

School of Biomedical Sciences

**Investigations into the Therapeutic Potential of
Antimicrobial Peptides: Applications for Treating Topical
Staphylococcus aureus Infections**

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Degree of Doctor of Philosophy
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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

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Date: 12/05/2015

Abstract

The introduction of antibiotics into medicine has protected patients from pathogenic infection acquired in the community or the hospital. Global misuse of these antibacterial drugs has encouraged resistance to develop and disseminate. The isolation of community-acquired total drug resistant pathogens forewarns that antibiotic-therapy may soon become ineffective at treating infections, and medical practice may retrogress to amputation of infected limbs to prevent the systemic spread of disease-causing microbes. Methicillin resistant *Staphylococcus aureus* has become ubiquitous in hospitals and general communities. Antibiotic-therapy is becoming less effective against these bacteria, creating an urgent need for the development of new antimicrobial agents to control these and other antibiotic resistant microorganisms. Antimicrobial peptides may become a viable treatment option, either alone or in combination with other antibacterial agents, for controlling MRSA infections. It is believed that because the mechanism of action of these peptides targets the fundamental physiology of the bacterial cell, resistance development is unlikely to occur. However, conflicting reports place doubt on this hypothesis. Exploratory experiments investigating their effectiveness under physiological conditions as well as the possibility of using them with other antimicrobial drugs or devices will allow researchers to better judge the therapeutic potential of antimicrobial peptides.

A simple and rapid technique for homogenising and extracting bio-active compounds from plant, fungal and invertebrate material was developed. Using this technique, 31 different aqueous extracts from 24 different species were tested for antibacterial activity. Fourteen of the thirty-one extracts showed antibacterial activity. Proteinase treatment of these extracts showed that only *Juniperus communis*, *Capscium chinese* and *Elettaria cardamomum* had antibacterial activity with proteinaceous origin. Isolation and characterisation of these potential antibacterial peptides were planned, but not conducted due to time constraints

Clinical application of antimicrobial peptides, as with conventional antibiotics, may be compromised by the development of bacterial resistance. Using the antibacterial peptide melittin as a model, we found that three strains of methicillin resistant *S. aureus* and one methicillin sensitive strain became equally resistant to melittin when grown in sub-lethal concentrations over seven days. The minimal

lethal concentration of melittin increased from 5 $\mu\text{g/ml}$ to 170 $\mu\text{g/ml}$ in bactopectone medium depending on the strain. The same experiments repeated with three *S. aureus* strains and an un-related peptide, bac8c, showed bacteria developed resistance to bac8c to levels comparable with strains which developed resistance to melittin. Scanning electron microscopy revealed that resistance to melittin did not change the morphology of the cells. Melittin-naïve cells treated with melittin appeared shrivelled or disintegrated, whereas resistant cells treated with melittin appeared to retain their original structure, with a few cells showing surface blebbing or a shrivelled morphology.

Eight subcultures of a melittin-resistant strain without melittin reduced the minimal lethal concentration to 30 $\mu\text{g/ml}$, and 24h growth with 12 $\mu\text{g/ml}$ melittin restored it to 100 $\mu\text{g/ml}$. However, for melittin-resistant bacteria, the minimal lethal concentration in phosphate buffer never exceeded 3 $\mu\text{g/ml}$. Further investigations with stock cultures of MRSA showed that the minimal lethal concentration of melittin was proportional to NaCl and protein content of the media. Survival of parental strain bacteria in phosphate buffer with melittin also decreased when incubation temperatures were increased.

Exposing melittin naïve and resistant bacteria to a cationic fluorophore, and measuring the binding affinity of this probe to the cells using flow cytometry, indicated that melittin resistance coincides with a decreased net negative charge on the bacterial surface. The increased binding affinity of the anionic dye eosin to the melittin resistant cells appeared to support this hypothesis. Pulsed-field electrophoresis was used to separate small digested DNA from antimicrobial peptide-naïve and resistant bacteria to investigate whether resistance coincided with any major gain or loss of genetic material. The four *S. aureus* strains made resistant to melittin and three strains made resistant to bac8c showed no difference in banding pattern compared to their wild-type counterparts. This suggested that resistance may already be intrinsic, or may be related to the up-regulation of normal metabolic pathways, in some *S. aureus* strains.

It was hypothesised that melittin-resistant bacteria which mask the anionic charges on their membrane may reduce their tolerance to other antimicrobial agents, especially those with an anionic charge. Although the anionic antimicrobial peptide, AP1, was ineffective against wildtype and melittin-resistant bacteria, resistance to melittin in the MRSA strain WBG 8287 coincided with an increased sensitivity to eleven antibiotics with different physiochemical and

electrostatic properties. The melittin-resistant bacteria appeared especially sensitive to fusidic acid, so for further investigations, four *S. aureus* strains were made resistant to fusidic acid by exposure to sublethal doses of the antibiotic. These strains were then made resistant to melittin. Two of the four strains showed an increased sensitivity to fusidic acid after being made resistant to melittin. In strain W17S, resistance to melittin, and subsequent loss of resistance to fusidic acid, appeared to coincide with the loss of the fusidic acid resistance genes FusB and FusC.

Melittin was haemolytic at low concentrations. A truncated analogue of melittin, melfrag12-26, has been reported to have the same antibacterial activity as full length melittin but without the haemolytic potential. This study found that melfrag12-26, and other analogues derived from melittin, had no antibacterial activity but were also non-haemolytic. Electroporation of the bacteria in the presence of 50 µg/ml of melfrag12-26, killed off 99.3% of the cells. The antibacterial activity of whole melittin and bac8c also improved when inserted into the bacteria using electroporation. Using a low concentration of the mild ionic detergent, Triton X-100, to permeabilize the cells also improved the peptides bactericidal activity and, together with the electroporation experiments, suggested that the peptides used in this study may have intracellular antibacterial activity.

The silver nanoparticle-loaded antibacterial gauze, Acticoat, is routinely used in the hospital to prevent infection of vulnerable wounds. This study found that, in a semi-solid agar medium, the silver particles were able to diffuse off the Acticoat and inhibit growth around the gauze. However, when Acticoat was overlain on agar with a blistered surface, bacteria were able to grow underneath the antibacterial strips. In addition, Acticoat that had been overlain on inoculated agar over three days and blotted on fresh agar indicated that bacteria were possibly growing on the surface of Acticoat. Scanning electron microscopy confirmed this, and when the number of surviving bacteria growing on inoculated pig skin underneath, and on the surface of Acticoat were quantified, it appeared that the antibacterial activity of Acticoat may not be sufficient to prevent MRSA infection. However, when Acticoat was coated with glycerol, lysozyme and an antimicrobial peptide, the gauze was able to prevent the survival of bacteria on a densely inoculated pig skin sample.

Results from this study indicated that attempts to treat topical MRSA infections with antibacterial peptides could be most effective with prolonged application of warm, low ionic strength solutions without extraneous proteins. The substantial resistance developed in protein-rich saline environments suggests that attempts to use cationic peptides to treat systemic bacterial infections could allow rapid development of resistance, possibly sufficient to render treatment ineffective. However, resistance does not appear stable in a population without selection pressures. The mechanisms by which bacteria use to resist antimicrobial peptides may also sensitise them to other antimicrobial drugs, and the peptides were shown to enhance the bactericidal activity of an antibacterial gauze. Therefore, it appears that antimicrobial peptides may become a powerful tool to prevent pathogenic infection, especially when used in conjunction with another antibacterial drug or device.

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List of Abbreviations

ACCESS	Australian Collaborating Centre for <i>Enterococcus</i> and <i>Staphylococcus</i> species Typing and Research
ABC	ATP-binding cassette superfamily
AGP	Antibiotic growth promotant
AMP	Antimicrobial peptide
ASP	Antimicrobial stewardship program
BHI	Brain heart infusion
CA-MRSA	Community associated methicillin resistant <i>Staphylococcus aureus</i>
CA-MSSA	Community associated methicillin sensitive <i>Staphylococcus aureus</i>
CDS	Calibrated dichotomous sensitivity
CHEF	Contour clamped homogenous electric field
CRAMP	Cathelicidin-related antimicrobial peptide
EF-G	Elongation factor G
E-MRSA	Epidemic Methicillin resistant <i>Staphylococcus aureus</i>
FDA	Food and Drug Administration
HpH ₂ O	Highly purified water
LB	Lysogeny broth
LDL	Low density lipoprotein
LTA	Lipoteichoic acid
MATE	Multidrug and toxic compound extrusion transporter

MFP	Membrane fusion protein
MFS	Major facilitator super family
MIC	Minimal inhibitory concentration
MLC	Minimal lethal concentration
mprF	Multiple peptide resistance factor
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
OMF	Outer membrane factor
PB	Phosphate buffer
PBS	Phosphate buffered saline
PKC	Protein kinase C
PSBS	Periplasmic substrate binding site
PVC	Panton valentine leukocidin cytotoxin
RND	Resistance nodulation division superfamily
RNP	Resistance nodulation protein
SAE	<i>S. aureus</i> acquired endocarditis
SEM	Scanning electron microscopy
SIC	<i>Streptococcal</i> inhibitor of complement
SMR	Small multidrug resistance family
TCRS	Two component regulatory system

1. Literature Review

1.1. Staphylococcus aureus

By the end of the 19th century human understanding of the nature and cause of disease had allowed many medical advances, such as vaccination and antiseptic techniques, which have saved many lives worldwide (1). In 1880 the Scottish physician Sir Alexander Ogston opened a patient's putrefied abscess and prepared a smear slide of the subcutaneous exudate (2). What he observed under the microscope were lines and bunches of round organisms which he termed *Staphylococcus* after the Greek terms *staphyle*, for bunch of grapes and *kokkos* for berry (2). In 1884 Dr Anton Julius Rosenbach isolated two strains of *Staphylococcus*, one with a golden pigment and one with a white pigment and sub-classified them *aureus* and *albus* respectively (*S. albus* has since been renamed *S. epidermidis*: 2, 3). *Staphylococcus aureus* is a Gram-positive coccoid bacterium which colonises the epidermal surfaces of human nares, skin, rectum, axillae and oropharynx (Figure 1.1: 4, 5). Generally, *S. aureus* is commensal or asymptomatic with human hosts (5, 6). However, if host homeostasis is disrupted *S. aureus* can cause pathogenic infections that range from benign to life threatening (5, 6).

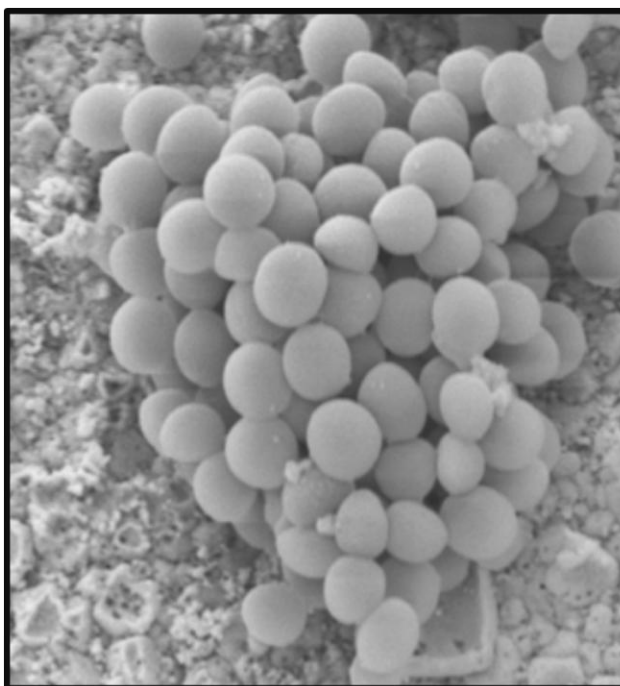


Figure 1.1 Scanning electron microscopy image of *Staphylococcus aureus* in a grape-like cluster

1.1.2. *Staphylococcus aureus* Infections and Diseases

Staphylococcus aureus commonly colonises the epidermal layers of the skin, nares and throat asymptotically (7, 8). People carrying *S. aureus* can suffer from benign medical conditions such as boils, blisters, and impetigo (9). However, if the bacteria overcome the superficial barriers of the body and host defence mechanisms, the infection can spread into the blood stream and become life threatening (10, 11). The development of *S. aureus* bacteraemia, via access through the respiratory system or infected wounds, can lead to pathologies such as osteomyelitis, endocarditis and pneumonia (7, 10). Individuals most at risk of invasive *S. aureus* infections include those suffering from leukaemia, diabetes mellitus type 1 or cystic fibrosis. Invasive surgery, haemodialysis or implantation of indwelling medical implants such as catheters, stents, pacemakers, breast implants and prosthetic valves also put patients in serious risk of infection (10, 12, 13). Prior to the introduction of antibiotics into clinical therapy, over 80% of invasive *S. aureus* infections were fatal (14). During this time only one study described the recovery of a patient suffering from *S. aureus*-acquired endocarditis (SAE:15). The patient was hospitalised for 42 weeks and was confined to a wheel-chair for most of her treatment (15). In contrast, advances in medicine have reduced the mortality rate of SAE to around 40% with non-invasive therapy periods of 4-6 weeks (16). However, development of antibiotic resistance in pathogens is greatly reducing the effectiveness of treatment for infectious pathogenic diseases such as endocarditis and pneumonia (17, 18).

1.2. Antibiotic resistance

Penicillin was serendipitously discovered by Alexander Flemming in 1928 and eventually received much interest from the medical community as it proved to be highly effective against *S. aureus* infections (19, 20). A team of Oxford researchers, under the direction of Sir Howard Florey, Professor Ernst Boris Chain, and Dr Norman Heatley, vastly expanded the research into penicillin and in 1941, North American pharmaceutical companies began mass production of the antibiotic (19, 20). In 1940 the penicillin deactivating β -lactamase enzyme was identified in *Escherichia coli* (21). By the mid-1940s almost 40% of all *S. aureus* strains isolated from hospital patients carried the enzyme (22). Methicillin

resistant *S. aureus* (MRSA) is resistant to all broad spectrum β -lactam antibiotics as well as many other classes of antibiotics (23, 24). The U.S Centres for Disease Control and Prevention reported that, in 2011, of the 80,000 reported cases of MRSA infections in North America, 11,000 were fatal: a higher mortality rate than *Pseudomonas aeruginosa* and *Clostridium difficile* combined (25). Most pathogens can now only be treated with a limited range of antibiotics (24, 25). The problem is exacerbated by the dearth of new antimicrobial agents in the development pipeline (17, 26). Without proper intervention strategies to combat antibiotic resistance, humanity could face apocalyptic epidemics leading to global recessions and decimation of populations (26, 27).

1.2.1. Mechanisms of Antibiotic Resistance

The different classes of antibiotics can target a multitude of bacterial enzymes to disrupt metabolic pathways (28). Consequently, bacteria have a sizeable arsenal of resistance mechanisms which can be broken down into five categories (Figure 1.2): mutation of the target site of the antibiotic, development of new metabolic pathways that are un-affected by the antibiotic, enzymatic modification or destruction of the antibiotic, efflux of the antibiotic from the cytoplasm using active transport proteins, and reducing the permeability of the cell membrane to the antibiotic (29).

Mutation of the target site of the antibiotic can render the target insensitive to the antibiotic, reducing bacterial susceptibility (30). Furthermore, alternative steps in metabolic pathways can be generated to circumvent the process affected by the antibiotic (29, 31). However, because these enzymes and pathways are generally critical to cell survival, mutation may reduce bacterial fitness, and other adaptations arise to ensure normal metabolism (30, 32, 33).

Some bacteria produce transferase and kinase enzymes specific for certain antibiotics, which modify their structure to prevent them from binding to their target (34). Other resistance enzymes such as β -lactamase, macrolide esterase, and epoxidase, chemically cleave the antibiotic and neutralise its activity (34). Bacteria have also been shown to inhibit antibiotics using enzymes that catalyse the oxidation of active sites of the antibiotics (34). For example, the TetX monooxygenase produced by some *Bacteroides* species catalyses the hydroxylation of the Mg^{+} binding site of tetracyclines. This hydroxylation inhibits

tetracycline activity because chelation between tetracycline and divalent cations is required for the antibiotic to target and disrupt ribosome activity (35). As with most antibiotic resistance proteins, these transferases, hydrolases and redox enzymes share similarity to bacterial proteins with functions that are not related to antimicrobial resistance. Therefore, it has been suggested that antibiotic resistance enzymes originated from metabolic enzymes and increased exposure of microbes to antibiotics recently has led to the preservation of alterations that has allowed them to function as resistance proteins (34).

Efflux pumps are protein-based transport systems which can expel toxic and non-toxic substances from the cytoplasm of a cell (36, 37). Antibiotic efflux pumps can be specific to one class of antibiotic or to a broad range of antimicrobial agents, allowing multiple drug resistance to be developed through mutations in the gene of a single protein (36, 37). These pumps are innate to both Gram-positive and Gram-negative bacteria and antibiotic resistance through the action of efflux pumps results from over-expression of the pump mechanism, rather than acquisition of new genetic material (36, 37). Although efflux pumps are generally not associated with high levels of resistance to antibiotics, it has been suggested that this intrinsic method allows bacteria to survive in low levels of a broad spectrum of antimicrobial agents, creating the potential to generate more effective resistance mechanisms (36).

The outer membrane of Gram-negative bacteria can be modified to reduce its permeability to antibiotics: restricting access of the drug to intracellular targets (38). An outer membrane densely packed with hydrophilic lipopolysaccharides has been shown to increase resistance to large hydrophobic antibiotics such as aminoglycosides and macrolides (38, 39). Small hydrophilic antibiotics gain entry to the cell by passively diffusing through the membrane gateway proteins: porins (38, 39). Loss of the porin proteins, modification of their structure, or closure of the porin channel can increase resistance of the bacteria to hydrophilic antibiotics such as β -lactams (38, 39).

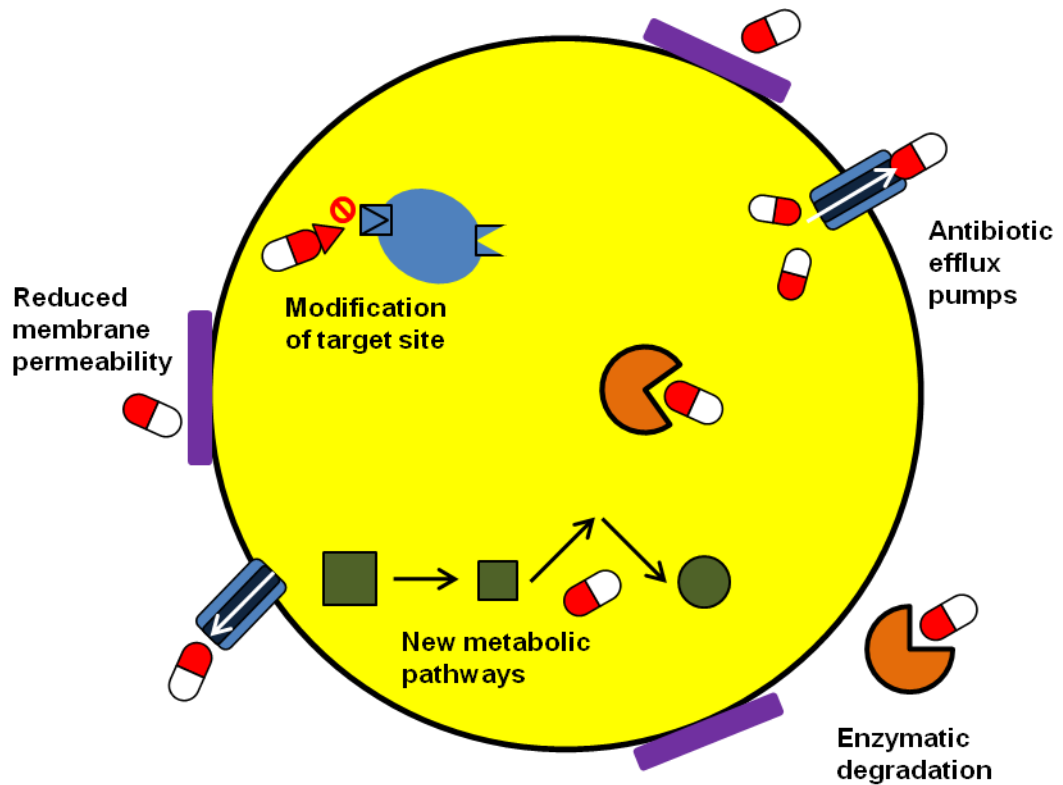


Figure 1.2 Antibiotic resistance mechanisms of bacteria

1.2.2. Dissemination of Antibiotic Resistance

The success of many medical procedures such as open surgery, organ transplantation and cancer chemotherapy would be severely compromised without antibiotics. However, resistance to antibiotics and their related compounds is not a new phenomenon (40). Sequencing of ancient bacterial DNA revealed that as far back as 30,000 years, bacterial genomes contained sequences which appear to confer resistance to β -lactams, tetracyclines and glycopeptide antibiotics (41). The introduction of antibiotics into medical practice and the agricultural industry and their subsequent misuse has conferred a selective advantage on those bacterial populations already resistant to antibiotic compounds. This evolutionary pressure, combined with the development of global markets, increased international travel and migration has caused antibiotic resistance to spread rapidly throughout hospitals and the general communities within the past 50 years (42).

1.2.2.1. Healthcare Industry

The effectiveness of antibiotic treatment against pathogens has led to their overuse in the healthcare industry. The threat of litigation from patients demanding relief from illness may cause clinicians to disregard their better judgement and prescribe antibiotics inappropriately.(43, 44). In China, 50% of the revenue of hospitals comes from drug sales and Chinese physicians can receive upward of 20% commission on drug prescriptions. This has undoubtedly contributed to China having one of the highest consumption rates of antibiotics by inpatients in the world (45). To compound the problem, medical professionals who may be inexperienced or inappropriately trained are prescribing broad spectrum antibiotics for viral infections such as the common cold world-wide (45, 46). The over-prescription of antibiotics through negligence or corruption exposes potential pathogens to antibiotics: encouraging resistance development in microbial communities whilst having no effect on the causative agent (46). The emergence of the HIV pandemic has also greatly contributed to the development and spread of antibiotic resistant pathogens. The impaired host defence system in immunocompromised patients makes them more susceptible to infection from pathogenic organisms (47, 48). Constant prophylactic treatment with antibiotics to prevent these infections can replace the patient's normal antibiotic-sensitive flora with more difficult to treat antibiotic resistant bacteria (49). In addition, the complimentary antimicrobial activity of antibiotics and the immune system may not be as effective in patients with impaired immunity. Therefore, bacteria infecting these patients may be more likely to survive antibiotic treatment than in healthy patients, which may compromise future use of the antibiotic.

