism also exists regarding growth on glucose and glycerol. Growth on glucose represses transcription even in the presence of methanol. Only when the yeast is grown in glycerol (and methanol) will there be optimal induction of the *AOX1* promoter and best possible expression levels of the fusion protein [Cereghino *et al.*, 2002].

CPGRP was originally discovered by Kappeler *et al.*, [2004] in camels' lactating mammary glands. Crystallization of the protein by Sharma *et al.* [2008] revealed its structure and provided details about its binding properties and characteristics. All previous work characterising this protein was carried out after purification from camel's milk [Sharma *et al.*, 2008; Sharma *et al.*, 2011a] and has in fact never been artificially synthesised and expressed.

P. pastoris is considered a first-rate expression system for production of proteins from multiple sources. However, it has been suggested that the feasibility of production appears to be target-protein dependent [Liu *et al.*, 2013]. Codon optimization is an important step to improve translational fidelity to ensure optimal expression of proteins [Hutterer *et al.*, 2012]. This was carried out on CPGRP for this study. Reducing the expression temperature has also been shown to improve protein production [Yang *et al.*, 2013]. However, this was not successful in this study.

Even with the use of different expression systems CPGRP was never visibly expressed or detected. Possible explanations for this could be the size of the protein, the complexity of the molecule, and the fact that it was a multimer. Proteolysis could also play a role in degrading or truncating the product [Macauley-Patrick *et al.*, 2005]. It was most likely an accumulation of factors which contributed to the non-expression of CPGRP.

When CPGRP was unable to be produced using the yeast expression system, a second bacterial binding protein, SP-D, was included in the study to provide a comparison to CPGRP. The decision was also made to move both proteins into a bacterial expression system.

E. coli physiology is well documented as this species has long been used in many aspects of molecular biology and biotechnology and is considered a reliable 'workhorse' [Berlec and Štrukelj, 2013]. The arabinose inducible expression vector, pBAD, is well characterised and considered a reliable regulator of protein production with a strong promoter and normally high levels of expression [Guzmann *et al.*, 1995]. The *E. coli* expression system is easier to use than the *P. pastoris* expression system and does not require as much incubation and expression time to produce proteins.

SP-D expression has been described in multiple papers from the same research group in both bacterial and mammalian cell expression systems [Crouch *et al.*, 2005; Crouch *et al.*, 2006; Hartshorn *et al.*, 19996; Wang *et al.*, 2008].

Once again both proteins, CPGRP and SP-D, were codon optimised for use in *E. coli* as certain condons especially for arginine are rare which causes mis-translational errors and lower protein expression [Calderone *et al.*, 1996]. However, this did not make a difference in the production of CPGRP which was never visibly detected. SP-D was produced but only at very low levels.

Production of high protein yields is vital in order to characterise and utilise a protein. The reasons behind the extremely low protein yields of SP-D are multifactorial. High-level gene expression has been shown to stress cells and adversely influence cellular physiology [Berlec and Štrukelj, 2013]. Intracellular and extracellular factors can also produce unwanted physiological consequences which may affect protein production [Chou, 2007]. Another contributing factor could be that the proteins are bacterial binding so they could be damaging the bacterial expression system when they are first produced. This may account for the non-detectable and very low levels of protein production.

Ultimately, it was decided that this project was not suitable to pursue and efforts were refocused towards other scientific goals.

Conclusion

Even though the production of these bacterial binding proteins was not successful, the idea behind the project is still sound. The possible use of a bacterial binding and fluorescent fusion proteins to detect and image bacterial infections has merit. This is especially true for difficult cases in which clinical presentation of infection and traditional laboratory tests produce unclear results leading to an unconfirmed diagnosis, as is often the case with prosthetic joint infections. However, before this could be implemented the problems encountered in this chapter would have to be overcome.

Chapter 6: Conclusion

The use of multiple different bacterial species has shown that the alginate bead method is valid for use to investigate biofilm development. Formation of biofilms by the different species appears similar, with rapid non-specific attachment followed by the development of a mature biofilm with significant resistance to different antibiotics. One of the main advantages of the alginate bead method over the Calgary Biofilm Device is the cost. The reagents and equipment required for the alginate bead method are readily available and inexpensive. However, the alginate bead method is more labour intensive compared to the Calgary Biofilm Device. Even considering these factors, the alginate bead method is a suitable alternative to the Calgary Biofilm Device. Moreover, it lends itself to more flexible assay development, for example, exploring mixed community interactions and abiotic antimicrobials.

Stable biofilms formed on the alginate beads within four hours of inoculation, though they continued to develop after this time, presumably with planktonic cells still attaching and additional replication of the biofilm based cells. This indicates that infections in clinical environments need to be identified quickly in order to have an effective treatment regimen. As the biofilms became established, antibiotic resistance developed. The time this took depended on the specific antibiotic and its mode of action as well as the bacterial species forming the biofilm. However, after eight hours incubation all species' biofilms showed significant resistance to antibiotics.