1.2.2.2. Pharmaceutical Industry

Investment from private companies and public funds into medical research has created therapeutic agents and strategies to address the problems created by antibiotic resistant pathogens (50). However, the competitive nature of the global economy and strict policies controlling the approval of therapeutic agents for use in humans and animals have caused a significant reduction in funding from pharmaceutical companies, leading to a decline in antimicrobial agent research and development (51, 52). The development of antibiotic resistance by pathogenic organisms reduces the therapeutic effectiveness of the antibiotic which limits its applications in medicine and ultimately decreases its potential as

a profitable investment within a relatively short time. Additionally, a course of antibiotics is prescribed to eliminate an infection, rather than treating the symptoms, and once the infection is eliminated the patient stops purchasing the antibiotics. Therefore, it appears more lucrative for a company to invest in a therapeutic agent that treats a chronic illness, for example: insulin or insulin mimics for diabetics, or statins for patients with high LDL levels. Conversely, investment in pharmaceutical research may be a double edged sword as it may encourage some companies to report misleading or erroneous results if it is in the company's interest (49).

1.2.2.3. Agricultural Industry

In North America it is estimated that approximately 80% of available antibiotics are given to livestock (53). The majority of these are administered in the feed to promote growth in cattle, poultry and swine (54) and as a prophylaxis to prevent disease transmission in unsanitary farms (53, 54). Both practises expose microorganisms to low doses of broad-spectrum antibiotics which can encourage resistance development in native and exogenous gastrointestinal flora and leave the livestock susceptible to colonisation by antibiotic tolerant pathogens (54). In 2013 the U.S Food and Drug Administration (FDA) released two documents recommending that food animal production groups and pharmaceutical companies stop the use of antibiotic growth promotants (AGP:55). Since these were only recommendations, the FDA is not playing a regulatory role in phasing out AGP and is instead relying on these groups to act voluntarily. Additionally, the report encourages farms to continue to use antibiotics as prophylactics if a veterinarian considers the animals at risk of infection (55). Prophylactic medication does not fix the underlying problem of unsanitary, overcrowded farms. Perhaps a more prudent move would be to provide incentives for farms to rely on good animal husbandry practice, to avoid AGP and only used them as a last resort. In 1999 Denmark banned the use of AGP and now regulates antibiotic use in animal food production (56). Since then, the incidence of antibiotic resistant *Enterococci* has declined significantly in Danish livestock without any serious deterioration in animal health or farmers' profit (56).

1.2.3 Global Strategies to Counteract Antibiotic Resistance

In 1940, a follow-on study from Alexander Fleming's original work was released which described bacteria that could tolerate penicillin by producing an enzyme which inhibited the antibiotic (21). Consequently, in his acceptance speech for the 1945 Nobel Prize, Alexander Fleming warned that "an ignorant man may easily underdose himself and, by exposing his microbes to non-lethal doses of the drug, make them resistant" (57). Despite the early observations of resistance development in pathogens (21, 22, 58), only recently have strategies been put in place to address the problem. Antimicrobial stewardship programs (ASPs) have been developed and implemented in many countries to audit the use of antibiotics in the clinic and community and ensure appropriate treatment is given to patients (59). Originally, ASPs were designed to ensure that infections were properly managed based on treatment effectiveness, toxicity to the patient and cost (60). Many countries also structure ASPs to maximise the longevity and effectiveness of available antibiotics by monitoring antibiotic prescriptions, rotating the use of effective antibiotics, educating clinicians in antibiotic resistance and using specialised equipment and techniques to rapidly identify the disease-causing organism (60-62). To address the problem of diminishing stocks of effective antibiotics the U.S FDA has established the Antibacterial Drug Development Task Force (63). This group encourages research and development of antibacterial compounds by working with entrepreneurs in biotechnology to design clinically relevant trials for their product, thereby replacing the daunting and expensive clinical trials currently applied to antibiotics (63). Whether this new scheme is an effective strategy to promote drug development or simply a revision of old FDA policies remains to be seen.

1.3 Antimicrobial Gauze

An open wound, resulting from cutaneous-burn or impact trauma, is a breach of the epithelial barrier that prevents microbes from invading the body and causing systemic infections. Burn wounds account for the highest fatality rate in all injury related deaths (64). Depending on the area of the body affected, the trauma of the burn may be sufficient to cause death. However, in most cases the principal complicating factor potentially leading to patient mortality is colonisation of the avascular necrotic tissue at the centre of the burn, or the burn eschar, by

pathogenic organisms and subsequent transmission of the infection systemically (64).

The burn eschar is at high risk of infection for a number of reasons. In the innate immune system, burn injury represses the activation and biocidal activity of macrophages and natural killer cells, disrupts neutrophil chemotaxis, oxidative burst and phagocytic capabilities, and inhibits complement cascade-initiation of the membrane attack complex (64-66). Thermal injury also significantly impairs the adaptive immune system by decreasing T-lymphocyte proliferation, increasing spontaneous apoptosis, and inhibiting the T-cell activation of immunoglobulin synthesis (67, 68). Although immunodeficiency of the burn patient is systemic, the eschar is at particular risk of infection because the wound exudate provides an ideal microbial growth medium and the extracellular matrix proteins secreted by the wound healing response of the body enables microbial attachment and biofilm formation (64). It has therefore become common practice to surgically excise the eschar and surrounding tissue from the patient, following serious burn trauma, to reduce the risk of systemic infection and to decrease scarring (64, 69).

Generally, the excision is performed within the first few days following trauma, although surgery is sometimes delayed for a week or more (70). Burn patients are generally kept in the hospital prior to surgery, so the risk of an infection in the burn wound by an antibiotic resistant pathogen is high. Antibiotics are sometimes administered to the patient systemically and may be an effective prophylactic for invasive infections. However, it is unlikely that these antimicrobial agents will be able to penetrate the damaged eschar tissue and reduce bacterial load if the wound is already infected (64). Therefore, to prevent infection and long term discomfort to the patient, the burns are wrapped in antimicrobial bandages which may or may not be co-administered with additional microbicidal compounds such as antibiotics (64, 71-73).

1.3.1 Silver Nanoparticle-Loaded Wound Dressings

Wound dressings have been used for centuries to absorb exudate, decrease blood loss and allow normal wound healing to proceed (74). Advances in nanotechnology and materials science has allowed the development of gauzes with bioactive compounds incorporated into polymer networks, which have been

optimised to be absorptive, insulate the wound while still allowing gas exchange, and provide a scaffold to augment wound healing (74). The dressing with the embedded bioactive compounds can be used for debridement of the wound, elimination of odorous gases, microbial clearance, initiation and maintenance of wound healing or, more commonly, a combination of all four (64, 74). The use of silver nanoparticle-loaded bandages has now become commonplace for the prevention of infections in burn patients and those with open wounds (72, 73, 75). The disease-fighting effects of silver have been known for millennia and, as such, silver has been used in many different items such as drinking vessels, eating utensils, and surgical equipment, to preserve the health of the owner or patient (76).

1.3.2 Antibacterial Properties of Silver

Although the mechanism of action of silver against microbes has not yet been fully elucidated it seems that the metal ions have multiple toxic effects both intracellularly and extracellularly. It seems the main property of silver that confers antibacterial activity is its ability to bind weak bases such as sulphur and phosphate (77). The intramolecular disulphide bonds between the cysteine residues of proteins are generally critical for their stability and secondary structure formation (78). Silver has been shown to disrupt disulphide bonds, which is likely to result from its binding of sulphur molecules, resulting in protein misfolding (79). Many proteins, especially those with β -sheet structures that are secreted extracellularly, require disulphide bonds to maintain their structural integrity. This means silver can have multiple disruptive effects on bacteria such as membrane integrity and metabolic processes (77, 79).

The aerobic respiratory system of bacteria generates reactive oxygen species by enzymatic reduction in the oxidative state of oxygen (80). Reactive oxygen species are extremely damaging to cells, as they can cause lipid peroxidation resulting in increased membrane permeability, sever the phosphate-sugar backbone of DNA and RNA, and denature proteins. Therefore, prokaryotic and eukaryotic cells have developed defence mechanisms such as antioxidant synthesis pathways, oxidative-damage repair mechanisms, and dismutase enzymes that convert reactive oxygen back into its benign (O_2) state (80). Silver therapy is believed to exploit the sensitivity of the cells to reactive oxygen species by disabling superoxide dismutase and similar catalase enzymes, which

increases the concentration of reactive oxygen species in the cell (77, 81). Silver has also been shown to bind DNA and, depending on the concentration of silver, cause a structural transition to one of three denatured, bio-inactive states (82). Silver can bind to nucleotide bases, although it was originally believed that the metal interacted with the phosphate backbone of DNA. Detailed investigations have shown that, at low concentrations, silver will only bind guanine nucleotides. In contrast, high concentrations will cause the ions to bind between pyrimidine and purine base-pairs (82). In both cases it is likely that in these condensed states, normal DNA replication and protein synthesis may be impeded.

1.3.3 Wound Healing Properties of Silver

Topical silver therapy has, for the most part, centred on clearing an infected wound of the infecting organisms and not about improving the healing rate. In an international consensus report detailing the positive effects and proper use of medicinal silver, it was suggested that silver has no influence on wound healing (72). However, multiple *in vitro* and *in vivo* studies contradict this notion and instead suggest that the bioactivity of silver extends to injury recovery (83-86). For example, excessive matrix-metalloproteinase activity, resulting from inflammation or from bacterial secretion, can degrade the extracellular matrix in the wound bed and delay healing (83). Silver has been shown to inactivate metalloproteinases through an, as yet, unknown mechanism (83). However, it is possible that silver ions could displace the zinc ion in the catalytic domain of the metalloproteinase: leading to unfavourable energetics between the nucleophile-water complex which is critical for hydrolysis (83, 87).

The initial inflammation response to wounding is an important phase of wound repair, as it allows closure of the wound and begins the process of sterilising the vulnerable tissue (88). However, continued inflammation of the wound is detrimental to wound healing, as this encourages the release of tissue degrading matrix-metalloproteinases and toxic reactive oxygen species (88). Additionally, the increased permeability of the vascular network around the wound exposes the body to increased risk of systemic infection (88, 89). In rodent and *in vitro* model wounds, silver treatment was shown to suppress the release of the proinflammatory cytokines: interleukin 6 and tumour necrosis factor- α (85, 90). Furthermore, in mice, silver treatment increased the production of the angiogenic cytokine: vascular endothelial growth factor. Collectively, these observations

correlated with reduced inflammation and improved wound healing in mice (85). The antibacterial effect of silver also provides dual benefits to the patient in that it protects against invasive infection and clears the wound of pathogenic organisms which stimulate proinflammatory cytokine production, cause further tissue damage and prolong the wound healing process (86). The wound healing ability of silver nanoparticle gauzes seems to be related to the antibacterial activity of silver as well as its modulation of inflammatory response and inhibition of metal-associated proteolytic enzymes.

1.4 Antimicrobial Peptides

Antimicrobial peptides (AMP) are a structurally diverse class of proteins which are integral to the innate defence systems of a wide array of organisms (91, 92). Most AMPs are multifunctional and generally perform roles linked to immunity such as chemo-attraction of immune cells and wound healing (91). As a host defence molecule, AMPs can protect against bacterial, fungal and viral pathogens (91-93). It has also been reported that some AMPs are selectively cytotoxic to cancerous cells and may be able to inhibit the growth of tumours that have become resistant to chemotherapy (94). Although the exact mechanisms behind the mode of action of AMPs have not yet been verified, it appears that cationic peptides are attracted to anionic residues on the target membrane. Once bound, the peptides form transmembrane pores which can kill or inhibit the metabolic processes of the cell, either by lysing the membrane or by disrupting intracellular processes after gaining access to cytoplasmic organelles (93, 95). This rapid, broad spectrum and non-specific targeting of cells has led researchers to hypothesise that bacterial resistance to AMPs is unlikely to occur (93, 96). Development of resistance to new antibiotics is of serious concern to healthcare workers and pharmaceutical companies, as increased use could desensitize the target organism and render the product ineffective as a marketable therapeutic. As a result of this, plus an urgent need for effective therapeutic agents against multidrug resistant organisms, extensive research and developmental studies have been conducted into the therapeutic potential of AMPs (93, 97).

1.4.1 Structure-Function Relationship in Antimicrobial Peptides

Antimicrobial peptides are produced by nearly every known organism and have multiple roles including protecting the host against a broad spectrum of pathogens (92, 93, 97, 98). Antimicrobial peptides are of various sizes, tertiary structures, and physicochemical properties (92, 95, 99). However, certain characteristics are common to most AMPs. These include a length of less than 100 amino acids, a net cationic charge, and a high proportion of hydrophobic amino acids (92, 95, 99). Antimicrobial peptides have a disordered or incomplete folded structure in solution but generally fold to form amphiphilic secondary structures when bound to the cell membrane (98, 99). The amphiphilic configuration of amino acids segregates charged residues and hydrophobic residues to separate regions of the peptide (99). Once bound to the membrane, most AMPs form an α -helical conformation, while some cyclic AMPs form β -sheet conformations and others may remain as unfolded linear or loop peptides (98). The amphipathic secondary structures of AMPs allow the peptides to insert into the cell membrane, form pores and kill or inhibit the growth of the cell through one or more proposed mechanisms (95, 98, 99). At present there are multiple models which describe the pore-forming mechanisms of AMPs. However, details of the exact mechanisms involved remain unclear and, although some studies have provided evidence as to how a specific peptide may function to create transmembrane pores, no unifying theory has been developed to link the structure of AMPs with their pore forming mechanism (92, 99-101).

1.4.1.1 Net Electrostatic Charge of Antimicrobial peptides

Bacterial membranes are composed of phospholipids arranged in an amphipathic bilayer. In contrast to eukaryotic cell membranes, bacterial membranes contain hydroxylated phospholipids: phosphatidylglycerol, phosphatidylserine and cardiolipin (Figure 1.3: 102). The phosphate group of phosphatidylglycerol and phosphatidylserine each give the molecules a charge of -1 (103, 104). Phosphatidylserine also has a carboxylate anion group but it is electrostatically balanced by an ammonium group (104). Cardiolipin is formed from two phosphatidylglycerol molecules and, therefore, has a charge of -2 (103). The high proportion of these three phospholipids in bacterial membranes gives the cells a net anionic surface charge (105). Most AMPs contain a higher proportion of basic amino acids than acidic amino acids, which gives the peptides a net positive

charge at physiological pH (99, 105). It is theorised that the anionic teichoic acids in the cell wall of Gram-positive bacteria and lipopolysaccharides in the outer membrane of Gram-negative cells may draw the cationic peptides towards the surface of bacterial cells (102, 106-109). The peptides are then drawn to regions of anionic phospholipids on the bacterial membrane through electrostatic interaction (105). This may partly explain the selective toxicity of the peptides to prokaryotes, as the cationic peptides are electrostatically attracted to anionic membranes and have little electrostatic affinity to zwitterionic, eukaryotic cell membranes (105).

Although the cationic charge appears important for the initial attraction and binding between peptides and bacterial membranes, it has been shown that this relationship is not linear. Increasing the ratio of basic residues in AMPs above a critical threshold can reduce the selectivity and antibacterial activity of the peptides (110). An increased positive charge of the peptides may increase the affinity of the peptides for the anionic phosphate groups on phospholipids, which may increase the cytolytic effects of the peptides on eukaryotic cells. Interestingly, this may also cause the peptides to bind to the membrane of bacteria with greater force, which could reduce the propensity of the peptide to translocate into the membrane interior which is required for the formation of transmembrane pores (105). Despite the predominant focus on cationic AMPs, recent studies into anionic AMPs have shown that this class of peptides is an important component of innate immunity in eukaryotes and may show promise for antibacterial therapy (111). For example, the anionic peptide, dermcidin from human sweat has been shown to have antibacterial activity against a broad spectrum of topical pathogens in acidic environments with high salt concentrations (112). Generally, anionic AMPs require divalent cations or other cationic AMPs to function effectively (111, 113). Negatively charged molecules will naturally be repelled by anionic groups on bacterial membranes. However, it is believed that divalent cations may mask the charge of the anionic phospholipids, or form salt-bridges between the peptides and phospholipids, which allow the anionic AMPs to insert into the phospholipid bilayer through hydrophobic interactions (111, 113). The peptides are then able to diffuse through the membrane and interfere with intracellular processes (111, 113-116).

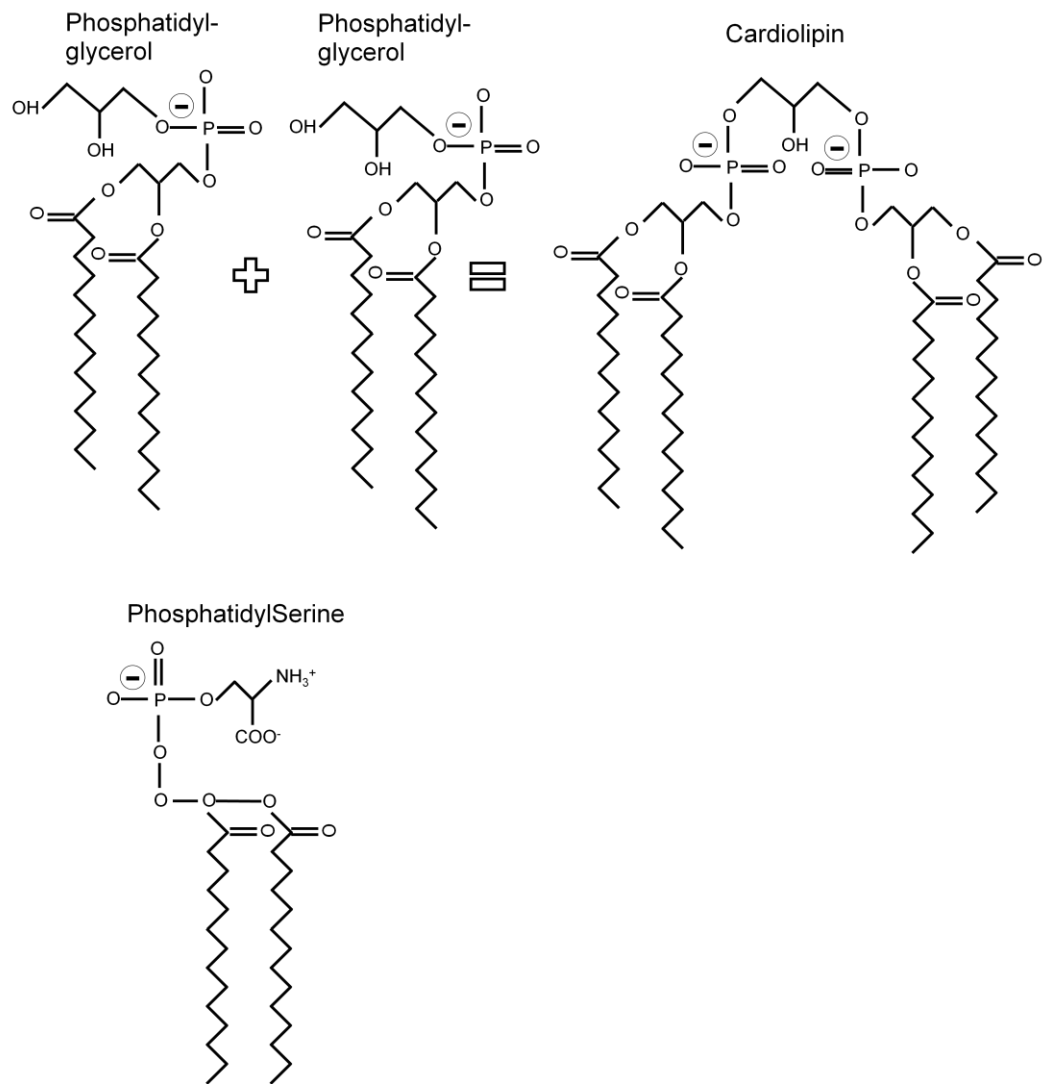


Figure 1.3 Anionic phospholipids of bacterial membranes

1.4.1.2 Hydrophobicity and Amphipathicity

Antimicrobial peptides generally have a higher proportion of hydrophobic than hydrophilic residues. When the peptides have folded into their secondary structures, hydrophobic regions are separated on separate faces to hydrophilic regions. This structural asymmetry of the peptide, with hydrophobic and hydrophilic residues polarised on opposite faces, is referred to as amphiphilicity (110, 111). With this amphiphilic configuration, the cationic residues attract the peptides to anionic regions of the bacterial membrane. When in close proximity to the bacterial membrane, the hydrophobic face of the peptides overcomes the electrostatic affinity of the cationic face for the anionic components of the membrane, causing insertion of the peptide into the membrane and disruption of

the phospholipid bilayer (117). It could be assumed that increasing the hydrophobicity of a peptide would increase its antimicrobial and haemolytic activity because an increased number of hydrophobic residues should intensify the hydrophobic interactions between the peptide and the hydrophobic interfacial membrane. This may be true for zwitterionic, cholesterol stabilised eukaryotic membranes since the main interaction between the peptide and the membrane is hydrophobic (118). However, it appears that peptides with greater amphiphilicity have a higher affinity for interfacial binding and insertion into membranes than do peptides with higher hydrophobicity (110, 113). This observation may aid in peptide drug design because increasing the amphiphilicity of a peptide without increasing the number of hydrophobic residues could create peptides with greater antimicrobial activity and lower eukaryotic cytotoxicity.

1.4.1.3 Alpha-helical Peptides

Alpha-helical peptides are the most extensively studied class of AMPs because they are abundant in nature, easy to synthesise and simple to characterise using spectroscopy techniques such as circular dichroism and attenuated total reflectance infrared spectroscopy (119). In solution, α -helical peptides have a linear structure. Once bound to a membrane, the hydrophobic nature of the lipid bilayer induces a conformational change in the peptide causing it to adopt an α -helical conformation with a hydrophobic face and a polar cationic face segregated along a perpendicular plane (99). If the hydrophobic regions of a peptide lie outside the hydrophobic core of the bacterial membrane, intermolecular forces cause a change in the conformation of the peptide and the phospholipid structure of the membrane, to shield these regions from the aqueous environment. Hydrophobic interactions force the helices of the peptides to tilt towards the hydrophobic acyl-core, causing the peptides to insert deeper into the membrane. In addition, the acyl chains of membrane lipids stretch, compress or tilt to align with the hydrophobic regions of the peptides (120).

1.4.1.4 β -Sheet Peptides

Unlike α -helical AMPs, most β -sheet peptides have a cyclic structure with a partially folded β -hairpin or β -sheet conformation in solution and form stabilised secondary structures in membranous environments (99). The cyclic structure increases the tendency for β -sheet structures to form by reducing the entropy and the number of possible conformations of the peptide (116). Generally, the β -structure is secured by intramolecular disulphide bridges between cysteine residues. For some peptides, such as porcine protegrins, the disulphide bridges are essential for antimicrobial activity (121). However, multiple studies have shown that disulphide bridges are not necessary for some peptides to form β -sheets when in contact with the membrane (121, 122). Furthermore, removal of the cysteine residues also reduced the haemolytic potential of some peptides while retaining antimicrobial activity (122).

1.4.1.5 Extended and Loop Structured Antimicrobial Peptides

Peptides that do not form secondary structures are referred to as extended peptides. Generally, these contain a high proportion of one type of amino acid. An example is the bovine neutrophil peptide indolicidin, which contains 40% tryptophan residues (99). Unlike other antimicrobial peptides, for most extended peptides transmembrane pore formation does not appear to be the functional mechanism. Instead, many of the smaller extended peptides diffuse through the membrane, accumulate in the cytoplasm and interact with intracellular machinery (99). However, the neutrophil-derived peptides indolicidin and tritrpticin are the exception to this phenomenon, as they are highly membrane active and can form transmembrane pores in a variety of organisms (99, 123, 124). Loop peptides are linear peptides which form one or more loops stabilised by a single cysteine-cysteine disulphide bridge when in a membranous environment (93). Some loop AMPs, such as the gramicidins, have proved excellent model peptides for ion-channel investigations as they form well-defined selective transmembrane channels and can be easily modified to test various parameters of defective channels (125).

1.4.2 Mechanisms of Action of Antimicrobial Peptides

Antimicrobial peptides, like antibiotics, have many different methods by which to stop the growth and replication of their target organisms, or kill the cell directly (92, 99, 105). Model membrane studies have shown that most AMPs interact with the cell membrane to form transmembrane pores (95). It has been proposed that these pores are multifunctional, in that they induce non-selective leakage of cytoplasmic components and allow free AMPs access to intracellular targets (126). The majority of studies into AMP-induced pore formation have used *in vitro* assembled lipid membranes (127). The disadvantage of this is that such studies can overlook secondary effects of the peptides on live bacteria. For example, Patrzyk *et al.* (128) demonstrated that a hybrid peptide of pleurocidin and dermaseptin (P-Der) could diffuse across the cell membrane and inhibit macromolecular synthesis at the minimal inhibitory concentration (MIC) without forming transmembrane pores. However, at ten times the MIC, P-Der was able to form transmembrane pores and cause cell death (128). In addition to this, studies from other groups have shown that the antimicrobial activity of AMPs is more complex than pore formation and may include disruption of other cellular processes such as the synthesis of peptidoglycan, nucleic acids and proteins (105, 126). Therefore, some AMPs are able to target multiple organelles, in addition to the cell membrane, potentially reducing the chance of resistance developing in microorganisms by increasing the complexity of change required to make the organism resistant to the peptide. Although pore formation may not be the exclusive mechanism of action, it still appears a critical step in the peptide-induced pathway to cell death: especially at high peptide to membrane lipid ratios (105, 114, 128, 129). Exactly how AMPs form transmembrane pores is still unclear. However, techniques such as nuclear magnetic resonance imaging of the peptides and analysis of artificial lipid membranes has allowed the proposal of multiple models of pore formation, such as the barrel-stave, toroidal pore and carpet method to explain the peptide-lipid interactions (99, 105, 130).

1.4.2.1 Models of Pore Formation: Barrel-Stave

The barrel-stave model depicts pores as transmembrane channels of aggregated AMP oligomers arranged in a cylindrical or “beer barrel” structure (99, 105). This model proposes localised binding of multiple peptides to the membrane surface with the hydrophobic surfaces of the peptides facing towards the hydrophobic

core of the interfacial membrane. Once bound, the hydrophobic residues insert into the membrane: repelling the phosphate head-groups of the phospholipids. As more peptides bind and embed, the membrane surface area expands and the void between the extracellular and intracellular lipid layers is filled by the acyl chains of phospholipids surrounding the peptide. The induced negative-curvature on the membrane surface thins the interfacial space of the lipid bilayer and allows the hydrophobic face of the peptides to insert further into the hydrophobic core (Figure 1.4a: 131).

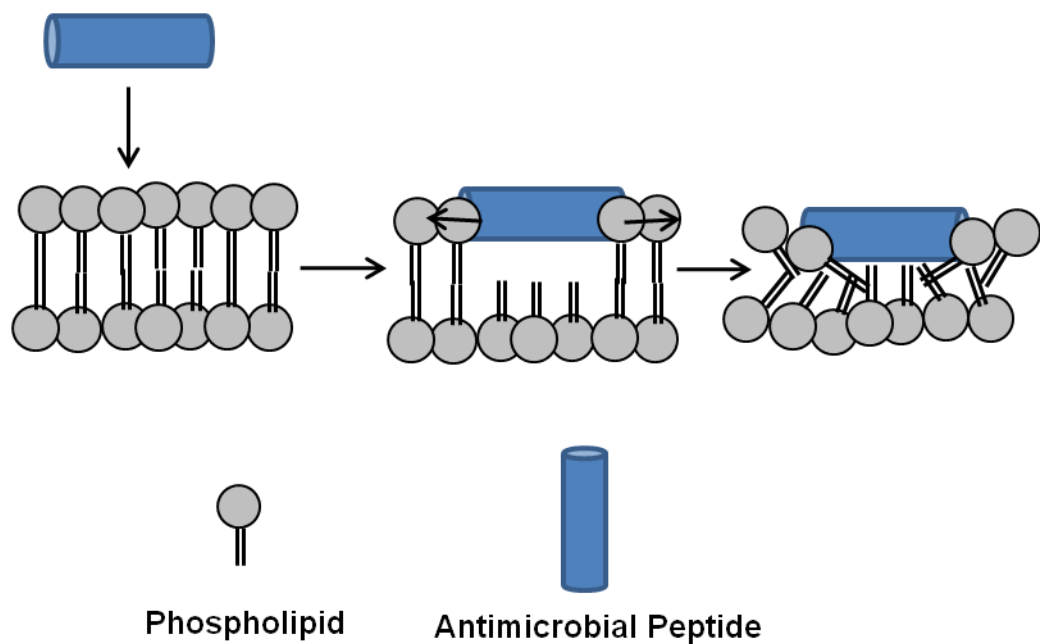


Figure 1.4a Membrane thinning from antimicrobial peptide hydrophobic interactions

If the number of peptides bound to the same area reaches a threshold concentration, the peptides will aggregate and translocate perpendicularly across the membrane to form a transmembrane pore. The translocated AMPs position their hydrophilic faces away from the hydrophobic membrane core which creates a pore lumen and allows indiscriminate leakage of the cell content. The pore is expanded by unbound peptides inserting themselves into the transmembrane complex in a manner resembling staves in a barrel (Figure 1.4b: 105). Despite earlier studies regularly using the barrel-stave model to describe AMP pore formation, only one naturally occurring peptide has been shown to use this

mechanism (132). More recent studies have shown that a majority of AMPs use a variant of the barrel-stave mechanism which has been described as the toroidal or wormhole pore model (105, 133, 134).

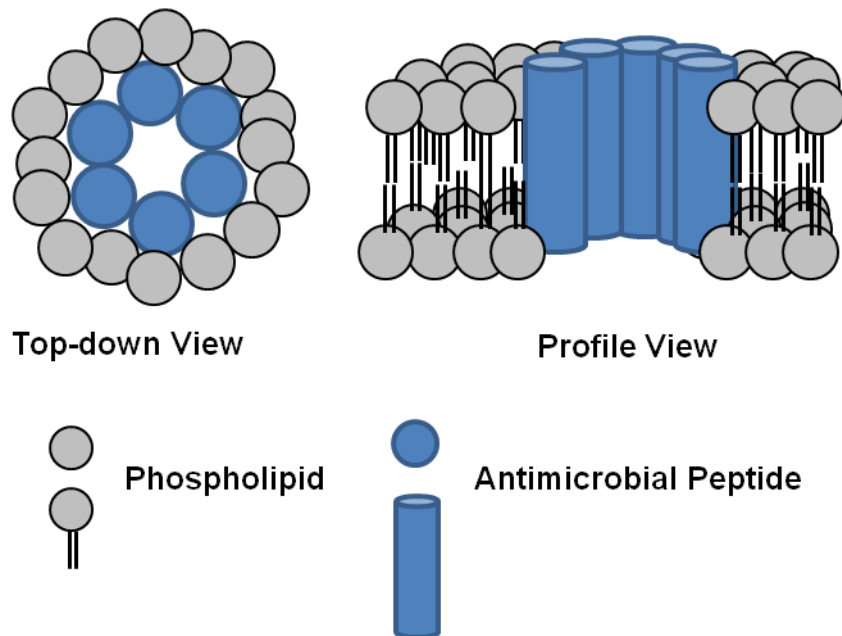


Figure 1.4b Illustration of a barrel-stave pore in a phospholipid membrane in top-down and profile view

1.4.2.2 Models of Pore Formation: Toroidal Pore

The configuration of a toroidal pore differs from the barrel-stave pore in that the peptide residues intercalate with phospholipid head-groups to form a peptide-membrane supramolecule (Figure 1.4c). As in the barrel-stave model, toroidal pores are formed by peptides binding to bacterial membranes and displacing the phospholipid head-groups by inserting their hydrophobic faces into the membrane (105). Monomer peptides aggregate on the bacterial membrane through binding of their hydrophobic N-terminal regions. These peptide aggregates can then saturate areas of the cell membrane, increasing the tensile forces between peptide hydrophobic residues and membrane phospholipids (135). At a critical peptide to membrane lipid ratio, the curvature-strain induced on the membrane is great enough to force phospholipids apart and allow the peptides to insert perpendicularly into the membrane (105, 135). Once inside the membrane, hydrophobic forces and electrostatic interactions between the anionic

phospholipid heads and the basic peptide residues cause the peptides to embed within the outer leaflet of the bilayer, creating a peptide-phospholipid complex (135). This forms a torus shaped transmembrane pore lined with the hydrophilic AMP regions and phospholipid head groups (105, 135). An alternative model of the toroidal pore proposes that only one or two peptides insert deep into the membrane while other peptides bind to the opening of the pore and stabilise the channel (136). Toroidal pores may have much shorter half lives than barrel-stave pores. Electrostatic repulsion of the positively charged side-chains of the AMPs can cause the pores to collapse in on themselves and release embedded peptides into the cytoplasm (105). This has been suggested as a method of AMP translocation across cellular membranes (105, 135).

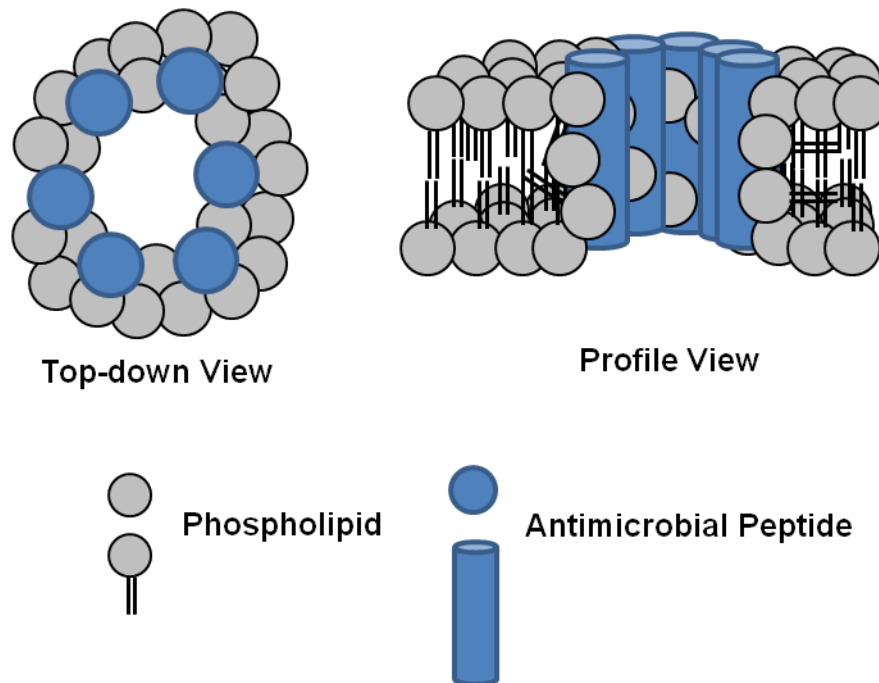


Figure 1.4c Illustration of a toroidal pore in a phospholipid membrane in top-down and profile view

1.4.2.3 Models of Pore Formation: Carpet Model

The carpet model does not rely on cells inserting into the membrane or the formation of peptide-stabilised transmembrane channels (137). Instead, this model proposes that localised sections of the membrane become saturated with AMPs, leading to membrane disaggregation and the formation of large holes (Figure 1.4d). This is achieved by large numbers of peptides binding parallel to

the membrane surface which increases the surface area covered and enhances the electrostatic forces between peptide and phospholipid layers (105, 138). Once a high enough concentration of peptides has bound to a region, the electrostatic forces between phospholipid head groups and the basic residues of the AMPs cause the packed configuration of the lipid bilayer to become disrupted. This causes a loss of surface tension between phospholipids resulting in segments of the lipid bilayer dispersing as micelle particles and the formation of holes in the membrane (99, 105, 138). It has been suggested that the carpet model may be applicable to most AMPs as long as the concentration of peptides is high enough (137). However, as the carpet model requires a very high peptide to membrane lipid ratio to form transmembrane holes, it appears unlikely that this mechanism occurs naturally *in vivo*.

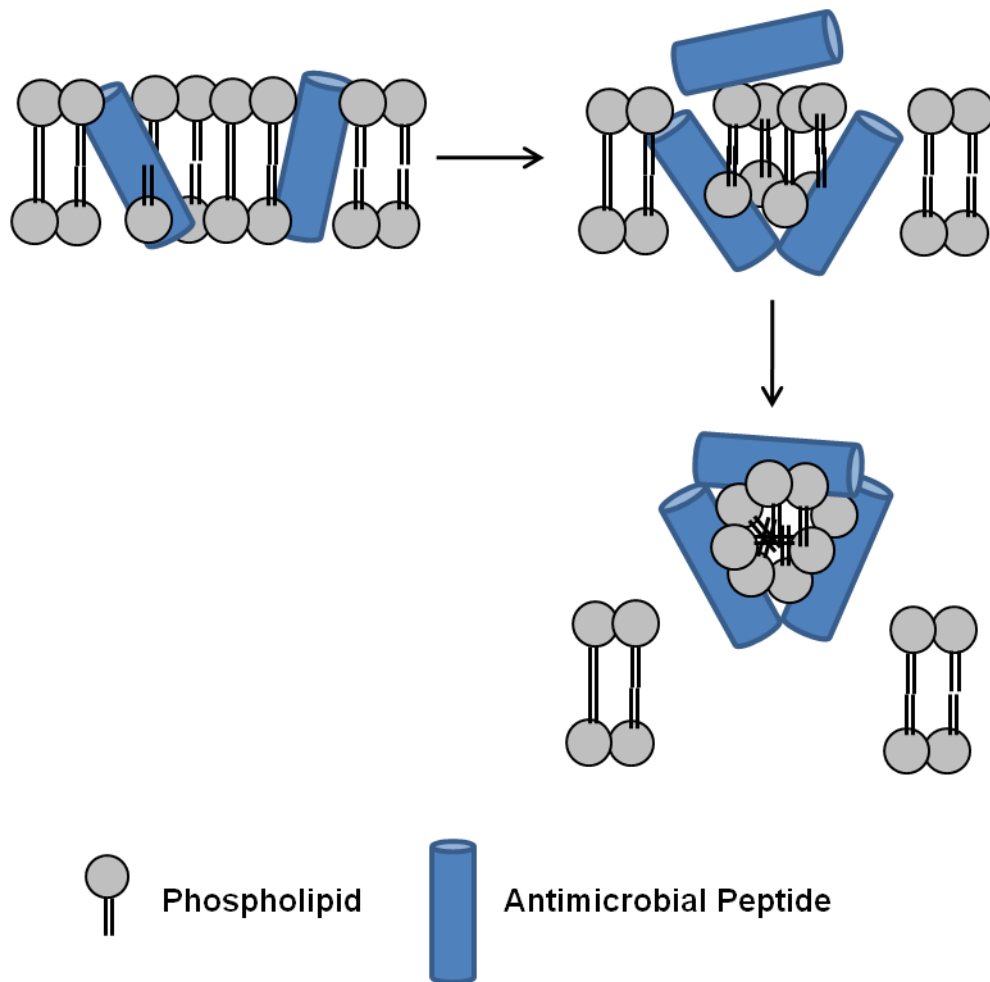


Figure 1.4d Carpet model of pore formation in bacterial membranes

1.4.2.4 Extracytoplasmic Targets of Antimicrobial Peptides

As described above, the lipid membrane is not the only target of AMPs. A study by Bierbaum and Sahl (139) has shown that the AMPs nisin and Pep5 can associate with lipoteichoic acids and activate autolysins, which hydrolyse peptidoglycans resulting in cell lysis. In addition, nisin and other lantibiotics contain a set of lanthionine rings which form a glove like structure known as the pyrophosphate cage. The pyrophosphate cage is able to bind the lipid II transferase, which is essential for transmembrane transport of cell wall subunits, and to inhibit cell wall assembly (140). Understanding the multiple bactericidal mechanisms of AMPs can help to improve drug design by removing or replacing residues that could influence patient cytotoxicity. For example, protegrin-1 is a peptide with broad spectrum antimicrobial activity and a high potential for haemolysis of human erythrocytes (141). Srinivas *et al.* (142) have shown that an analogue of protegrin-1 can kill *Pseudomonas aeruginosa* by binding to the lipopolysaccharide membrane protein D and impair correct outer membrane formation, without forming transmembrane channels. The peptide was also more effective against *P. aeruginosa* at lower concentrations and had a reduced haemolytic capability compared to full length protegrin-1 (142).

1.4.2.5 Intracellular Targets of Antimicrobial Peptides

Multiple studies have shown that AMPs can diffuse across cell membranes or enter through transmembrane pores to disrupt intracellular metabolite synthesis and other crucial bacterial processes (105, 143). Linear peptides rich in proline and arginine generally follow this process (144). The high proportion of proline residues inhibits the formation of amphipathic α -helices and β -sheet structures in the peptides. This is due to a lack of free amine groups on proline residues, which prevents hydrogen bonding between amino acids and the formation of an α -helical or β -sheet backbone. The folding of β -sheets is also inhibited by the limited spectrum of available phi angles on this cyclic amino acid (145). The lack of an amphipathic structure may explain why these peptides have little tendency to produce transmembrane pores and instead rely on the membrane transport system of the bacteria to enter the cell and attack intracellular targets (144, 146). So far, the mechanisms by which proline/arginine rich peptides affect the cell are still unclear. It has been proposed that once inside the cell, these peptides may inhibit the growth of the microorganism by disabling chaperone proteins,

inhibiting protein synthesis or directly binding to nucleic acids (147). Otvos *et al.* (148) have shown that pyrrolicorin, apidaecin, and drocosin specifically bind to the chaperone protein DnaK and inhibit its ability to assist in protein folding. Further studies have shown that mutants lacking DnaK are also susceptible to pyrrolicorin and similar peptides, which suggests that these AMPs may have multiple internal targets (144). Some peptides such as indolicidin and the neutrophil AMP PR-39 can inhibit the cell from dividing and cause filamentation of the bacteria (146, 149). The mechanism behind this has not yet been elucidated but is believed to be a result of the peptides either blocking DNA replication or inhibiting the membrane proteins required for the division septum to form (149).

The diversity of AMPs with intracellular functions extends beyond proline rich linear peptides. Buforin-2 is a histone-derived alpha helical peptide with two helices separated by a proline residue (150). It is a non-lytic peptide that penetrates the cell membrane and binds to DNA and RNA (150, 151). It appears that buforin-2 binds to nucleic acid by non-specific interactions of the basic residues of the peptides and the anionic phosphate backbone of DNA and RNA (150). However, there is some evidence that the peptide may also have a specific nucleic acid sequence to which it strongly associates (151). Although it is not known how binding DNA or RNA kills the cell, the link between the antimicrobial activity of the peptides and their affinity for nucleic acids has been established (151). It can therefore be theorised that aggregation of buforin-2 with DNA or RNA could broadly inhibit protein synthesis and retard cellular metabolism. Understanding the multiple mechanisms of microbial inhibition or destruction by AMPs is of great interest to researchers, as it may provide insight into new techniques that could substantially improve our arsenal of antimicrobial agents, drug delivery vectors, wound healing drugs, and anti-cancer cell therapeutics.

1.4.2.6 Immunomodulatory Activity of Antimicrobial Peptides

In vivo AMPs are believed to be critical to the nonspecific clearing of pathogens. However, physiological factors inside the body such as high ionic concentrations, exogenous peptides, peptidases and acidic environments can inhibit the bactericidal action of the peptides (152, 153). Additionally, in a healthy host the concentrations of peptides are far lower than those required to achieve effective inhibition of the microorganisms *in vitro* (154). Therefore, AMPs in nature may

need to work in conjunction with additional host defence mechanisms, such as extracellular enzymes, leukocytes or other AMPs, to inhibit invading pathogens. Direct targeting and lysis of bacteria may not be the principal role of these peptides in host defence (155). In fact, numerous immunomodulatory roles have already been identified in two broad families of mammalian AMPs: the defensins and cathelicidins.

Defensins are a class of AMP, segregated into groups α , β and, in old world primates, θ which show broad spectrum antimicrobial activity (156, 157). In addition to the direct antibiotic action of these peptides, it has been demonstrated that defensins are also regulatory molecules of the immune system (154, 158). Human defensins have multiple roles in the direct, non-specific clearing of localised epidermal and systemic infections. These molecules also serve the innate and adaptive immune systems by recruiting T-lymphocytes, macrophages, dendritic cells and mast cells.

The α -defensin subfamily recruits T-lymphocytes by acting as chemotaxins in areas of infection and inflammation (159-161). Alpha-defensins have also been shown to enhance the efficiency of leukocyte phagocytosis by stimulating the release of tumour necrosis factor- α and interferon- γ molecules from macrophages (162). The AMP LL-37 is derived from the cathelicidin polypeptide hCAP18. To date, it is the only functional cathelicidin AMP identified in humans (163, 164). In addition to its pore forming activity, LL-37 induces the release of chemotactic cytokines from cells at areas of infection. The peptide can also act as a pro-inflammatory molecule by degranulating mast cells, resulting in the release of histamine and prostaglandin (155). Conversely, LL-37 can act as an anti-inflammatory molecule by neutralising inflammation-inducing endotoxins such as lipoteichoic acids and lipopolysaccharides released by dead bacteria (165). Furthermore, LL-37 can enhance phagocytosis in neutrophils by interacting with extracellular kinase receptors to stimulate interleukin-8 production (166).