The antibiotic resistance is likely to be due to multiple reasons. Specific resistance mechanisms, such as selection of genetic mutations against the antibiotics probably only played a small role in the overall resistance over the timescale examined. Resistance may rely on factors including a certain cell density, innate cell mechanisms, such as active export and drug sequestering which prevent the antibiotic from reaching its target, and most importantly, the biofilm structure and its properties. The aspects of a biofilm which provide resistance include heterogeneous gene and protein expression, reduced diffusion into the biofilm matrix, EPS, presence of persister cells, reduced metabolism and different growth rates [Archer *et al.*, 2011; Hogan *et al.*, 2015].

Some of the most serious biofilm based HCAs are prosthetic joint infections. These types of infections often require surgical intervention and long term, high dose antibiotics. When examining treatment regimens for prosthetic joint infections, gentamicin is the most common and is incorporated in the bone cement used in the joint replacement surgery [Neut *et al.*, 2005]. Due to increasing resistance a second

antibiotic is often combined with the gentamicin in the bone cement. The presence of the antibiotic pre-colonisation is thought to reduce infection rates. However, the results gathered in this thesis indicate that gentamicin might not be a suitable antibiotic to use in this manner.

The effect of gentamicin on biofilms, of not just *Staph. aureus* but also other clinically relevant bacteria (both Gram-positive and Gram-negative species) is minimal especially with established biofilms. Even a concentration of 1024µg/ml only reduced the *Staph. aureus* biofilm load by 3 log orders. While this is a significant reduction the end point of any clinical treatment would be the complete eradication of a biofilm, as any remaining cells would be able to reseed the site and reactivate the infection.

The clinical treatment dosage of gentamicin is recommended at 5mg/kg per day, whereas prophylaxis is 3 mg/kg per day for adults. Ideally administered once a day, gentamicin should not be used in cases of myasthenia gravis or in patients with renal impairment [NHS, 2010]. Ideally when administered into a patient, antibiotics would be present at inhibitory concentrations. However, over time and with inactivation, chemical decay and elimination from the host, the concentrations would fall. At this stage, the antibiotics may promote the development of antibiotic resistant strains and even further biofilm formation.

Indeed aminoglycosides, especially at subinhibitory concentrations, have been shown to stimulate biofilm formation in both Gram-positive species (*Staph. aureus*) and Gram-negative species (*Ps. aeruginosa/ E. coli*) [Hess *et al.*, 2011; Hoffman *et al.*, 2005]. This raises the question about whether gentamicin is an appropriate antibiotic for use. However, until a replacement antibiotic which has an equal or better activity and range, and is heat stable, is developed, the use of gentamicin persists.

It is usual in a clinical situation, especially with severe infections, to combine different classes of antibiotics to enhance their activity range and effects and to prevent to emergence of resistant mutants. The effects of different antibiotics on the activity of gentamicin was investigated (Figure 3.9).

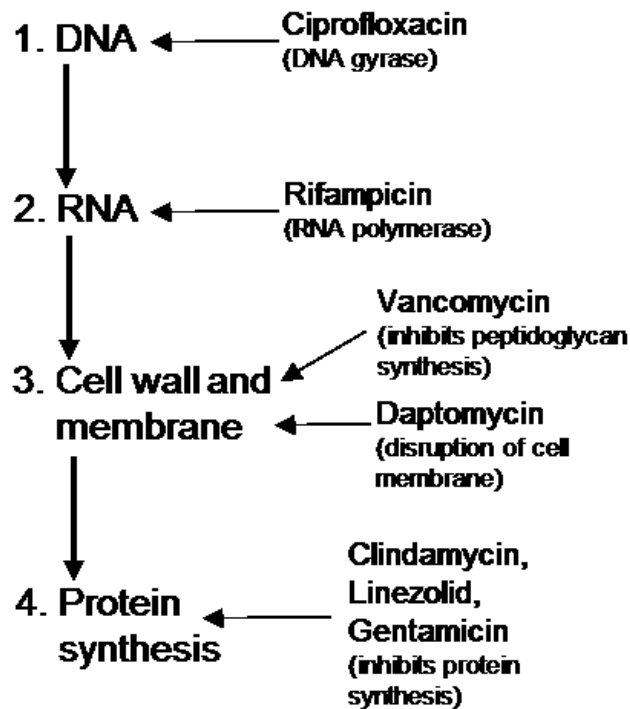


Figure 6.1: Antibiotic Targets within the Bacterial Cell. There are four main target areas within the bacterial cell which antibiotics can target. 1) DNA, 2) RNA, 3) Cell wall and membrane and 4) Protein synthesis.