1.4.3 Mechanisms of Resistance against Antimicrobial Peptides

The jawless ancestors of modern vertebrates lacked an adaptive immune system. Instead they relied on innate defences such as physical barriers, non-adaptive phagocytes, natural killer cells and cytotoxic molecules (167). A study

by Uzzel *et al.* (167) found that hagfish contain peptides strongly related to mammalian cathelicidin AMPs. This suggests that AMPs are an ancient defence mechanism that has protected organisms for hundreds of millions of years (168). Given the amount of time pathogens have been exposed to these molecules and their abundance in nature, it seems likely that resistance mechanisms have already been developed. Multiple reports have shown that bacteria can become resistant to the peptides *in vitro* and in some cases quite rapidly (169-171). It may be possible that some populations of bacteria have already developed resistance mechanisms that are either activated on exposure, or that intrinsically resistant bacteria grow to dominate the population when it is exposed to the peptides. Other studies report that bacteria only have a limited ability to become resistant to AMPs, which supports the idea that the non-specific targeting of fundamental organelles restricts the development of resistance (172, 173). The reasons behind these contradictory results have not yet been fully explored. However, it may be that mechanisms of resistance against AMPs are not universal and some bacteria are better equipped to develop, or may have already developed, these survival methods.

1.4.3.1 Modification of Cell Membrane Molecules

Regardless of the site of bactericidal activity of an AMP, in most cases the cell membrane is the initial site of interaction. Bacteria which are exposed to sublethal concentrations of a peptide can subsequently survive higher concentrations than their wild-type counterparts. This tolerance can be achieved by adding, altering or removing different membrane molecules (173-176). Lipopolysaccharides are major constituents of the Gram-negative bacterial outer membrane. They consist of three major components: a distal anionic polysaccharide referred to as the O-antigen, a zwitterionic oligosaccharide at the core of the molecule, and the hydrophobic, negatively charged lipid A endotoxin which anchors the lipopolysaccharide to the outer membrane (177). In the presence of AMPs, the *PhoPQ-PmrD* systems in *E.coli* and *Salmonella typhimurium* increase the expression of the membrane bound enzymes *ArnT* and *EptA* (178, 179). These transferases modify the core and lipid A moieties of lipopolysaccharides with ethanolamine and aminoarabinose to mask their anionic charge (180, 181). A reduction in the negative surface charge probably reduces the electrostatic affinity of cationic peptides for the membrane. Similar

modifications of lipid A by the addition of cationic residues are observed in *Bordetella pertussis*, *E.coli* and *Neisseria meningitidis* (182-185).

PhoPQ-PmrD activation is also initiated by temperature fluctuations. Studies have shown that *Yersinia* species are more sensitive to AMPs at higher temperatures (186, 187). The many arms of the innate immune system of vertebrates includes the initiation of fever, an increase in body temperature above that of homeostatic levels, in response to pathogenic invasion (188). Besides strengthening many aspects of the immune system, increasing the core body temperature may make some Gram-negative bacteria more susceptible to AMPs because it has been suggested that the *PhoPQ-PmrD* regulatory system is inhibited at higher temperatures (186). However, suppressing lipopolysaccharide modifications also helps the cell evade recognition by the immune system.

Some Gram-negative bacteria may increase their tolerance to AMPs and reduce the immune response of the host by inhibiting the action of their inner membrane phosphotransferase: *LpxT* (179, 189). This enzyme catalyses the transfer of a phosphate residue to the 1' position of the glucosamine backbone of lipid A (190). This creates a pyrophosphate anion on lipid A which increases the negative charge and the immunogenicity of the bacterial membrane. Therefore, inhibiting the action of *LpxT* increases resistance to AMPs and assists the cells in evading the immune system (179, 189).

The lipoteichoic acids (LTA) are a major anionic constituent of Gram-positive cell walls. These phosphate rich molecules give the cells an overall negative charge. In addition, LTA are believed to be important structural molecules because mutants lacking LTA show morphological defects and sensitivity to temperature fluctuations (191). Gram-positive bacteria such as *S. aureus*, *Enterococcus faecalis* and *Streptococcus* species can become resistant to cationic antimicrobial peptides by adding cationic D-alanyl residues to LTA. This is mediated by the *dlt* operon and is believed to confer resistance by reducing the net negative charge of the cell surface, thereby diminishing the electrostatic attraction of the peptides (174, 176, 192). However, Saar-Dover *et al.* (174) have shown that there is no difference in the amount of peptide bound to *Streptococcus agalactiae* mutants that lack the *dlt* operon and to wild-type bacteria (174). Instead, they propose that D-alanylation of LTA masks their anionic charges and allows the polysaccharides to pack more closely together and increase the density of the cell wall (174).

A similar resistance pathway involving the masking of anionic membrane charges in both Gram-negative and Gram-positive bacteria is mediated by the multiple peptide resistance factor protein (*mprF*: 126, 174, 193, 194). Phosphatidylglycerol is an anionic phospholipid that constitutes the bulk of bacterial membranes and contributes significantly to the net negative charge of the membrane (195). In the inner leaflet of the cytoplasmic membrane, the *mprF* protein switches the anionic charge of phosphatidylglycerol molecules to a cationic charge by attaching L-lysine residues using a synthase domain located in its hydrophilic C-terminus (193, 194). A translocase domain, located at the N-terminus of the *mprF* protein, flips the cationic lysyl-phosphatidylglycerol molecules to the outer leaflet of the membrane, thereby repelling cationic AMPs (193). The *mprF* enzyme is found in a broad range of both Gram-negative and Gram-positive bacteria and can also confer resistance to the antibiotic daptomycin (193, 196, 197). Strategies to inhibit or counteract the activity of *mprF* may therefore increase the sensitivity of pathogens to a large array of antimicrobial agents.

1.4.3.2 Direct Inhibition of Antimicrobial Peptide Action

Membrane modifications can improve the effectiveness of the barrier between the extracellular environment and cytoplasmic constituents in keeping out AMPs. However, a concentrated dose of AMPs may overcome these resistance mechanisms. Therefore, bacteria have evolved extracellular resistance strategies to inhibit the action of the peptides before they damage the membrane (102, 198). A range of bacterial extracellular proteases have been identified which cleave AMPs before they can interact with the cell membrane (199-201). To date, peptidases which degrade AMPs have been identified in the three major classes of bacterial proteases: metalloproteases, cysteine proteases and serine proteases (200, 202, 203). Elastase is a zinc-dependent metalloprotease that has been shown to contribute to the virulence of *Pseudomonas aeruginosa* by degrading the extracellular matrix of host tissue, as well as immune molecules such as cytokines, antibodies and lysozyme (204). Elastase from these cells has been shown to rapidly hydrolyse the human cathelin-like peptide hCAP18 and its C-terminal AMP, LL-37, specifically at hydrophobic residues (200). Peptidases with similar structures from *Enterococcus faecalis* and *Proteus mirabilis* were also shown to cleave LL-37 at its hydrophobic amino acids (200). Interestingly,

neutrophil-derived elastases have been shown to be essential for releasing the LL-37 peptide in its active form from the hCAP18 propeptide (205). Furthermore Zaiou *et al.* (206) have shown that the N-terminal domain of hCAP18, which also has antibacterial activity, is capable of inhibiting extracellular cysteine proteases from *E.coli* after it is cleaved from HCAP18. This may protect AMPs in the vicinity from degradation while at the same time inhibiting peptidase-mediated tissue damage in the area. It seems then, that in infected wounds, a proportionate balance between the cytotoxicity of neutrophil-elastases and their AMP-modulatory roles, exists to clear the area of infection while still allowing the wound to heal.

Oligopeptidase B is a serine peptidase, identified in Gram-negative bacteria, spirochaetes and some protozoan species which have been shown to have hydrolytic activity that appears specific to short cationic peptides. This enzymes activity appears to be restricted to hydrolysing the carboxyl side of basic residues in short peptides (around 40 amino acids in length), which suggests its main function could be to deactivate AMPs (203). Additionally, oligopeptidase B has been shown to play other roles in the virulence of *Leishmania* and *Trypanosomal* protozoa by regulating entry into host cells and stimulating the formation of lesions in the circulatory system (207). A mammalian orthologue of oligopeptidase B has not yet been discovered, which means that treatment with a peptidase inhibitor specific for this enzyme may selectively decrease the invasive capacity and haemorrhagic activity of protozoa from the *Leishmania* and *Trypanosoma* genus (208). Furthermore, peptidase inhibitor treatment may increase the effectiveness of the patients' innate antibacterial peptides.

Another mechanism that bacteria employ to inhibit the action of AMPs is the extracellular secretion or membrane surface attachment of peptide binding molecules. Sequestration and aggregation of AMPs mediated by these binding molecules reduces their concentration around the cell's surface, which limits peptide clustering on the membrane and pore formation (198, 209, 210). The concept appears simple, but a range of different mechanisms of peptide sequestering have been identified in a variety of different bacteria (211-214). For example, the staphylokinase enzyme is an important virulence protein that activates the plasma-protein cleaving enzyme plasmin. In a healing wound, the fibrin clot prevents entry of pathogens into the circulatory system, so plasmin activation and subsequent degradation of fibrin allows *S. aureus* access into the body (215). Staphylokinase has also been shown to bind and inactivate α -

defensins (211). The presence of multiple α -defensin binding sites on the enzyme allows a low concentration of staphylokinase to inactivate a large quantity of defensins (215). It seems then, that this virulence protein not only increases the invasive potential of the bacteria, it also allows them to evade immune cell molecules.

Another potent secreted inhibitor of AMPs is the *Streptococcal* inhibitor of complement (SIC) protein (216). As the name suggests, SIC was first discovered in *Streptococcus pyogenes* as a mechanism employed to prevent the formation of the membrane attack complex by the complement pathway of the immune system (216). However, more recent studies have shown that SIC is far more effective at binding and inhibiting the action of other immune molecules, such as lysozyme and leukocyte proteinase inhibitor (217, 218). It also has a high binding affinity for a range of cationic AMPs which is probably due to its anionic surface charge and its hydrophobic nature (218, 219).

Streptococcal M1 protein has also been shown to sequester and inhibit AMPs (212). The M1 protein is one of a family of surface-associated virulence factors which contribute to the invasive capacity and pathogenicity of group A *Streptococcal* species (212, 220). Lauth *et al.* (212) have also demonstrated that this protein inhibits the action of LL-37 by binding the peptide to its hypervariable N-terminal region. In a separate study, bacterial pilli of *Streptococcus* and *Lactococcus lactis* were shown to trap LL-37 before it reached its target organelle (213). In the presence of bactericidal molecules, using cell surface entrapment molecules such as those described in the previous studies may be a more metabolically efficient means of obstructing AMP activity, instead of secreting inhibitory molecules, although it is likely that bacteria use a combination of the mechanisms described. For example, Schmidtchen *et al.* (214) have shown that the elastases, gelatinases and cysteine proteases of various bacteria are able to release dermatan sulphate from decorin: a human proteoglycan that constitutes the major portion of collagenous connective tissue. Dermatan sulphate sequesters α -defensins which protect the bacteria from AMP activity (214). Therefore, bacterial extracellular enzymes may play multiple roles in inhibiting the action of AMPs.

1.4.3.3 Intracellular export of antimicrobial peptides

The majority of studies investigating AMP resistance have focused on extracellular or membrane-associated modifications. This is understandable because the initial target of AMPs appears to be the bacterial membrane. However, the identification of internal resistance mechanisms specific to AMPs provides evidence that some peptides may be effective intracellular bacteriocides. Antimicrobial efflux pumps are membrane bound transporter proteins which can be activated to expel toxic molecules from the cytoplasm (36). These ancient resistance proteins can be specific to one compound, such as a particular antibiotic or peptides of a specific sequence, or can have broad substrate selectivity so that a single class of efflux pump can provide resistance to multiple antibiotics, disinfectants or other toxic molecules (36).

So far five different families of efflux pumps have been identified in bacteria:

1. major facilitator super family (MFS)
2. resistance nodulation division superfamily (RND)
3. small multidrug resistance family (SMR)
4. multidrug and toxic compound extrusion transporter (MATE).
5. ATP-binding cassette superfamily (ABC)

The MFS is the largest transporter group dependent on the cells proton motive force to drive antimicrobial efflux. In Gram-positive and some Gram-negative bacteria these pumps are single protein antiporters with twelve transmembrane α -helical domains that use a “rocker-switch” mechanism to bind toxins in the cytoplasm and expel them by switching their orientation so their substrate binding site opens to the extracellular environment (Figure 1.5a: 221). These efflux pumps are described as antiporters as, generally, the movement of the toxic molecule out of the cytoplasm is coupled with the influx of hydrogen ions (222, 223). In Gram-negative bacteria, most MFS have been shown to use a trimeric system where the drugs are transported into the periplasm by the MFS protein and are then removed by membrane fusion proteins (224, 225).

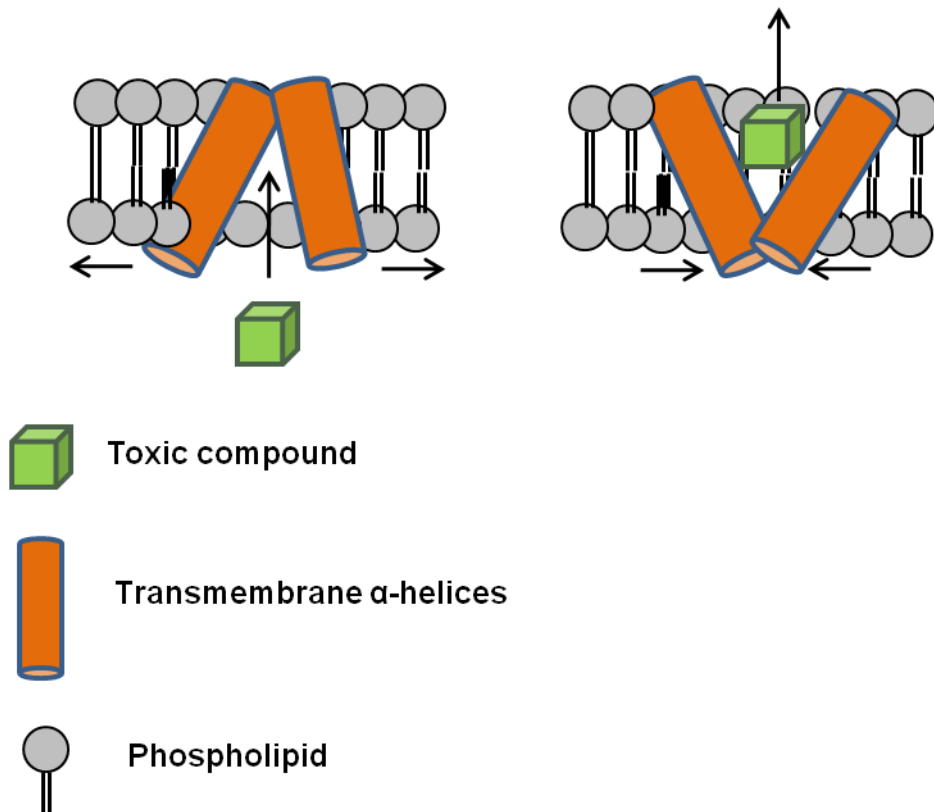


Figure 1.5a Illustration of the proposed “rocker-switch” mechanism used by the major facilitator super family efflux pumps

In contrast to the narrow spectrum of drugs transported by the MFS, the RND super family, which is also dependent on ion gradients, can confer resistance to a broad range of antimicrobial compounds (226). Similar to some MFS systems, RND transporters comprise three separate components: a trimeric RND-drug efflux protein complex, membrane fusion proteins and an outer membrane channel to export antibacterial agents from the cytoplasm (224, 225). In this system, the periplasmic substrate-binding site of the RND protein is protonated, which induces a structural change in the protein, allowing AMPs and other drugs to diffuse across the plasma membrane bound region of the RND protein and bind to the substrate binding site. The RND-protein is then de-protonated which causes the toxic molecule to lose affinity with the binding site and diffuse across the cell wall, through a linked outer membrane channel, and into the extracellular environment (Figure 1.5b: 227). Although the RND-systems are generally found in Gram-negative bacteria, these systems have been found in many other species including Gram-positive bacteria, plants, animals and humans (224,

227). A study by Lee *et al.* (228) has also shown that RND transporters can work synergistically with MFS to remove antibiotics from the cell (228).

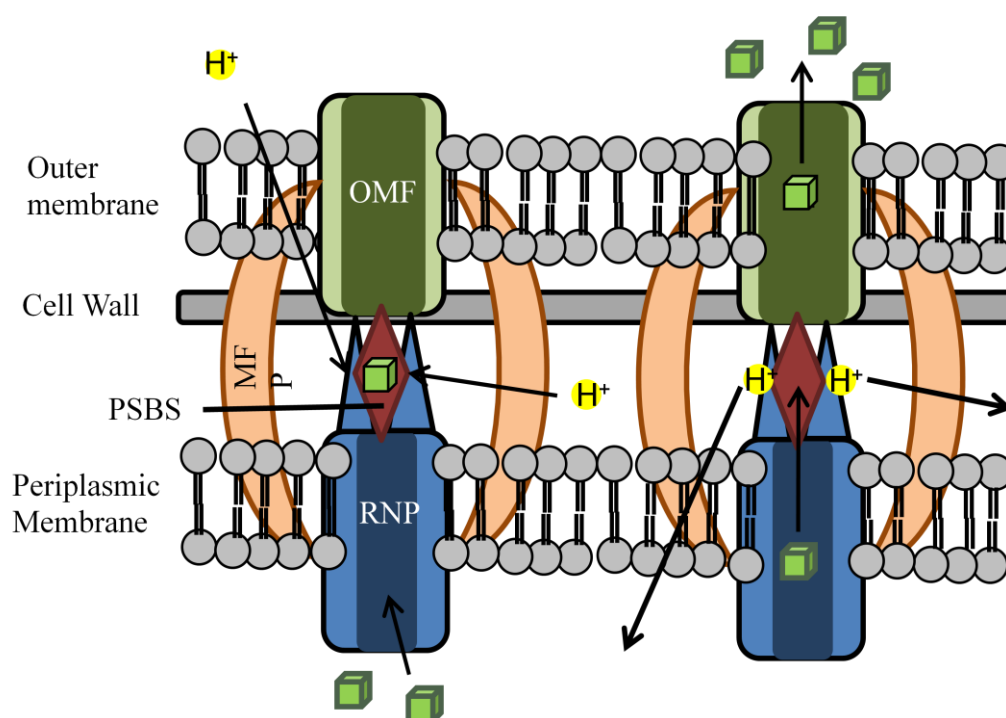


Figure 1.5b Illustration of resistance nodulation pumps of Gram-negative bacteria. The acronyms RNP, PSBS, MFP and OMF stand for resistance nodulation protein, periplasmic substrate binding site, membrane fusion protein and outer membrane factor, respectively.

Members of the SMR family consist of four α -helical protein domains, which span the inner membrane of bacteria (229). However, the SMR pumps function as a homodimer with two proteins, each made up of four α -helices, bound together in an antiparallel configuration. The mechanism behind SMR efflux is still not clear. However, multiple studies describe models in which a lipophilic toxic compound is taken into a protonated hydrophobic pocket of the protein. This de-protonates the binding site, causing the protein to alter its configuration so that the inner pocket is exposed to the external environment. The toxic compound is exchanged for proton ions in the extracellular environment and the SMR protein returns to its active intracellular state with its hydrophobic pocket facing the cytoplasm (230, 231).

The last proton motive force-dependent family, the MATE family, confers resistance to multiple cationic toxic compounds and can be found in both prokaryotic and eukaryotic species (224, 232). The MATE complex consists of an internal cation binding site which is open to the external environment. When a

cation, such as hydrogen or sodium ion, binds to the protein, it flips its configuration so that the binding site and cation is open to the cytoplasm. The cation is then exchanged for a cationic molecule, such as an AMP, the protein flips again, and the toxic molecule is expelled from the cell (Figure 1.5c:233) . In human renal cells, the MATE transporters have been shown to exchange sodium and hydrogen cations for harmful cationic molecules which are then expelled in the urine (234). Likewise, bacterial MATE proteins confer resistance to cationic compounds, such as fluoroquinolones and ethidium bromide, by exchanging these molecules with extracellular sodium ions (224, 235).

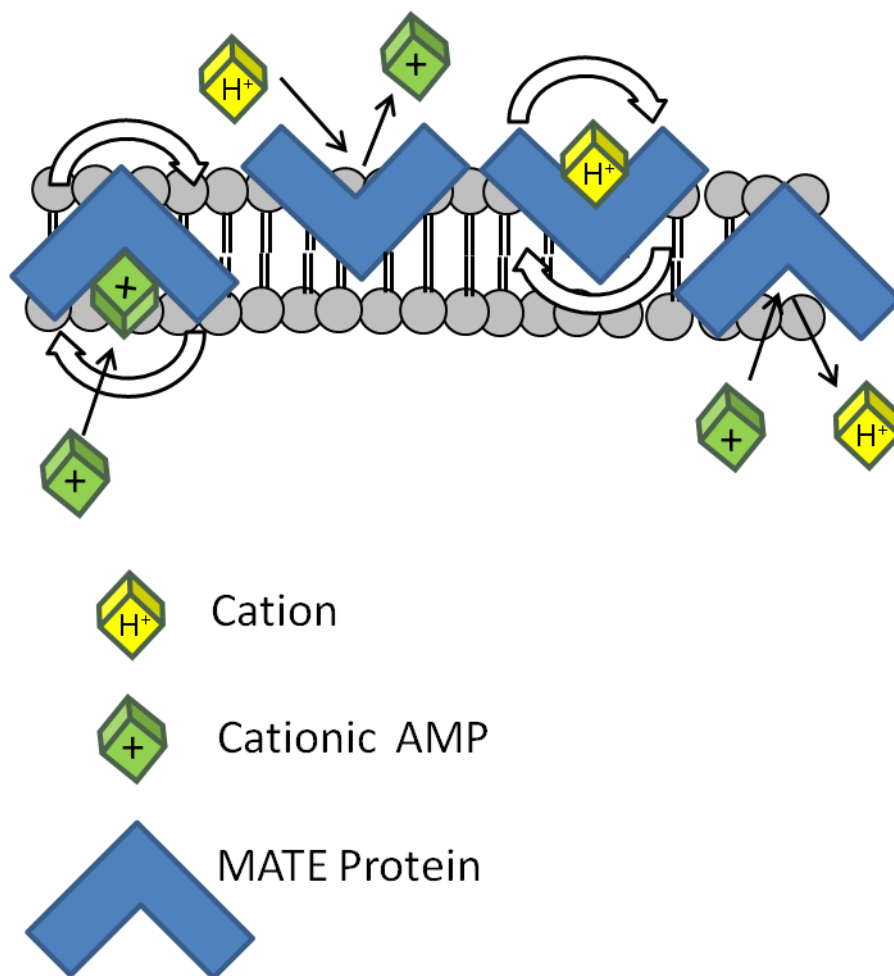


Figure 1.5c Illustration of the MATE efflux pump mechanism

ATP-binding cassette transporters differ from the other four classes of transporters because these transporters use the direct hydrolysis of ATP to drive efflux. Typically, ABC transporters are found in Gram-positive bacteria, where they remove endogenously produced toxic compounds (224). Their structures consist of two hydrophobic membrane-integrated domains and two hydrophilic

nucleotide binding domains which are situated in the cytoplasm (236). When a target substrate binds to the hydrophobic transmembrane domains, the nucleotide binding domains switch to an ATPase-active state. ATP is bound to each of the two nucleotide binding regions causing dimerization of the nucleotide binding regions. This forces the cytoplasmic-facing region of the transporters to close and the externally-facing region to open. ATP is hydrolysed, releasing phosphate molecules and ADP which allows the structure to return to its native state (Figure 1.5d: 237, 238).

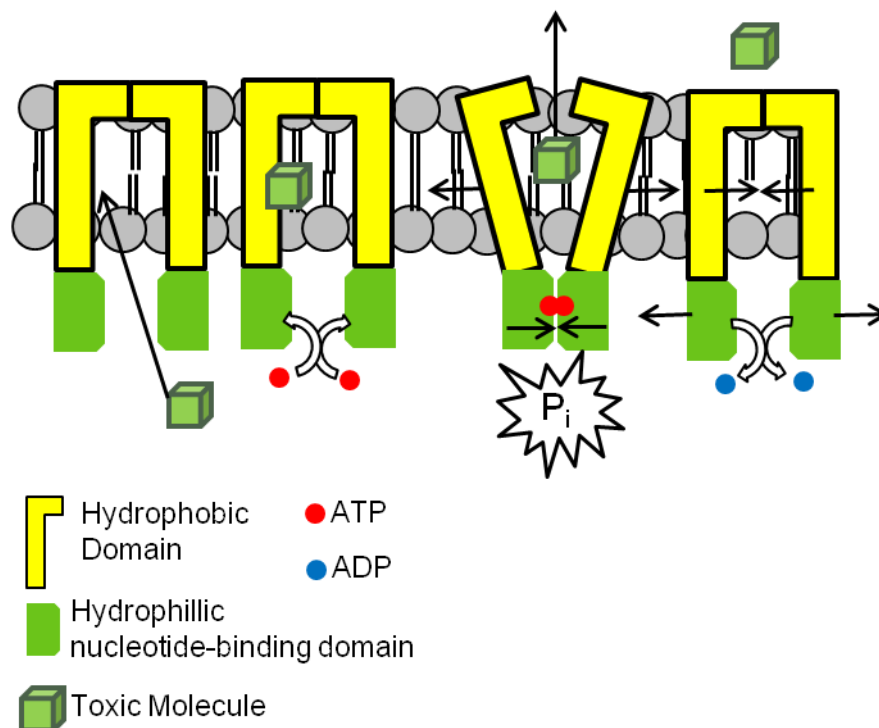


Figure 1.5d Illustration of the ABC-transporter mechanism

Efflux pumps provide a broad spectrum mechanism of resistance to antibacterial compounds in pathogens. Some of these drug exporters, such as the ABC transporters, which are over expressed in malignant cells and provide immunity to anticancer drugs, are also clinically relevant in normal human cells (239). Therefore, efflux pump inhibitors could have multiple roles in medicine. The multifaceted components of activation and mechanism of action of bacterial efflux pumps gives researchers a range of targets on which to base inhibitory molecules. Such targets include the expression of the efflux proteins, ATP for ABC-pump activation, proton motive force pathways and competitive binding to drug affinity sites of efflux transporters (240, 241). Combinational therapy with

efflux inhibitors and antimicrobial agents might be used effectively to treat bacteria which have developed broad resistance. It is possible that a novel mechanism might be created by covalently linking the antimicrobial agent with an efflux inhibitor. In the case of antimicrobial peptides, this could enable the inhibitor to readily enter the cell, while at the same time protecting the peptide from cellular efflux. However, it is unclear how the pharmacokinetics of this relationship would affect the pore forming ability of the peptide, or whether the complex would be able to separate in the cytoplasm so that each component can reach its target.

1.4.3.4 Two component regulation of resistance

The five basic senses of humans and animals make up our perception of the external environment and allow us to respond to changes accordingly. In most cases, bacterial perception of external stimuli comes from transmembrane regulatory proteins such as those in two-component regulatory systems (TCRS). Despite the name, TCRS can consist of two or more protein domains. However, the system generally consists of a membrane-bound histidine kinase sensor and a cytoplasmic phosphatase response regulator (242, 243). If the sensor domain of the histidine kinase is stimulated, it causes phosphorylation of a histidine residue in the carboxyl or transmitter terminal of the protein. The phosphoryl group is then attached to an aspartate residue in the receiver domain of the regulatory phosphatase. This causes the protein to undergo a conformational change, allowing it to affect its target organelle or gene and induce a proportionate cellular response to the external stimulus (242, 243). Most of the time these systems serve to regulate gene expression but they can also activate cellular responses to variations in the external environment through enzymatic reactions (243).

Two-component regulatory systems have been shown to regulate antimicrobial peptide and antibiotic-resistance pathways in multiple bacterial species, including *S. aureus* (244-246). In *S. aureus* the *graRS*, or antibiotic peptide sensor, TCRS has been shown to mediate resistance to a select range of AMPs and also to induce partial tolerance to vancomycin (247, 248). If a *graS* receptor is stimulated, *graR* phosphatase responds by inducing increased expression of the *vraFG*, *mprF* and *dlt* genes (244). As discussed above *mprF* and *dlt* mediate resistance to AMPs by decreasing the net anionic surface charge of the cells. This was achieved by modifying various constituents of the plasma membrane

(174, 192, 193, 249). The *vraFG* locus encodes an ABC transporter protein composed of an extracellular transmembrane domain (*vraG*) and an intracellular ATP-binding domain (*vraF*: 250). Falord *et al.* (250) have shown that *vraFG* permease responds to the presence of AMPs by signalling the *graS* histidine kinase to initiate resistance. As *graS* is an intracellular transferase, it is likely that *vraFG* stimulation induces *graRS*-mediated resistance rather than direct interactions of the AMP with the *graS* sensor domain (250). As a result, *vraFG* expression creates a positive feedback loop for itself in the presence of antimicrobial peptides. A similar resistance pathway, mediated by interactions between a TCRS (*bceRS*) and the permease of an ATP-binding cassette transporter (*bceAB*) has been detected in other *firmicute* bacteria: *Bacillus subtilis* and *Streptococcus mutans*(251).

Some Gram-negative bacteria also use multiple TCRS to resist AMPs. In *Salmonella enterica*, the *phoPQ* TCRS responds to the presence of AMPs by initiating the expression of the PmrD protein: which in-turn activates the PmrAB TCRS (252). The chief role of PmrAB is to increase the activity of the *ArnT* and *EptA* transferases and decrease the activity of the *LpxT* phosphotransferase. Collectively, this decreases the anionic charge of lipopolysaccharides and reduces the affinity of cationic AMPs for the bacteria (179). Although PmrAB-mediated resistance is observed in various bacteria, *phoPQ* activation may not be a necessary cofactor in every species (179, 245).

1.4.5 Limitations of Antimicrobial Peptides

Despite the intrinsic and developed AMP-resistance mechanisms of bacteria, in many cases AMPs remain an effective protective barrier against invasion by microorganisms. Despite this, the limitations of AMPs in their native forms suggest that it is unlikely that the peptides can be used therapeutically without physicochemical modifications. For example, AMPs are susceptible to enzymatic degradation and environmental conditions such as alkaline pH, salt and temperature extremes (93, 253). Peptide toxicity, antigenicity and the potential for cross-resistance against host peptides is also of concern to drug developers and therapeutic regulatory agencies. Consequently, only three out of the 2531 discovered AMPs, all of which come from the polymyxin group, have been approved for clinical use so far (254, 255). However, structure-function

relationship studies and research into hybrid synthetic peptides is providing strategies to overcome these limitations.

1.4.5.1 *In vivo* Stability of Antimicrobial Peptides

Antimicrobial peptides can either be constitutively released or secreted in response to infection (256, 257). Generally, high concentrations of AMPs are stored in the vesicles of granulocytes (257) presumably to protect the peptides from digestive enzymes and inhibitory molecules present in biological fluids. As discussed above, bacteria already have a host of proteolytic enzymes to evade AMPs. In addition, the half-life of peptides in the body is substantially reduced by host trypsin-like enzymes which cleave proteins adjacent to basic residues (258). To improve the resistance of cationic AMPs to enzymes that cleave at basic residues, Rozek *et al.* (258) added a cysteine residue to both ends of an indolicidin analogue. This circularized the peptide by disulphide bridge formation between the cysteine residues and protected the cationic arginine residues from enzymatic cleavage. Other strategies to reduce the affinity of enzymes for AMPs include using shorter peptide fragments or modified peptides with fewer potential enzyme binding sites, using d-enantiomers to limit interactions with proteases, and acetylation of the N-terminal and amidation of the carboxy terminals of the peptide (259, 260).

In the presence of salts and cationic organic molecules the activity of most AMPs is also greatly reduced (261, 262). It is believed that positively charged ions may compete with the peptides for anionic binding sites on the bacteria. Furthermore, monovalent ions such as sodium have been shown to bind deep within the lipid bilayers, near the lipid ester oxygens. This causes the lipids to cluster closer together, which increases membrane organisation and reduces lipid head group displacement by the peptides (262). Most AMPs have reduced activity at physiological salt levels. Consequently, diseases that disrupt salt homeostasis, such as cystic fibrosis, can inhibit peptide activity entirely (263, 264). Salt sensitivity appears to be dependent on two physiochemical characteristics of the peptides: structural stability, and cationic charge cluster density (264). The ability for peptides to form stable secondary structures when in the presence of a bacterial membrane is critical for their bactericidal activity. Multiple studies have shown that increasing the structural stability of the peptides by adding cysteine pairs, or by end capping α -helices with stabilizing motifs, increases the

antibacterial activity of the peptides under high salt conditions (264, 265). Although it is not clear how salts and cations affect the structure of the peptides, it appears that excess positively charged ions can change the conformation of some peptides which reduces their bactericidal activity (115, 265). Incorporating cationic motifs into an AMP sequence also increases the antibacterial activity of the peptides in high salt environments (264, 266). The higher density of cationic residues on the peptides may displace sodium ions that are shielding anionic regions of the membranes phospholipids (266). In a similar manner, overcoming the negative effects of salts on the binding efficiency of the peptides could be achieved by adding hydrophobic amino acids to the ends of the peptides. It is suggested that this increases the tendency for the peptides to bind and insert into the hydrophobic lipid bilayer, regardless of anion shielding by salts and other cations (267). Replacing smaller aromatic residues with bulky amino acids, such as substituting alanine with phenylalanine, L-(4,4'-phenyl)alanine or β -naphthylalanine, increases the antibacterial activity of the peptides in media with high salinity but also slightly increases their haemolytic activity (268). The bulky amino acid variants of alanine (Figure 1.6) are extremely hydrophobic and, therefore counteract the inhibitory effects of salt in the same manner as adding hydrophobic motifs to the ends of the peptides.

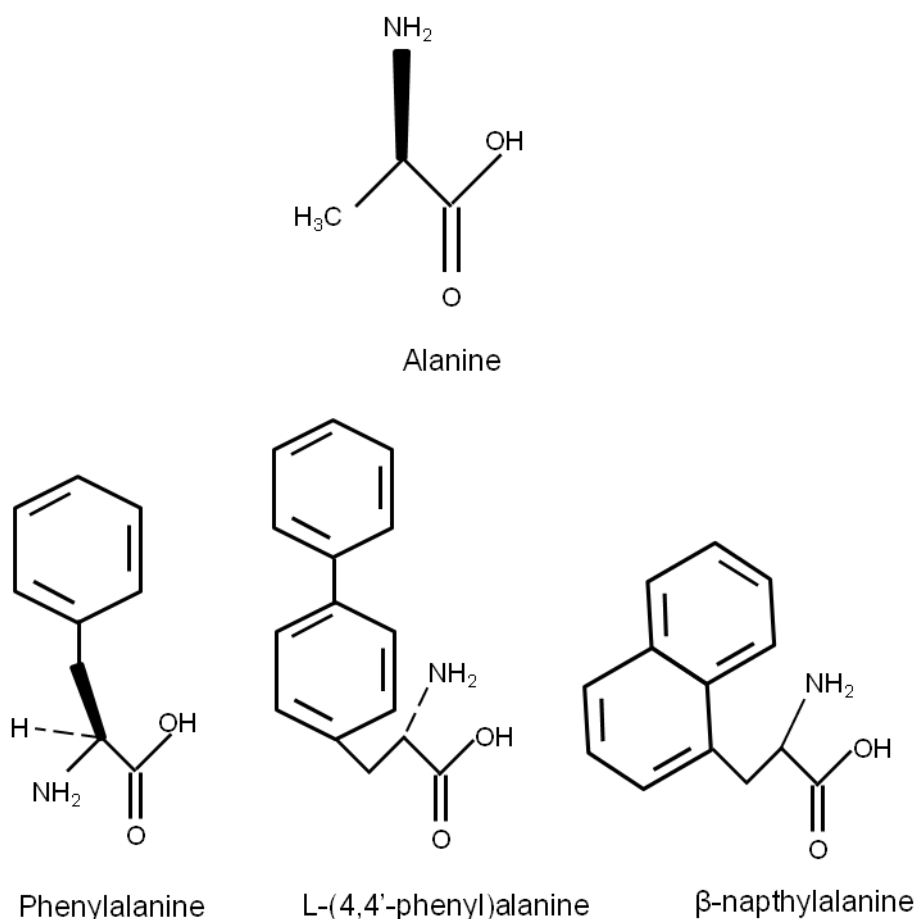


Figure 1.6 Bulky amino acid derivatives of alanine

The acid dissociation constant (K_a) measures the tendency of hydrogen atoms to dissociate from a molecule at different pH values (269). Multiple studies have shown that the bactericidal activity of AMPs, with varying pK_a values, is influenced by pH (270, 271). Antimicrobial peptides rich in histidine residues ($pK_a \approx 6$) such as the histatins and clavanins may be ineffective for therapeutic use in humans because physiological pH (≈ 7.4) reduces the overall cationic charge of the peptide (270). On the other hand, clavanins have been shown to have increased antimicrobial activity in acidic media (270). Therefore, to increase the antibacterial efficacy of peptides *in vivo*, it may be best to select those which have pK_a values one, or preferably two, units above that of the environmental pH in which they will be used. For example, in more alkaline environments such as the pancreas, AMPs which have a large proportion of amino acids with side chains that have high pK_a values, such as arginine (12.48), lysine (10.53), and

tyrosine (10.07), may be more likely to retain their cationic charge and therefore be more effective at targeting bacteria than peptides with low pK_a values.

1.4.5.2 Antagonistic potential of *in vivo* antimicrobial peptide treatment

Antimicrobial peptides seem to be semi-selective in that they do not interact with specific receptors but are structured to interact with the anionic charge and hydrophobic environment of cell membranes to bind to their target (272). The zwitterionic and cholesterol stabilised nature of mammalian cell membranes protects them from peptide attack (273). However, some AMPs can form lethal membrane pores in human cells and disable mitochondrial function if used at high concentrations (274). The cytotoxicity of AMPs comes mainly from their hydrophobicity, amphiphilicity and α -helical structures (118, 275, 276). Therefore, artificial peptides which are optimised to balance these characteristics, for optimum effect against bacterial membranes, could reduce the injurious side-effects of AMPs *in vivo*. Polyanski *et al.* (277) have shown that reducing the hydrophobicity of the N-terminal region of α -helical peptides, by adding cationic or polar amino acids, substantially reduces the toxicity of the peptides to erythrocytes and leukocytes (277). These modifications did not affect the antibacterial activity of the peptides against wild-type *E.coli* and *B. subtilis*. However, the modified peptides had reduced antibacterial activity against *S. aureus*. It was suggested that the *S. aureus* strain used in their study had a greater tendency than the other bacteria to reduce its outer surface negative charge so that its membrane charge more closely resembled a zwitterionic eukaryotic membrane than the other bacteria, which allowed the strain to evade the modified peptides (277).

Leucine zipper motifs are sequences of leucine and isoleucine residues that are spaced seven amino acids apart. As one α -helix has approximately 3.5 residues per turn, two leucine residues spaced seven amino acids apart will be located on approximately the same sides of the helix (278). It is believed that these motifs promote dimerization between peptides through the hydrophobic interactions of the leucines and isoleucines between peptides (Figure 1.7: 279). In a study by Asthana *et al.* (280) the leucine zipper motif of melittin was disrupted by substituting the sixth and thirteenth leucine residue with an alanine residue. This did not affect its antibacterial activity. However, on zwitterionic membranes, the modified melittin failed to form α -helices which resulted in reduced haemolytic

activity (280). It could be that the dimerization of melittin by leucine zippers influences secondary structure formation, membrane clustering and subsequent pore formation. On a zwitterionic membrane, the lack of electrostatic attraction and peptide dimerization mediated by leucine zippers could reduce clustering of these modified AMPs on the membrane and explain the absence of pore-forming activity.

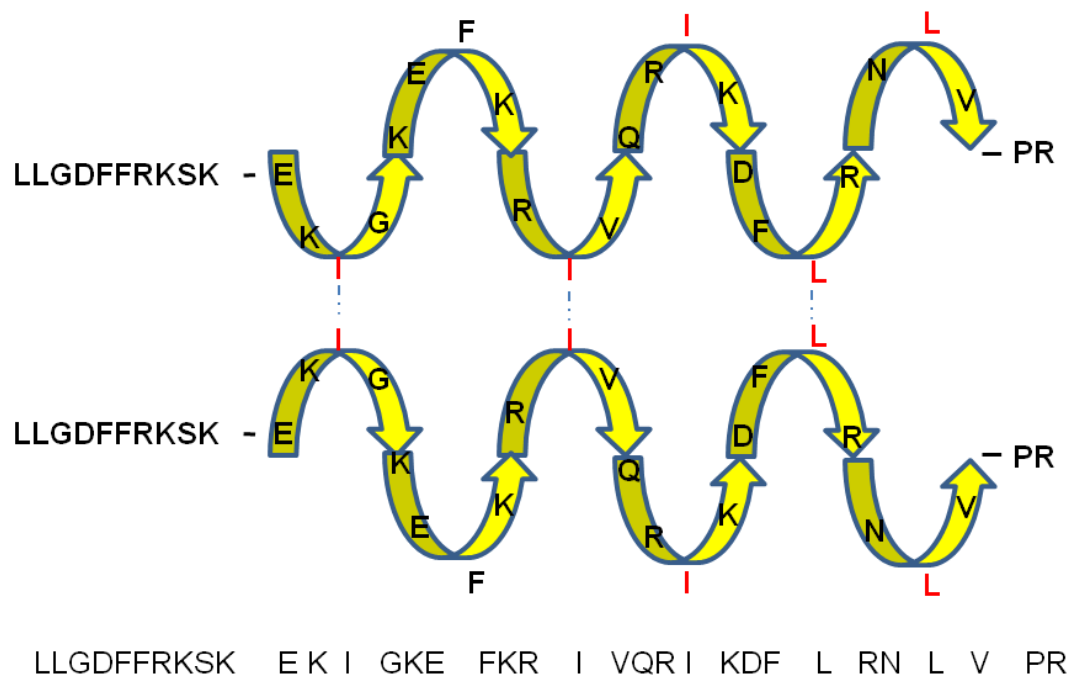


Figure 1.7 Leucine zipper dimerization between two LL37 peptides. Two LL37 peptides are shown in a potential dimer caused by leucine zipper motifs. Leucine (L) and isoleucine (I) which are coloured red indicate residues that likely participate in the zipper dimerization. Although the secondary structure of LL37 is α -helical, only the regions affected by the leucine zipper are shown as a yellow helix.

The multiple roles AMPs perform in the immune system illustrate the importance of these molecules to the host. Most AMPs target the bacterial membrane, which allows them to attack a broad variety of pathogens (99, 102). However, having a mode of action which is shared among the majority of AMPs also enhances the risks of developing widespread cross-resistance. This has already been demonstrated in *S. aureus* species which developed point mutations in their

mprF domain for daptomycin resistance and, in turn, became resistant to a variety of structurally distinct AMPs (281). In addition, resistance to pexiganan conferred intermediate levels of resistance to α -defensin: a human defence peptide with contrasting structure and mechanism of action (282). Pathogens that have invaded a multicellular organism have probably developed means to evade AMPs. If AMP-based therapeutics encourages wide-spread development of resistance, this could result in the weakening of a critical part of the immune system, resulting in an increased incidence of recalcitrant systemic infections. The antigenicity of foreign or synthetic AMPs could also complicate clinical treatment by activating the immune system. However, it appears that some AMPs have little or no antigenic potential, which may be due to their small size (283-285). Surprisingly, this is not the case when the peptides are used as an adjuvant for an antigenic molecule for vaccine therapy. Co-administration of an AMP with a subunit antigen has been shown to increase the production of antibodies by the humoral immune system as well as augmenting the activity of cytotoxic T cells (286-288). The risks of AMP use *in vivo* are serious and some consequences, like cross resistance to other peptides, will be difficult to mitigate as they relate directly to the mode of action of the peptides. However, in the context of an almost inevitable epidemic of total drug resistant pathogens (24, 25, 289), the availability and effectiveness of AMPs is too valuable to dismiss without trying to offset their negative characteristics.