As can be seen from Figure 6.1, most of the antibiotics target processes upstream of the gentamicin target. While cell wall and membrane inhibition is not directly upstream of protein synthesis, if these processes are disrupted the cell dies and there is no protein synthesis. The other two antibiotics (clindamycin and linezolid) which target protein synthesis do so at different sites (as explained in Chapter 1) and are bacteriostatic rather than bactericidal. The only antibiotic to show enhanced inhibitory effect was daptomycin, this may be due to the completely different target sites acted on in the cell simultaneously by the two antibiotics.

All the tested combinations delayed the emergence of gentamicin resistance. The main reason behind this may be that the target on which the gentamicin acts is downstream of the second antibiotic target. Thus, gentamicin activity and possible resistance cannot develop until resistance to the second antibiotic has developed giving the gentamicin a target.

Within a clinical environment, biofilm infections may be caused by multiple species from the same host site, acting synergistically [Burmølle *et al.*, 2014]. *Staph. aureus* and *Ps. aeruginosa* dual infections have been shown to delay wound healing resulting in worse patient outcomes [DeLeon *et al.*, 2014; Rosenbluth *et al.*, 2004]. In the alginate bead model, a mixed species biofilm consisting of *Staph. aureus* and *Ps. aeruginosa* developed evenly (Figure 4.6) and conferred substantial protection against antibiotics upon the component species. Antibiotic resistance developed earlier and lasted longer than the individual biofilms formed by each species (Figures 4.7-4.9).

In both single and mixed species biofilms, antibiotic resistance is due to a multitude of factors. The discussions of Chapters 3 and 4 detail these aspects and propose that the resistance should not be considered permanent as there is no genetic basis for it, as when the cells return to a planktonic state the antibiotics are once again effective.

The antibiotic concentrations required to reduce biofilm loads in both single and mixed species biofilms are immense and not practical in a clinical setting due to the difficulty in administration and the possible side effects on the patients. Therefore, alternative approaches are required to disrupt the biofilms and act in conjunction with conventional therapies.

Many alternative approaches to disrupt biofilms, especially those caused by staphylococcal species, have been proposed (Table 1.7) including sugar metabolites, bacteriophage therapy, D-amino acids and activation of the quorum sensing system [Kiedrowski and Horswill, 2011]. Some of these approaches show more promise than others, but a recurring concern with many of the therapies is that they are strain specific which would make development of a universal treatment difficult [Pincus *et al.*, 2015]. Nevertheless, due to the nature of the treatments acting on a certain target or species, there may be diminished side effects compared with antibiotics [Wu *et al.*, 2003].

A potential alternative approach that was explored involved the use of D-amino acids to prevent stable biofilm formation (Figure 4.11) or to disrupt an established biofilm (Figure 4.12). The effects of D-amino acids was first reported in *B. subtilis* [Kolodkin-Gal *et al.*, 2010] and then in *Staph. aureus* [Hochbaum *et al.*, 2011]. Disruptive effects were also reported for *Ps. aeruginosa* and some *Staph. epidermidis* strains [Kolodkin-Gal *et al.*, 2010; Ramón-Peréz *et al.*, 2014]. However, it has since been discovered

that the original experiments were not reproducible [Leiman *et al.*, 2013; Sankar and Pires 2015]. Experimental results from this thesis supported that conclusion (Chapter 4). Consequently, the use of D-amino acids as an alternative therapy is unlikely.

The use of sugar metabolites, especially fructose, is worth further investigation though the effects published by Allison *et al* [2011] were not reproduced in this thesis (Figures 4.13 and 4.14). Other publications have reported that different sugar combinations have a detrimental effect on biofilm formations, not just in *Staph. aureus* but also in other species [Bucior *et al.*, 2013, Durmus *et al.*, 2012]. The administration of sugars should have minimal or no side effects as they are naturally occurring molecules which can be found in the human system. Another advantage connected to this would be the lack of any immune response, as could be the case with lysostaphin for example, as the sugar metabolites would not be recognised as foreign. Furthermore, as the cells do not revert back to a metabolically active (pathogenic) state there is not a risk of seeding other sites and systemic infection.

As the sugar metabolites are not strain specific, immediate treatment could begin upon clinical diagnosis. However, they are primarily aminoglycoside specific so could not be used for infections caused by certain bacteria. Such species would include anaerobes, upon which the gentamicin cannot act as the antibiotic requires oxygen to function. The issue of innate, genetic antibiotic resistance would also have to be considered and lack of such verified, before treatment could begin.

As biofilms are such a huge issue within the clinical environment, especially in regards to promoting antibiotic resistance and inducing treatment failure, the current therapy protocols need to be revisited. Current use of gentamicin, especially in bone cements is an excellent example of this, as the antibiotic is not very effectual and no longer fit for the purpose. Alternative approaches, including the promising use of sugar metabolites, urgently need to be developed and introduced in order to counteract biofilm infections.

Chapter 7: References

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