1.4.6 Antimicrobial Peptides used in this Study

In this study, various aspects of three antibacterial peptides: melittin (280, 290), bac8c (291), and the anionic peptide, AP1 (292), were investigated. Melittin is a highly lytic peptide that has similar primary and secondary structure to many other peptides (132, 290, 293, 294). Two truncated analogues of melittin, which are claimed to retain bactericidal activity, but to have reduced haemolytic activity, were also investigated (295, 296). Bac8c is a truncated variant of bactenecin and is one of the shortest AMPs with potent, broad-spectrum activity (297). Peptide AP1 is an anionic synthetic peptide that was designed to form oblique α -helical secondary structures (292). In this study a variety of different parameters that have relevance in the design and implementation of AMPs in clinical therapy were investigated using peptides with different physicochemical and functional characteristics.

1.4.6.1 Melittin

Melittin is a 26 amino acid peptide, which constitutes the main component of honey bee venom (290). It has a cationic charge of +5 with four of its five basic residues situated at positions 21-24 in a lysine-arginine repeat. The N-terminal region of the peptide has a high proportion of hydrophobic residues with the first six amino acids being hydrophobic and the remainder scattered through the central region of residues 7-20. This pattern of a hydrophobic N-terminal and a cationic C-terminal is common among pore-forming α -helical peptides and appears to be an important factor in maintaining the amphiphilic α -helical configuration adopted when bound to lipid bilayers (132, 290). The amphiphilic α -helices allow melittin to insert into and lyse eukaryotic and prokaryotic membranes (110). Many groups have attempted to improve the specificity of melittin for bacterial membranes by hybridisation with other AMPs, truncating the peptide, and/or inserting or substituting amino acids at critical sequence points (295, 296, 298). A hybrid peptide composed of the first eight amino acids from the N-terminal of cecropin A, which has four basic and four hydrophobic residues, and the twelve amino acid hydrophobic N-terminal of melittin, was shown to have greater antimicrobial activity, a broader spectrum of prokaryotic targets than either of the wild-type AMPs, and reduced haemolytic activity (299). Cecropin A does not adopt a secondary structure in the presence of zwitterionic membranes. Similarly, the melittin-cecropin A hybrids show only a slight tendency to adopt α -helical configuration on neutral membranes (300). This may explain why the hybrid peptide shows reduced lytic activity on erythrocyte membranes. However, Schlamadinger *et al.* (300) have shown that the partially folded structure of the hybrids can still insert into zwitterionic lipid vesicles, which suggests that the peptide may still be toxic to some eukaryotic cells at high concentrations. Sun *et al.* (296) have reported that deleting eleven residues from the hydrophobic N-terminus of melittin eliminated its haemolytic activity but did not affect its bactericidal activity. In the same study, removal of the two glutamate residues from the carboxyl terminal did not affect the haemolytic or bactericidal activity of the truncated peptide (296). Collectively, these studies concluded that optimisation of melittin sequences can generate analogues that have a better therapeutic index than wild-type peptides and the same principals could be applied to other peptides to produce effective therapeutics.

1.4.6.2 Bac8c

The benefits of developing derivatives of natural peptides are demonstrated by the truncated and modified bactenecin peptide, bac8c. Bactenecin is a cyclic AMP, found in the granules of bovine neutrophils (301). Bac8c consists of residues 4-11 of bactenecin, with multiple substitutions, including replacement of the carboxyl terminal cysteine with an alanine residue (291). Removal of the N-terminal and carboxyl terminal cysteine residues from bactenecin linearises the peptide in solution and on bacterial membranes. This change in secondary structure is believed to be the reason for the increased antimicrobial activity of the peptides against Gram-positive bacteria and reduced haemolytic activity (302). The main mechanism by which bac8c damages bacterial cells is by membrane destabilisation and permeation. At concentrations below its minimal lethal concentration (MLC) it has also been shown to arrest cell growth by disrupting the proton motive force across the membrane and reducing the synthesis of ATP, NAD/NADH and proteins (297). Bac8c has also been shown to effectively inhibit the growth of cells in biofilm. Additionally, in *Streptococcus mutans*, bac8c represses the *gtfBC* genes, that encode glycosyltransferase which metabolizes sucrose for biofilm formation. It also represses expression of *LuxS* and *ComDE* genes, which allow interspecies and intraspecies interactions in biofilms respectively (302). This suggests that the peptide can inhibit the growth of cells in the protective biofilm environment and can also suppress the development of biofilm by gene repression.

1.4.6.3 AP1

The abundance of cationic AMPs in nature appears to have selected for resistance mechanisms in pathogens that seem specifically evolved to counteract the positive charge of the peptides. In response, organisms susceptible to pathogenic infection have developed anionic antimicrobial agents that complement the action of cationic peptides which have become ineffective against bacteria with zwitterionic membranes (292, 303). In humans, anionic AMPs are used by the innate immune system to protect the brain, respiratory tract, skin and gastro intestinal tract from a narrow spectrum of pathogens (303). Compared to research focused on cationic AMPs, research into the mechanisms

of action of anionic AMPs has been limited. However, it has been documented that some anionic AMPs have membranolytic activity as well as antibacterial mechanisms that operate inside the bacterial cell (106, 303). The peptide AP1 was chosen as a model anionic AMP for investigations of antibacterial function. AP1 is a synthetic α -helical peptide with a glutamic acid residue at each terminal and a predominantly hydrophobic carboxyl terminal region, which gives the peptide a net charge of -2. Apart from the negative charge, AP1 is similar to other α -helical peptides and appears to rely on its uneven distribution of hydrophobic residues to form amphiphilic secondary structures on bacterial membranes. This asymmetrical hydrophobic gradient of the helices allows the peptide to insert into the membrane and form lethal transmembrane pores (292). AP1 has been shown to be bactericidal to *S. aureus* and *E. coli* only at relatively high concentrations (4.2 mg/ml: 292). However, to effectively target the anionic bacterial membrane most anionic AMPs require divalent cationic cofactors, such as zinc or magnesium, to form cationic salt bridges between the peptide and negatively charged membrane components (114, 115, 303). This may explain why a concentration of 4.2 mg/ml of AP1 was required to kill *S. aureus* and *E. coli* in the study conducted by Dennison *et al* (292). In addition, bacteria which mask their anionic membrane charge to evade cationic AMPs could become more susceptible to anionic peptides even without functional cofactors. Therefore a combinational, but separately administered, treatment of anionic and cationic AMPs might effectively eliminate an infection and reduce the likelihood of developing resistance.

1.5 Aims and Significance of Project

Antibiotic resistance is not an emerging threat but rather a current impediment to medical treatment (26). Global strategies to counteract antibiotic resistance by reducing usage and promoting novel drug development are steps in the right direction. However, the continued exposure of pathogens to antimicrobial drugs, as a consequence of continued abuse in medicine and agriculture, is perpetuating the development and dissemination of more efficient resistance strategies, against a broader range of antimicrobials (26). The broad spectrum and rapid killing mechanism of action of AMPs could turn around this current dearth in the production of effective antimicrobial agents. However, there are conflicting reports on whether resistance to peptides could develop in the same

way that antibiotic resistance in pathogens developed. In this study the aim was to investigate important features that could govern the therapeutic potential of a variety of model peptides against MRSA and, if possible, to identify antibacterial peptides in a variety of plant and invertebrate species. A primary interest was to examine the potential for *S. aureus* to develop resistance to AMPs, identify changes that mediated resistance, and to investigate how this resistance might be counteracted. In addition, this project also examined the possibility that current bactericidal strategies such as antibiotic therapy and silver bandage treatment of wounds might be augmented with AMPs. From these experiments, new strategies were developed and explored to enhance the bactericidal potency of the peptides by themselves and as adjuncts with other antimicrobial agents and devices against wild-type and AMP resistant MRSA. The results from this study will hopefully assist in identifying potential limitations and risks of using AMPs as therapeutic agents, and whether these peptides may become a potent tool against pathogens in the post-antibiotic era.

2. Materials

2.1 Chemicals, Reagents and Equipment

Table 2.1 Chemicals and suppliers

Chemical/Reagent	Supplier
Agarose (Molecular Grade)	Electran
Attune Focusing Fluid	Life Technologies
Bacteriological Grade Agar	VWR Chemicals
Bactopeptone	Oxoid
Bacterisense 645 Molecular Probes	Perkin Elmar
Brain Heart Infusion Media	Oxoid

Brij 58®	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
Boric Acid	Sigma-Aldrich
Chloroform	Chem-Supply
Chromosomal Grade Agarose	Bio-Rad
Dimethyl Sulphoxide (DMSO)	Fischer Bioreagents
DNA Marker (100bp-Ladder)	New England Biolabs
Eosin Y	Sigma-Aldrich
Ethidium Bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Ajax Chemicals
Gel Green	Biotium
Glutaraldehyde	Asia Pacific Specialty Chemicals
Glycerol	Ajax
HCl	Sigma-Aldrich
KCl	VWR Chemicals
KH ₂ PO ₄	Sigma-Aldrich
Lysine	Sigma-Aldrich
MgCl ₂	VWR
MyTaq Polymerase	Bioline
MyTaq Reaction Buffer	Bioline
NaCl	Amresco
NaH ₂ PO ₄	Sigma-Aldrich

NaOH	Sigma-Aldrich
Pierce BCA Protein Assay	Thermo Scientific
Trypan Blue	Astral Scientific
Pulse-Field Certified Agarose	Bio-Rad
Sodium deoxycholate	Merck Millipore
Sodium dodecyl sulphate (SDS)	MP Biomedicals
Sodium lauroyl sarcosinate	Sigma-Aldrich
Triton X-100	Ajax
Trizma® (Tris Base)	Sigma-Aldrich
Tryptone	Oxoid
Tween-20	Ajax
Undenatured ethanol	Scharlau
Xylene Cyanol FF	Sigma-Aldrich
Yeast Extract	Oxoid

Table 2.2 Enzymes

Enzyme	Supplier
Lysostaphin	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Protease OB	Omega Bio-Tek
Proteinase K	Roche
Restriction Endonuclease <i>Sma</i> 1	Promega

Trypsin

VWR

 All enzymes were supplied in solution and stored at -20°C prior to use

Table 2.3 Equipment and manufacturer

Equipment	Model	Manufacturer
Biopsy punch	BP-30F	Kai Medical
Blood collection vessel	Vacutainer Rapid Serum Tube	BD
Cell disrupter	HAIVA	Constant Systems
Cell homogeniser	FastPrep FP120	Thermo Scientific
Centrifuge	Allegra X-12R	Beckman Coulter
Cellulose membrane filter (0.2µm)	GSWP29325 MF-Millipore	Merck Millipore
Electrophoresis apparatus	PowerPac 3000	Bio-Rad
Electroporation cuvettes (0.1cm gap)	Gene Pulser	Bio-Rad
Electroporation system	Micropulser Electroporation Apparatus	Bio-Rad
Flow cytometer	Acoustic Focusing Flow Cytometer	Life Technologies
Freeze dryer	Scanvac Coolsafe	Labogene
Haemocytometer	Bright-Line Haemocytometer	Hausser Scientific

pH meter	Ultrabasic-10	Denver Instruments
Pulse field electrophoresis system	CHEF-DR Variable Angle pulse-field electrophoresis system	Bio-Rad
Scanning electron microscope	40ESB Crossbeam	Zeiss
Spectrophotometer	Nanodrop 1000	Thermo Scientific
Spectrophotometric plate reader	Victor 3V Multilabel Plate Reader	Perkin Elmar
Thermo cycler	Veriti 96-well	Applied Bioscience
UV transilluminator	Gel Doc XR+	Bio-Rad
Water purification system	Alpha Water Purification System	Ibis

2.2 Bacterial Strains

All *S. aureus* strains were obtained from Dr Frances O'Brien and Professor Warren Grubb, Australian Collaborating Centre for *Enterococcus* and *Staphylococcus* species Typing and Research (ACCESS), Faculty of Health Sciences, School of Biomedical Sciences, Curtin University, Perth Western Australia. A summary of the strains used, their origins and resistance profiles is presented in Table 2.4

Table 2.4 Characteristics of *S. aureus* strains

Strain	Epidemiology	Antimicrobial Resistance Phenotype	Reference
Aus3	<p>Mercuric acetate and mercuric chloride resistant clone of ST239-MRSA-III: a multidrug resistant epidemic-MRSA (EMRSA) predominantly found in central and eastern Australia. Aus3 is the most prevalent EMRSA isolated from Victorian and Tasmanian patients. It has a low frequency of identification ($\approx 1\%$) in Western Australia.</p>	<p>ERY,TCN, TMP, GEN, CIPX, HgOAc₂, HgCl₂,PMA</p>	304, 305
Bengal Bay (BB)	<p>ST772-MRSA-V origin. Originally a community associated (CA-MRSA) strain isolated in India in 2004. Bengal bay has spread globally and accounted for $\approx 1\%$ of CA-MRSA infections in Australian in 2011. Produces the Panton-Valentine leukocidin (PVL) cytotoxin.</p>	<p>OXA, GEN, ERY, COT</p>	306
WBG 8287	<p>Genetic lineage ST1-MRSA-Iva. The prefix WBG indicates the strains were obtained from Professor Warren Grubbs group. Ancestral clones of this group were identified in Aboriginal communities in the Kimberley region in the 1980s. First isolated from a diabetic foot ulcer in 1996, WBG 8287 became infamous as the first CA-MRSA to cause a nosocomial epidemic. It is now the most prevalent CA-MRSA in</p>	<p>OXA, ERY, CIPX, FA, RFP,GEN, TCN,MUP</p>	304

Western Australia. Contains
pWBG750 plasmid which encodes β -
lactamase, cadmium resistance and
bacteriocin production.

W17S	<p>ST93-MSSA lineage: most prevalent methicillin susceptible <i>S. aureus</i> (MSSA)-group identified in West Australian aboriginal communities. First isolated from the epidermal layers of a patients hand in 1995, W17S has now displaced most other CA-MSSA. Produces the PVL toxin.</p>	NR ^a	307
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Antibacterial Resistance: ERY: Erythromycin, TCN: Tetracycline, TMP: Trimethoprim, GEN: Gentamicin, CIPX: Ciprofloxacin, HgOAc₂: Mercury acetate, HgCl₂: Mercury chloride, PMA: Phenylmercuric acetate, OXA: Oxacillin, COT: Cotrimoxazole, FA: Fusidic acid, RFP: Rifampicin, MUP: Mupirocin

^aNone reported

2.3 Highly Purified Water

Water used in this study was purified using an Ibis Alpha water purification system which produces high purity water (HpH₂O) with resistivity readings around 15 MOhm/cm.

2.4 Buffers

Phosphate Buffered Saline (PBS)

NaCl	8.01 g/l
KCl	0.20 g/l
NaH ₂ PO ₄	1.78 g/l
KH ₂ PO ₄	0.27 g/l

Reagents were dissolved in 800 ml of H_2O , adjusted to pH 7.4 with HCl and then made up to 1 litre with H_2O . The PBS solution was sterilized by autoclaving at 121°C for 15 minutes.

Phosphate Buffer (PB)

KCl	0.20 g/l
NaH_2PO_4	1.78 g/l
KH_2PO_4	0.27 g/l

Reagents were dissolved in 800 ml of H_2O , adjusted to pH 7.4 with HCl and then made up to 1 litre with 200 ml of H_2O . The PB solution was sterilized by autoclaving at 121°C for 15 minutes.

Extraction Buffer JEB1

NaCl	5.85 g/l
Glycerol	25% v/v
DMSO	1% v/v

Extraction Buffer JEB2

NaCl	5.85 g/l
Glycerol	25% v/v
DMSO	1% v/v
EDTA	29.2 g/l

All extraction buffer reagents except DMSO were dissolved in 500 ml H_2O and autoclaved for 15 minutes at 121°C. After sterilization, DMSO was added and the extraction buffers were stored at room temperature prior to use.

20x SB Buffer

Boric acid	48 g/l
NaOH	8 g/l

Reagents were dissolved in 1 litre HpH₂O. When used in electrophoresis experiments, 50 ml of the stock solution was added to 750 ml of HpH₂O adjusted to pH8. The solution was then made up to 1 litre with HpH₂O.

EC Buffer

Tris Base	0.73 g/l
NaCl	58.44 g/l
EDTA	29.22 g/l
Brij 58	0.5% wt/v
Sodium deoxycholate	0.2% wt/v
Sodium lauroyl sarcosinate	0.5% v/v

Sodium lauroyl sarcosinate, Brij and sodium deoxycholate were dissolved in 800 ml warm ($\approx 60^{\circ}\text{C}$) HpH₂O. The rest of the reagents were added and the pH was adjusted to 7.5. HpH₂O was added to a final volume of 1 litre and the buffer was stored at room temperature.

EST Buffer

Tris Base	0.61 g/l
EDTA	146.12 g/l
Sodium lauroyl sarcosinate	1% v/v

Sodium lauroyl sarcosinate was dissolved in 800 ml warm HpH₂O after which, Tris and EDTA were added to the solution. The pH was adjusted to 7.5 and the solution was made up to 1 litre.

20x TBE Buffer

Tris Base	121.14 g/l
Boric Acid	61.83 g/l
EDTA	5.85 g/l

When used in contour clamped homogeneous field electrophoresis (CHEF) experiments, 50 ml of the stock solution was added to 1950 ml of H_2O .

Cell Lysis Buffer

Tris Base	1.214 g/l
EDTA	0.29 g/l
SDS	1% w/v
Triton X-100	2% v/v

Tris, EDTA and SDS were added to 800 ml H_2O and the solution was sterilised by autoclaving at 121°C for 15 minutes. Triton X-100 was added, the buffer was made up to 1 litre with H_2O and then filter sterilised through a 0.2 μm cellulose membrane filter (Merck Millipore).

DNA Loading Buffer (6x)

Glycerol	30% v/v
Bromophenol Blue	2.5 mg/ml
Xylene Cyanol FF	2.5 mg/ml

The reagents were dissolved in 7 ml H_2O . When the reagents had dissolved the volume was adjusted to 10 ml and stored at room temperature.

2.5 Growth Media

Lysogeny broth (LB) was made using 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl. The LB media components were mixed with 800 ml of HpH₂O, adjusted to pH 7.4 using an Ultrabasic-10 pH meter (Denver Instruments) and NaOH and then made up to 1 litre by adding 200 ml of HpH₂O. The agar growth medium used in these experiments was 25 g/l of bactopectone in HpH₂O, supplemented with 1.5% w/v bacteriological agar (VWR). Twenty millilitres of molten agar was poured into each 94 mm petri dish to form the solid bacteriological peptone agar plates. All growth media were autoclaved at 121°C for 15 min and stored at 4°C prior to use.

2.6 Antibiotics

All antibiotic discs were purchased from Oxoid. The antibiotics used and their concentrations are presented in table 2.5. Fusidic acid was supplied as sodium fucidin powder from (CSL Biotherapies). Once solubilised in HpH₂O, the stock and working solutions were stored at -20°C.

Table 2.5 Antibiotic discs

Antibiotic	Conc. (µg/disc)	Antibiotic	Conc. (µg/disc)
Ampicillin	10	Nitrofurantoin	5
Amoxicillin	30	Norfloxacin	10
Augmentin	60	Oxacillin	1
Cefaclor	30	Penicillin	9.91
Cefoxitin	10	Polymyxin B	35.7
Cephalexin	100	Rifampicin	1
Ciprofloxacin	2.5	Streptomycin	10

Chloramphenicol	10	Sulfamethoxazole	25
Erythromycin	15	Tetracycline	10
Fusidic Acid	10	Ticarcillin	75
Gentamicin	10	Tobramycin	10
Imipenem	10	Trimethoprim	5
Kanamycin	30	Vancomycin	5
Nalidixic acid	30		

2.7 Biological Tissue Tested for Antimicrobial Activity

Table 2.6 lists of all the plant, fungal and invertebrate tissue tested for antimicrobial activity. Also included is the location from where the sample was obtained, and the weight of the sample per ml of buffer used in the extraction.

Table 2.6 Plant, fungal and invertebrate samples

Species	Common Name	Tissue	Origin	Concentration (mg/ml)
<i>Agaricus xanthoderma</i>	Yellow-staining mushroom	Head	Curtin university grounds	667
<i>Agaricus xanthoderma</i>	Yellow-staining mushroom	Stem	Curtin university grounds	667
<i>Aloe vera</i>	Aloe vera plant	Leaf	Local Market	200
<i>Amanita xanthocephala</i>	Vermillion amanita	Head	Curtin university grounds	279

<i>Amanita xanthocephala</i>	Vermillion amanita	Stem	Curtin university grounds	222
<i>Armillaria luteobubalina</i>	Australian honey fungus	Head	Curtin university grounds	446
<i>Armillaria luteobubalina</i>	Australian honey fungus	Stem	Curtin university grounds	535
<i>Brassica oleracea</i>	Cabbage	Leaf	Curtin university grounds	667
<i>Capscium chinese</i>	Bhut jolokia chilli	Seeds	Wild Fire	106
<i>Capscium chinese</i>	Bhut jolokia chilli	Fruit	Wild Fire	403
<i>Citrus latifolia</i>	Persian Lime	Fruit	Local Market	300
<i>Cucumis sativus</i>	Cucumber	Fruit	Local Market	667
<i>Elettaria cardamomum</i>	Green cardamom	Seeds	Local Market	300
<i>Humulus lupulus</i>	Hop plant (Cascade)	Flower	Gage Roads Brewery	250
<i>Humulus lupulus</i>	Hop plant (Citra)	Flower	Gage Roads Brewery	250
<i>Humulus lupulus</i>	Hop plant (Chinook)	Flower	Gage Roads Brewery	250
<i>Humulus lupulus</i>	Hop plant (Summer)	Flower	Gage Roads Brewery	250

<i>Humulus lupulus</i>	Hop plant (Topaz	Flower	Gage Roads Brewery	250
<i>Juniperus comminus</i>	Juniper berry	Berry	Local Market	300
<i>Lucilia sericata</i>	Green bottle fly	Larva	Pathology West	100
<i>Mentha spicata</i>	Spearmint	Leaf	Local Market	200
<i>Musa acuminata</i>	Cultivated banana	Skin	Local Market	667
<i>Musa acuminata</i>	Cultivated banana	Fruit	Local Market	667
<i>Persea americana</i>	Avocado	Fruit	Local Market	1136
<i>Pisolithus tinctorius</i>	Horse dung fungus	Head	Curtin university grounds	667
<i>Pisolithus tinctorius</i>	Horse dung fungus	Stem	Curtin university grounds	667
<i>Pycnoporus coccineus</i>	Scarlet bracket	Head	Curtin university grounds	323
<i>Solanum lycopersium</i>	Common truss tomato	Fruit	Local Market	300
<i>Tulbaghia violacea</i>	Society garlic	Leaf	Local Market	200
<i>Tulbaghia violacea</i>	Society garlic	Bulb	Local Market	200

2.8 Synthetic Antimicrobial Peptides

Table 2.7 shows the AMPs used in this study, their amino-acid sequence and electrostatic charge. All peptides were synthesised and purchased from Mimotopes in powder form. Peptides were dissolved in H_2O and absorbance at 280nm was measured using a Nanodrop spectrophotometer (Thermo Scientific). The concentration of the peptide in solution was then calculated using the following formula: $X = (A^{280} \div MW^p) \div [(n^w \times 5500) + (n^y \times 1490)]$ where (X) is the concentration of the peptide, (A^{280}) is the measured absorbance of the peptide solution at 280nm, (MW^p) is the molecular weight of the peptide, (n^w) is the number of tryptophan residues in the peptide sequence and (n^y) is the number of tyrosine residues in the sequence. Using this formula, it was possible to accurately predict the concentration of the peptide in solution by accounting for the absorption bias of the aromatic rings of tryptophan and tyrosine. Peptide stock solutions were kept at -20°C prior to use.

Table 2.7 Antimicrobial peptides

Peptide	Sequence	Charge
AP1	GEQGALAQFGEWL-OH	-2
Bac8c	RIWVIWRR-OH	+3
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-OH	+5
Melfrag12-24	GLPALISWIKRKR-OH	+4
Melfrag12-26	GLPALISWIKRKRQQ-OH	+4
Melfrag12-26-L1	LLPALISWIKRKRQQ-OH	+4

2.9 Primers

Primers for *fusB* and *fusC* genes were obtained from Geneworks and were diluted to 100 μ M in HpH₂O before use. Table 2.8 shows the sequence of the primers as well as the Genbank accession number of their corresponding sequence and their location in the sequence. Forward primers were designed to match the sequence of the sense-strand, whereas the reverse primers were designed to be complimentary to the sense strand of the DNA.

Table 2.8 Primer sequences

Primer	Sequence (5'->3')	Accession Number	Location (nt #)
FusB-F	CGATATGAATTCCCATAAAACACA	CP003193.1	1414 1437
FusB-R	GAGTGAGGTACAAAATATAACAACCC	CP003193.1	2011 2036
FusC-F	GATAAATCTTGATTCGGATGCTG	KF527883.1	22073 22095
FusC-R	CTAAAGATCAGGGCTGTTTGG	KF527883.1	22990 23011

2.10 Pig Skin Preparation

Pig skin, which included the epidermis, dermis and sub-dermal fat layer was purchased from a local abattoir. The fat layer was removed from the dermis and the skin was cut into 2cm x 2cm squares. The skin was sterilised in 70% undenatured ethanol for 1h, washed with HpH₂O and allowed to dry under UV bactericidal lights of a biological safety cabinet. The skin was placed in molten bactopectone agar 2mm deep to immobilise and rehydrate the sterilised skin. The plates were stored at 4°C until needed.

2.11 Acticoat

Acticoat™ (Smith and Nephew) was generously donated by Dr Fiona Wood and the burns department of Fiona Stanley Hospital, Perth, Western Australia.

2.12 Details of suppliers

Table 2.9 Suppliers details

Name of Supplier	Suppliers Address
Ajax Chemicals	Supplied By: Rowe Scientific Pty Ltd 11 Challenge Boulevard Wangara, Perth, W.A, 6065 Australia
Amresco	Amresco LLC 6681 Cochran Road Solon, Ohio, 44139 USA
Applied Bioscience	Supplied By Life Technologies Australia Pty Ltd 30-32 Compact Circuit Mulgrave, Melbourne, VIC, 3170 Australia
Asia Pacific Specialty Chemicals	Asia Pacific Specialty Chemical Ltd 9 Short Street Auburn, Sydney, NSW, 2144 Australia
Astral Scientific	Astral Scientific Pty Ltd

	29 Mangrove Lane Taren Point, Sydney, NSW, 2229 Australia
Bioline	Bioline (Aust) Pty Ltd PO Box 122 Alexandria, Sydney, NSW, 2015 Australia
Bio-Rad	Bio-Rad Laboratories Pty Ltd Level 5, 446 Victoria Road Gladesville, Sydney, NSW, 2111 Australia
Bright-Line	Supplied by Sigma Aldrich PO Box 970 Castle Hill, Sydney, NSW 1765 Australia
Chem-Supply	Chem-Supply Pty Ltd PO Box 201 38-50 Bedford Street Gill Man, Port Adelaide, S.A, 5013 Australia
Constant Systems	Constant Systems LTD Low March Daventry Northants, NN11 4SD UK
CSL Biotherapies	CSL Corporate Registered Head Office 45 Poplar Rd

	Parkville, Victoria, NSW, 3052 Australia
Denver Instruments	Denver Instrument 5 Orville Dr Bohemia, New York, 11716 U.S.A
Electran	Supplied By: VWR International Pty Ltd Unit 1/31 Archimedes Place Murarrie, Brisbane, QLD, 4172 Australia
Fischer Bioreagents	Thermo Fischer Scientific Australia Pty Ltd 5 Carribbean Drive Scoresby, Melbourne, VIC, 3179 Australia
Gage Roads	Gage Roads Brewing Corporate 14 Absolon Street Palmyra, Perth, W.A, 6157 Australia
Geneworks	Geneworks Pty Ltd 28 Dalgleish St Thebarton, Adelaide, SA, 5031 Australia
Hausser Scientific	Hausser Scientific Company 935 Horsham Road: Suite C Horsham, Pennsylvania, 19044 U.S.A

Ibis Technology	<p>Ibis Technology</p> <p>24/70 Roberts Street</p> <p>Osborne Park, Perth, WA, 6017</p> <p>Australia</p>
Kai Medical	<p>Kai Europe GmbH</p> <p>Kottendorfer Str 5</p> <p>Solingen 42697</p> <p>Germany</p>
Labogene	<p>Supplied by Bio-Strategy Pty Ltd</p> <p>Unit 3, 21 Lakeside Drive</p> <p>Broad Meadows, Newcastle, NSW, 3047</p> <p>Australia</p>
New England Biolabs	<p>New England Biolabs</p> <p>240 Country Road</p> <p>Ipswich, Massachusetts, 1938-2723</p> <p>USA</p>
Omega Bio-Tek	<p>Omega Bio-Tek Inc</p> <p>400 Pinnacle Way, Suite 450</p> <p>Norcross, Georgia, 30071</p> <p>USA</p>
Pathology West	<p>A division of: Westmead Hospital</p> <p>Dept of Medical Entomology</p> <p>PO Box 533</p> <p>Level 3, ICPMR, Westmead Hospital</p> <p>Wentworthville, Sydney, NSW,2145</p> <p>Australia</p>

Perkin Elmar	Perkin Elmar Level 2, Building 5, Brandon Office Park 530-540 Springvale Road Glen Waverley, Melbourne, VIC, 3150 Australia
Promega	Promega Corporation Suite W3A, Level 3, Westside Building South Sydney Corporate Park 75-85 O'Riordan St Alexandria, Sydney, NSW, 2015
PRO Scientific	PRO Scientific 99 Willenbrock Road Oxford, Connecticut, 06478 USA
Roche	Roche Products Pty Ltd 4-10 Inman Road Dee Why, Sydney, NSW, 2099 Australia
Sigma-Aldrich	Sigma-Aldrich PO Box 970 Castle Hill, Sydney, NSW, 1765 Australia
Smith and Nephew	Smith and Nephew Pty Ltd Suite 7, 567 Newcastle Street

	West Perth, W.A, 6005
	Australia
VWR Chemicals	VWR International, Pty Ltd
	Unit 1/31 Archimedes Place
	Murarrie, Brisbane, QLD, 4172
	Australia
Wildfire Chilli	Wildfire Chilli Pty Ltd
	http://www.wildfirechilli.com.au/
Zeiss	Carl Zeiss Pty
	Microscopy
	Tenancy Office 4 Level 1, 40-52
	Talavera Road
	North Ryde, Sydney, NSW, 2113
	Australia

3. Methods

3.1 Selection of Plant, Fungal and Invertebrate Samples for Anti-MRSA Screening

Sample selection was based off ease of acquisition, exposure of the samples to microorganisms in nature and ethical considerations. Initial experiments focused on fruits, seeds, leaves and other material from commonly available plants. From these results, material from plants with more aromatic attributes, such as chilli seeds and hop flowers, more frequently showed antibacterial activity than other samples. Subsequently, samples were sourced from plants which displayed these characteristics. The natural habitats of fungi among damp and decaying material could be predicted to expose them to a wide variety of benign and

pathogenic microorganisms. Therefore it may be predicted that most fungi would synthesise broad spectrum antibacterial compounds as part of their innate immunity. Similarly, scavenging invertebrates which both feed and lay their eggs in carrion tissue would be expected to produce antibacterial compounds to prevent infection from pathogens growing in necrotic wounds. Consequently, multiple fungal samples commonly found in Western Australia as well as medical debridement larva: *Lucilia sericata* were tested for antibacterial activity.

3.1.1 Homogenisation of Biological Material

The materials tested for antimicrobial activity, their sources and the concentration of tissue tested are listed in table 2.6. Two slightly different methods were used to homogenise the different materials.

3.1.1.1 Freeze dry mortar and pestle

Whole samples were cut into fragments roughly 1 cm in length using sterile forceps and scalpel blades and transferred to a mortar bowl containing a pinch of sterile glass powder. Liquid nitrogen (5-10 ml) was added to the mixture, the sample was ground into a fine powder using a pestle and added to a 15 ml centrifuge tube. Five millilitres of JEB1 was added per 2 g of sample and the samples were centrifuged at 3000 x g for 15 minutes using a Beckman Coulter Allegra X-12R centrifuge. The supernatant was collected and stored at -20°C. The *L. sericata*, *M. spicata*, *S. lycopersium*, *T. violacea* and *Z. officinale* samples were homogenised using this method. All other samples were homogenised using the bladed homogeniser and cell disrupter method described below.

3.1.1.2 Homogeniser and Cell Disruptor

Samples were cut into fragments as described above and transferred to a 250 ml beaker containing 20 ml of JEB1. The samples were then homogenised using a 20 mm generator probe at 30,000 RPM. The homogenised extract was then passed through a HAIVA cell disrupter (Constant Systems) at 40 KPSI at a flow rate of 100 ml/minute. The extract was centrifuged at 3000 x g for 15 minutes. The supernatant was collected and stored at -20°C.

3.2 Antibacterial Analysis of Biological Extracts

The toxicity of the extraction buffers on MRSA was analysed by growth curve analysis. An overnight culture of WBG 8287 was counted and 10^5 CFU/ml were added to separate wells containing 0-50% v/v extraction buffers in 8 g/l. The plates were incubated for 16h in a plate reader at 37°C with 150 rpm orbital shaking for 30 seconds every 15 minutes. The absorbance of each well was measured at 600nm every hour over the 16 hour period. Each sample was tested in triplicate and the experiment was performed twice.

The effect of the biological extracts on MRSA was measured using a modified version of the calibrated dichotomous sensitivity (CDS) method developed by Sydney M. Bell (308). Follow-on experiments were conducted by measuring colony survival after treatment with increasing concentrations of the extracts.

3.2.1 Extract Sensitivity Testing Using CDS Method

Plate inoculation followed the method as described by Bell *et al.* (308). A single colony of WBG 8287, which had previously been streaked on a bactopectone agar plate for single colonies, was transferred to 2.5 ml of PBS using a sterile wire loop. The inoculated saline was spread evenly on a fresh agar plate by rocking. Excess media was removed and the surface was allowed to dry for 10-15 minutes. Holes, 3 mm² in diameter, were punched into the inoculated agar plates using a sterile tissue biopsy punch (Kai Medical) connected to a 1 ml automatic pipette (Figure 3.1). The agar inside the biopsy punch was aspirated off the petri disc using the pipette and discarded. Fifteen microlitres of sample extract was loaded into each well and the plates were incubated at 37°C for 24h. The annular radius from the edge of the well to the edge of the zone that begins to show bacterial growth, i.e. the zone of inhibition was measured for each sample. Extracts which showed antibacterial activity using the CDS method were tested another 2-3 times. The extracts which did not show activity were not tested again.

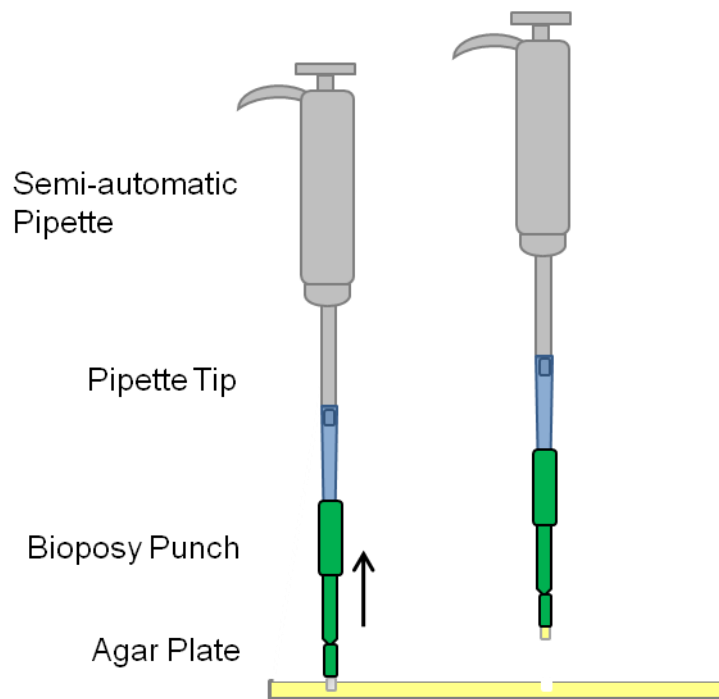


Figure 3.1 The hole punching apparatus used to create sample wells in agar plates

3.2.2 Colony Survival

WBG 8287, cultured in 8 g/l bactopectone medium for 24h, was diluted 1/50 in PBS. Twenty microlitres of cell suspension was added to 20 μ l of 0.4% trypan blue and the mixture was transferred to a haemocytometer chamber (Brightline), with graduations corresponding to 1/400 mm^2 , for cell counting. One million cells were treated with 10, 50 and 90% solutions of *A. luteobubalina*, *C. chinese*, *C. latifolia*, *E. cardamomum* and *J. communis* in 1 ml of 8 g/l bactopectone for 1 hour at 37°C with 150 rpm orbital shaking. Mock-treated controls were prepared in parallel with the test samples. After 1h the samples were diluted 1/1, 1/10, and 1/50 with PBS and 100 μ l of each suspension was spread on separate bactopectone agar plates using a sterile plate spreading rod. After 24h incubation, the number of surviving colonies was counted and the percentage of viable colonies relative to the untreated controls was calculated.

3.3 Testing of Digested Extracts for Antibacterial Properties

Aqueous extracts from *A. luteobubalina*, *C. chinese*, *C. latifolia*, *E. cardamomum* and *J. communis* were treated with 0.25 mg/ml trypsin and 1 mg/ml OB protease for 30 minutes at 37°C with 150 rpm orbital shaking. Mock enzyme-treated samples were prepared in parallel with the test samples. Following incubation, 180 µl of sample extract solution was added to 20 µl of a 10⁷ CFU/ml inoculum of WBG 8287 in 8 g/l bactopectone. Bacteria were also treated in the same way with mock-digested aqueous extracts. Survival of treated bacteria was tested by plating on agar and calculating the percentage colonies formed relative to mock-treated bacteria. Hop flower extracts were treated in triplicate with OB protease and Trypsin enzymes as described above. However, the sensitivity of MRSA to these extracts was tested using the CDS method (section 3.2.1).

3.4 Analysis of Protein Content and Peptide Precipitation in Growth Media

3.4.1 Quantitation of Media Protein Concentration

The concentration of proteins in the various media used in this study was measured using the Pierce BCA Protein Assay. This method relies on the peptide-peptide bonds catalysing the reduction of copper ($\text{Cu}^{2+} \Rightarrow \text{Cu}^{+}$) which interacts with two BCA molecules to form a purple compound that can be measured spectrophotometrically. The absorbance is proportional to the concentration of proteins in solution and, is calibrated against protein standards of known concentrations. Absorbance was measured at 660nm using a Victor 3V multilabel spectrophotometric plate reader.

3.4.2 Analysis of Peptide Precipitation in Different Media

Increasing concentrations of melittin and melfrag12-26 were added to 8 g/l bactopectone, PBS and PB (Table 3.1). Media without antibacterial peptides served as blank controls. Two microlitres of each solution was analysed at 630nm using a Nanodrop spectrophotometer. All samples were tested in triplicate.

Table 3.1 Concentration of melittin and melfrag12-26 in growth media and phosphate buffer

Melittin Conc. In Media ($\mu\text{g/ml}$)	Melfrag12-26 Conc. In Media ($\mu\text{g/ml}$)
50	50
75	100
100	150
200	200
500	250
	500

3.5 Minimal Inhibitory and Minimal Lethal Concentration Testing

3.5.1 Minimal Inhibitory Concentration: Growth Curve Analysis

An overnight culture of WBG 8287 was counted and diluted in 200 μl of 8 g/l bactopectone medium to 10^5 CFU/ml. Serial dilutions of AP1, melittin, melfrag12-24 and melfrag12-26-L1 ranging from 1-200 $\mu\text{g/ml}$ were added to separate inoculated wells. The plates were incubated for 16h in a plate reader at 37°C with 150 rpm orbital shaking for 30 seconds every 15 minutes. The absorbance of each well was measured at 600nm every hour over the 16 hour period. The minimal inhibitory concentration (MIC) of the peptides was defined as the lowest concentration in which the absorbance of the wells did not increase over that of the mock inoculated negative control. Unless otherwise stated, each sample was tested in triplicate and the experiment was performed twice.

3.5.2 Minimal Lethal Concentration: Colony Survival on Agar Plates

S. aureus was grown overnight at 37°C in 8 g/l bactopectone and cell numbers were counted the next day. The minimal lethal concentration (MLC) of synthetic AMPs was determined by treating 10^6 CFU/ml inoculum in 200 μl of medium with

a range of concentrations of the test samples for 1h at 37°C with 150 rpm orbital shaking. After incubation, 100 µl from triplicate samples were plated on individual bactopectone agar plates. The mock-treated control samples were diluted 1/50 with PBS prior to plating. The inoculated plates were incubated at 37°C for 24h after which the surviving colonies were counted. The MLC was defined as the lowest concentration of peptide or compound that reduced the amount of surviving colonies by at least 99.99% compared to the mock-treated control. The same methodology was used to test the MLC concentration of melittin, melfrag12-26 and bac8c in 8 g/l bactopectone with 0.005% and 0.1% v/v Triton X100. Unless otherwise stated, experiments were performed twice with triplicate samples for each peptide tested.

3.5.2 Antibacterial Activity of AP1 in media Supplemented with MgCl₂

WBG 8287 cells were suspended at 10⁶ CFU/ml in 200 µl of 8 g/l bactopectone medium supplemented with 10, 15 or 20 mM MgCl₂. The cells were then treated in triplicate with 50 or 100 µg/ml of AP1. Growth was measured over ten hours using a spectrophotometric plate reader (section 3.51).

3.6 Antibacterial Activity of Melittin at Different Incubation Temperatures

Cultures of MRSA strain WBG 8287 (10⁶ CFU/ml) were treated with 0.1 µg/ml or 0.5 µg/ml of melittin in 200 µl of PBS. The cells were incubated at 5°, 25°, 37° and 45°C for 1h with 150 rpm orbital shaking and then plated on bactopectone agar plates. The mock treated controls were diluted 1/50 prior to plating. After 24h incubation, the resulting colonies were counted and expressed as a percentage of untreated controls. Each sample was tested in triplicate and the experiment was performed twice.

3.7 MRSA Growth in Sub-inhibitory Doses of Antimicrobial Peptides

In the initial experiments, strain WBG 8287 was inoculated at 10⁶ CFU/ml into 200 µl of 8 g/l bactopectone medium containing 1 µg/ml of melittin and incubated overnight at 37°C with 150 rpm orbital shaking. This population was subcultured

for a further six days, with the melittin concentration increased by 2 µg/ml each day to a final concentration of 13 µg/ml. These cells were designated “melittin-resistant” bacteria and untreated controls, subcultured in parallel, were designated “melittin-naive” bacteria. After the six days of exposure to melittin, the MLC and MIC of melittin was tested as described above. WBG 8287 bacteria from these experiments were used to test the stability of melittin resistance in a population and were photographed using scanning electron microscopy (SEM).

In all other resistance-based investigations, overnight cultures of strains WBG 8287, W17S, Aus3 and BB were counted (section 3.2.2) and resuspended at 10^7 CFU/ml in 200 µl of 8 g/l bactopectone. The medium was supplemented with 2.5 µg/ml of melittin and the samples were incubated for 12h at 37°C with 150 rpm orbital shaking. After 12h the cells were counted and resuspended at 10^7 CFU/ml in 200 µl of 8 g/l bactopectone medium supplemented with 5 µg/ml melittin. The cells were then re-incubated for an additional 12h. This was repeated with melittin concentrations in the medium increasing to 8, 10, 15, 20, 40 and 60 µg/ml for the first three days followed by subculture every 12h in medium supplemented with 80 µg/ml of melittin for the next three days. Parallel experiments were performed with increasing concentrations of bac8c in cultures of WBG 8287, W17S and Aus3.

3.8 Scanning Electron Microscopy

3.8.1 Desiccator Method

Bacteria were suspended in 200 µl PBS at 10^6 CFU/ml and centrifuged at 12,000 x g for five minutes. The supernatant was removed and the bacteria resuspended in 1 ml PBS. The samples were then recentrifuged and the bacteria suspended in 100 µl PBS containing 10 µg/ml of melittin for 1h at 37°C. Control samples were prepared by the same process, without melittin. Aliquots of each sample (15 µl) were dispensed onto separate aluminium stubs. The stubs were covered and incubated for thirty minutes at 37°C. The cells were fixed by overlaying with 25 µl of a PBS solution containing 2.5% glutaraldehyde (Asia Pacific Specialty Chemicals) for 3 h at 4°C. The stubs were washed by gently applying H_2O to the tilted surface of the stubs, followed by a series of thirty minute incubations at 37°C in 70%, 90% and 100% ethanol respectively. The samples were transferred to a desiccator and dried for 24h at 37°C over silica gel. The stubs were

evaporatively coated with a 3 nm layer of platinum and viewed using a Neon 40ESB Crossbeam SEM. The desiccator method was used to obtain close-up images of melittin naive and melittin resistant WBG 8287 cells after melittin treatment.

3.8.2 Freeze Dry Method

WBG 8287 cells were seeded on bactopectone agar plates (section 3.2.1). Acticoat strips, cut to 1 cm² were carefully placed on the inoculated agar. The plates were then incubated at 37°C for 24h. After 24h the strips were removed from the agar and washed with PBS. The Acticoat strips were transferred to a 5 ml tube containing 2.5% glutaraldehyde supplemented with 75 mM lysine at 37°C for 10 minutes. Lysine fixation solution was washed off the Acticoat with HpH₂O and the cells attached to the Acticoat were fixed for a further 24h in 3% glutaraldehyde at room temperature. After fixing, the strips were washed with HpH₂O and then transferred to a tube containing 50% undenatured ethanol for 5 minutes. The ethanol was aspirated from the tube and the Acticoat strips were immersed for another 5 minutes in 70% ethanol. This step was repeated with 80% and 90% ethanol and then repeated twice with 100% ethanol for 10 minutes. After ethanol dehydration the Acticoat strips were frozen in liquid nitrogen and then transferred to a petri dish with a perforated lid. The petri dish was placed in a Scanvac Coolsafe freeze dryer and the cells were freeze dried at -55°C overnight. After the freeze drying process was completed the Acticoat was immobilised on the surface of aluminium stubs using conductive carbon glue (Figure 3.2). The stubs were then coated with platinum and viewed using the scanning electron microscope as described above (section 3.8.1). This process was repeated for WBG 8287 cells that were exposed to Acticoat on bactopectone agar plates for 48h and 72h.

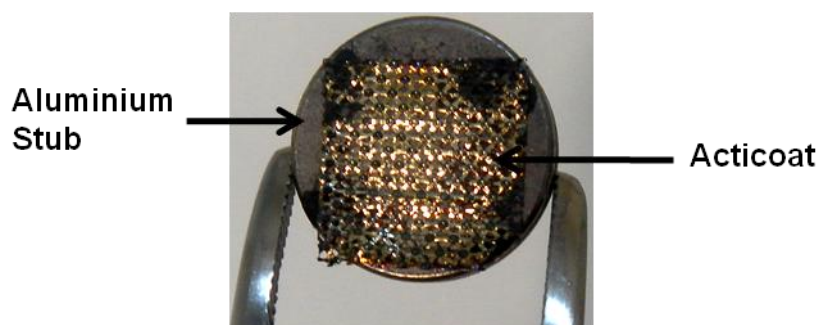


Figure 3.2 Acticoat, prepared for SEM using the freeze drying method, adhered to an aluminium stub

3.9 Qualitative Analysis of the Electrostatic Potential of the surface of Naive and Resistant Cells

3.9.1 Eosin Staining and Spectrophotometric Analysis of Cells

WBG 8287 cells were counted and resuspended at 10^8 CFU/ml in 1 ml HpH₂O. The cells were centrifuged at 8,000 x g for 10 minutes, the supernatant was removed and the cells were resuspended in 1 ml HpH₂O at concentrations of 2.5×10^7 , 1.5×10^7 , and 5×10^6 CFU/ml. The cells were recentrifuged and resuspended in 1 ml HpH₂O with 0.01% v/v eosin Y. The cells were incubated at 37°C with 150 rpm orbital shaking for 30 minutes. The cells were then recentrifuged, the supernatant was removed and the pellet was resuspended in 250 µl of HpH₂O and the absorbance of the cell suspension was measured spectrophotometrically at 490nm. Each test was performed in triplicate and the experiment was repeated three times.

3.9.2 Flow Cytometry Analysis of a Cationic Fluorophore

Binding of the cationic fluorescent probe Bacterisense 645 (Perkin Elmer) to the cell surface was compared between parental strain WBG 8287 and melittin resistant WBG 8287. Two hundred microlitre suspensions of both populations (10^7 CFU/ml) were washed twice in 1 ml PB with centrifugation at 8,000 x g for five minutes and resuspended in 1 ml of PB. A 100 µl aliquot (10^7 CFU/ml) was transferred to 400 µl of HpH₂O containing 2.08 picomoles of Bacterisense 645. The bacteria were washed three times for ten minutes each in PB, with shaking at room temperature, to remove unbound or weakly bound probe. Dye-binding to bacteria was measured using an Attune Acoustic Focusing Cytometer using Attune focusing fluid. All solutions including growth media used for flow cytometry were filtered through 0.2 µm membrane filters.

3.10 Removal and Return of Antimicrobial Peptide Selection Media

Melittin resistant bacteria (WBG 8287) and bac8c resistant bacteria (WBG 8287, W17S and Aus3) were subcultured daily for seven days in 8g/l bactopectone medium without AMPs. These were designated the “reversion” populations. After seven daily subcultures, the MLC of the peptides for the reversion bacteria was measured as described in section 3.5.2. The MIC of melittin against the reversion bacteria was also measured as before (section 3.5.1). Melittin reversion bacteria were returned to medium containing 12 µg/ml melittin for 24h and the MLC of melittin was measured.

3. 11 Internalisation of Antimicrobial Peptides by Electroporation

3.11.1 Preparation of Electro-competent MRSA

A 1 ml overnight culture of WBG 8287 (approximately 10^9 cells) was centrifuged at 8,000 x g for ten minutes and the cells were washed with 500 mM sucrose. The cells were recentrifuged and the pellet was resuspended in 500 µl of 500 mM sucrose. The cells were chilled on ice for thirty minutes and then recentrifuged and resuspended in 500 µl of 500 mM sucrose. The bacteria were then used in electroporation experiments or stored in 15% glycerol at -80°C.

3.11.2 Electroporation-Transfection of MRSA with Antimicrobial Peptides

Electro-competent WBG 8287 were counted and resuspended at 10^6 CFU/ml in 50 µl of 500 mM sucrose, in five separate Gene pulse™ electroporation cuvettes with a 0.1cm electrode gap. In two cuvettes, melittin was added at a concentration of 2.5 µg/ml and all cuvettes were chilled on ice for one minute. One cuvette containing cells with melittin and two cuvettes with cells but without melittin were pulsed using a Micropulser electroporator set at 25 µF, 2.5 kV and 100 Ω, which gave a pulse time of 2.5 ms. After electroporation, 2.5 µg/ml of melittin was added to a control, untreated electroporated sample and 450 µl of sucrose was added to each cuvette. The samples were placed on ice for fifteen minutes then spread on bactopectone agar plates (Figure 3.2). The untreated samples were diluted 1/50 prior to plating. The plates were incubated for 24h at 37°C. Colony survival was measured after incubation and expressed as a

percentage of an untreated, un-electroporated control. This method was also used to measure the effectiveness of transferring 50 µg/ml melfrag12-26 and 2 µg/ml bac8c into MRSA. All electroporation experiments were repeated twice with three replicates for each sample.

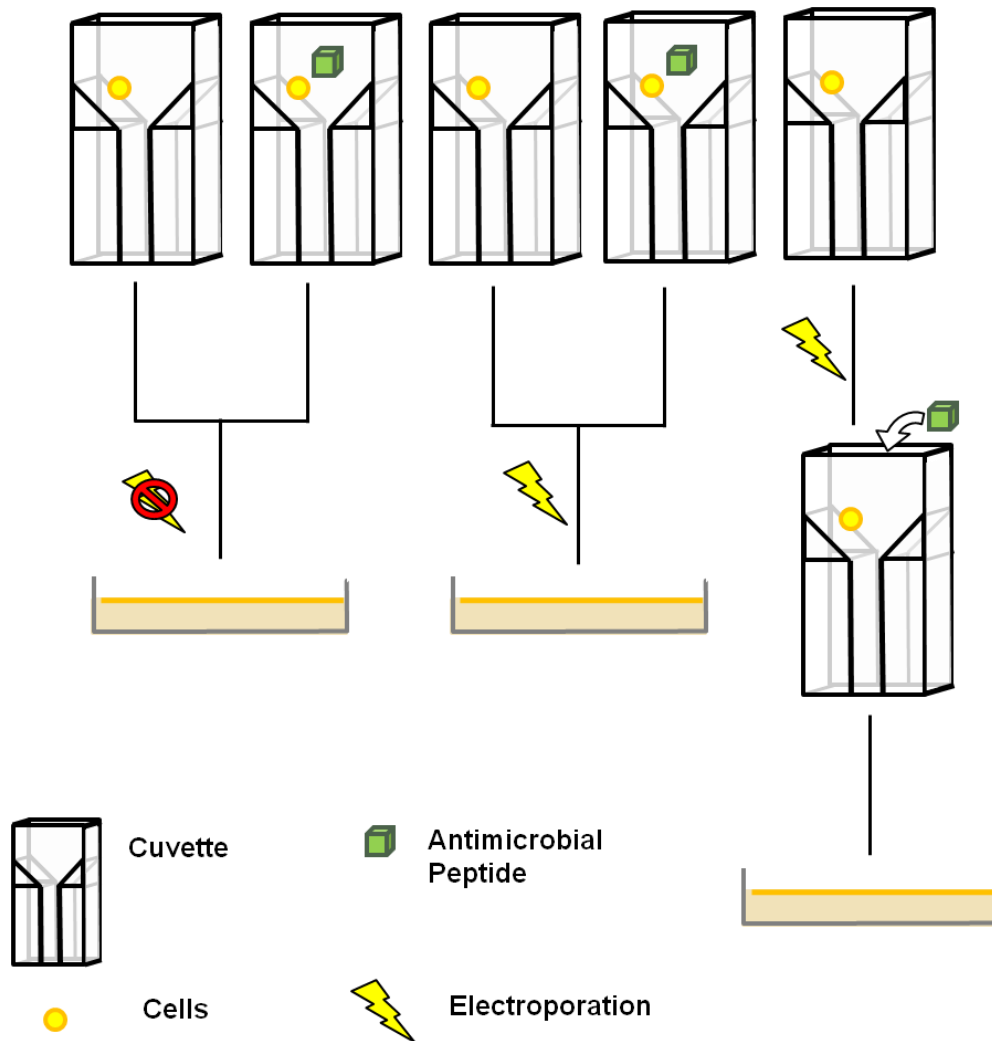


Figure 3.3 Flow diagram of cells exposed to antimicrobial peptides with and without electroporation. Some samples were electroporated before being plated on agar plates, while others were plated without being electroporated (lightning bolt with cross). One sample group which had been electroporated without the AMP, was exposed to the peptide before being spread on the agar plates.

3.12 Haemolysis Assays

3.12.1 Preparation of Erythrocytes

Fifteen millilitres of whole blood was drawn from the vein of a 26 year old male volunteer (ethical approval not required) and stored in an EDTA-coated Vacutainer. Erythrocytes were separated from whole blood by centrifuging at 500 x g for five minutes at 4°C. The supernatant was removed and 10 ml of ice-cold PBS was added. The cells were recentrifuged, the supernatant was removed and the pellet was resuspended in 10 ml PBS.

3.12.2 Haemolysis Assay

Red blood cells were counted and resuspended in PBS at 10^6 cells/ml. To measure haemolysis, melittin, melfrag12-26 or bac8c was added to each medium at different concentrations (Table 3.2). The erythrocyte suspension was incubated at 37°C for thirty minutes with 150 rpm orbital shaking and then centrifuged at 500 x g. The absorbance of the supernatant at 405nm was measured using a Victor Multilabel plate reader spectrophotometer. The haemolytic potential of the peptides was also measured in PBS supplemented with various combinations and concentrations of Triton X-100, glycerol and lysozyme (Table 3.2).

Table 3.2 Concentrations of the antimicrobial peptides, and other compounds, in PBS medium used to assess haemolysis

Sample	Peptide Conc. ($\mu\text{g/ml}$)	TX100 Conc. (v/v%)
	0	0
Blank	0	0.005
	0	0
Bac8c	6	0

	4	0.005
	80	0
Melittin	5	0
	2.5	0.005
Melfrag12-26	130	0
	80	0.005
	100	0

3.13 Antibiotic Sensitivity of Antimicrobial Peptide Naive and Resistant MRSA

3.13.1 Disc Diffusion

Antibiotic sensitivity of wild-type and melittin or bac8c resistant MRSA was tested using the calibrated dichotomous sensitivity method described by Bell *et al.* (308). Single bacterial colonies, grown overnight, were suspended in PBS and used to lawn inoculate bactopectone agar plates. Antibiotic discs were placed on the plates and incubated for 24h at 37°C, before measuring zones of inhibition as previously described (section 3.2.1). The antibiotic resistance profile of AMP-resistant bacteria was compared against wild-type populations to identify any change in sensitivity. The experiment was repeated 2-6 times for each antibiotic.

3.13.2 Growth of MRSA on Media containing Sub-inhibitory Concentrations of Fusidic Acid

Overnight-colonies of wild-type WBG 8287, W17S, Aus3 and BB were streaked on bactopectone agar plates containing 0.5 µg/ml fusidic acid. After 24h incubation a single colony was picked from each plate and streaked on agar containing 1 µg/ml fusidic acid and the plates were incubated for another 24h. This process was continued for a further five days with the concentration of fusidic acid increased by 2 µg/ml each day. A population of each MRSA strain exposed in this way to fusidic acid was made resistant to melittin and bac8c as

described above (section 3.7). The MLC concentration of fusidic acid to AMP-naïve and AMP-resistant populations was tested as described above (section 3.5.2).

3.14 PCR of FusB and FusC Genes

3.14.1 DNA Isolation

Two millilitres of MRSA culture, grown for 24h in 24 g/l bactopectone medium was centrifuged at 16000 x g for five minutes and the pellet was resuspended in 200 µl of lysis buffer. The cells were snap frozen in liquid nitrogen and then immediately thawed at 95°C for one minute. This process was repeated twice. A pinch of sterile glass beads (approximately 1/4 the volume of the cell suspension) and 200 µl of chloroform was added to the tubes. The tube lids were secured with parafilm and the samples were homogenised using a FastPrep FP120 cell homogeniser for 30s at 6.5 m/s. Two hundred microlitres of HpH₂O was added and the samples were mixed by inversion for 30s. The samples were centrifuged at 5000 x g for five minutes and the aqueous phase was collected in a fresh tube. An equal volume of chloroform was added to the extracts which were then mixed by inversion for two minutes and recentrifuged. The aqueous phase was collected and the chloroform extraction was repeated. A 1/10 volume of 1M NaCl and a 3 x volume of ice cold 70% undenatured ethanol was added to the aqueous phase. The tubes were placed on ice for 45 minutes. The extracts were then centrifuged at 16000 x g for ten minutes and the supernatant was removed. One millilitre of 70% ethanol was added and the tubes were centrifuged again. The supernatant was removed and the pellet was allowed to dry for approximately 5-10 minutes until no ethanolic aroma was detected from the tubes. The pellet was resuspended in 50 µl HpH₂O and the DNA concentration and purity was tested spectrophotometrically using a Nanodrop spectrophotometer.

3.14.2 PCR Amplification Primer Design

Primers were designed to match a partial sequence submitted to NCBI (*S. aureus*, fusB: Genbank accession number CP003193.1 and fusC: KF527883.1) using Primer3 software (309). The fusB gene begins at nucleotide 1336 of the

CP003193.1 sequence and continues for another 640 nucleotides (i.e. the gene is comprised of 641 nucleotides). The *fusB* forward primer was designed to match a 24 base-pair sequence on the sense-strand, beginning at nucleotide 1414 of the CP003193.1 sequence and ending at nucleotide 1437. The reverse primer was designed to compliment a sequence 34 bases downstream of the stop codon of the sense-strand (nucleotides 2011-2036) to reduce the proportion of AT repeats and the potential for mispriming. Likewise, the *fusC* gene contains many repeat sequences which limited the choice in the forward and reverse primer sequences. The *fusC* forward primer sequence was designed to match a sequence of the sense-strand of KF527883.1 that began at nucleotide 22073 and ended at 22095. The *fusC* reverse primer sequence was complimentary to the nucleotide sequence ranging from 22990 to 23011 of the sense-strand. The *fusB* primers produced an amplicon of 624 nucleotides and the *fusC* primers produced an amplicon of 939 nucleotides.

3.14.3 PCR Amplification

Table 3.3 shows the concentrations of the reagents used in each 20 µl PCR reaction. PCR reactions included the MyTaq DNA polymerase and reaction buffer. The reaction buffer has dNTPs and MgCl₂ at concentrations that are considered optimal for most PCR reactions. Template DNA was added at 5 ng/reaction and each primer was used at 2 µM. The reagents and reaction mixtures were kept on ice throughout the dispensing procedure.

Amplification was performed using a Veriti 96-well Thermal cycler. A gradient of annealing temperatures was conducted to optimise the PCR reactions (See Appendix). Cycling conditions used for amplification of *fusB/C* are shown in table 3.4.

Table 3.3 PCR reagents

Reagent	Volume/20µl Reaction	Concentration
5xMyTaq Reaction Buffer	4µl	1x
Forward Primer	0.4µl	2 µM

Reverse Primer	0.4µl	2 µM
Template	1µl	5 ng/reaction
Polymerase (5U/µl)	0.1µl	0.025 U/µl
HpH₂O	14.1µl	70.5%

Table 3.4 Thermal cycler protocol

Step	Temperature (°C)	Time (Seconds)	Cycles
Initial Denaturation	95	60	1
Annealing	59	15	
Extension	72	15	30
Denaturation	95	15	
Final Extension	72	60	1
Hold	4	∞	1

3.14.4 Visualisation of Amplified PCR Products

Amplification products were visualised on 1.5% agarose gels, with 0.6 g of molecular biology grade agarose in 40 ml SB buffer heated in a microwave oven. When the mixture had cooled to around 60°C, 5 µl of a 1000X concentration of Gel Green nucleic acid stain was added and the gel was cast in a plastic mould. The sample wells were loaded with 5 µl of amplified DNA, premixed with 4 µl of 6X loading buffer. A 100 base-pair DNA ladder ranging from 100-1500 base pairs, was added to the first and last well to allow calculation of the length of the amplicons. Electrophoresis used a PowerPac 3000 power supply, operating at 60V for ninety minutes. Following electrophoresis, the DNA was visualised using a Gel Doc XR+ UV transilluminator.

3.15 Assessment of Acticoat as a Antibacterial Dressing

3.15.1 CDS Analysis of Acticoat on Agar Medium

Acticoat layers were separated from the inner absorbent lining and cut into 1 cm x 0.5 cm rectangles using a scalpel blade and forceps (Figure 3.3). Bactopeptone agar plates were inoculated with wild-type WBG 8287 cells as described above (section 3.2.1). The Acticoat strips were aseptically placed on the agar and the plates were incubated for 24h at 37°C. Filter paper cut to the same size as the Acticoat strips was used as a negative control for growth inhibition. The zone of inhibition around the strips was measured after incubation. In subsequent experiments, the molten agar was textured using sterile moulds to create striated, blistered and creased textured surfaces. Each test and control sample was tested in a series of 9 replicates.

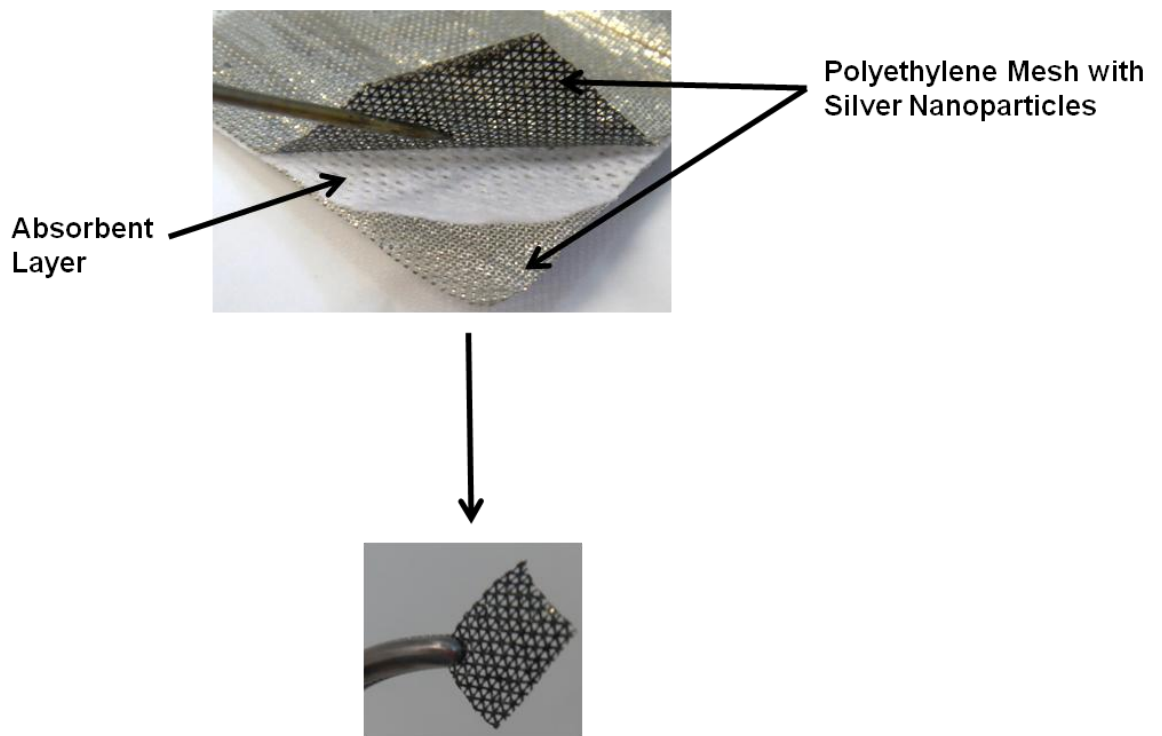


Figure 3.4 Preparation of Acticoat Strips. The silver nanoparticle loaded mesh was separated from the inner absorbent layer and then cut into 1cm x 0.5cm rectangles.

3.15.2 Measuring Bacterial Survival on Acticoat

WBG 8287 was overlain with Acticoat and filter paper on flat and textured bactopectone agar plates, which were incubated for 24, 48 and 72h as described above (section 3.15.1). After incubation, the strips were carefully removed and placed on fresh bactopectone agar for 5 minutes. The strips were then removed and the plates were incubated for 24h. The ability of MRSA to survive and transfer on Acticoat was assessed by observing growth within the regions exposed to Acticoat and the locations on which Acticoat was “blotted”.

Similar experiments were conducted with cells inoculated onto 2cm x 2cm squares of sterile pig skin immobilised in agar (section 2.11). WBG 8287 bacteria were counted and resuspended at 10^7 CFU/ml in PBS. Two microlitres of the suspension was placed in the middle of each square of pig skin and the plates were incubated at 37°C for 30 minutes. Acticoat strips were then applied to the middle of each inoculated square of skin and the survival of bacteria on the gauze was measured after 24, 48 and 72h as described above.

3.15.3 Measuring Bacterial Survival on Acticoat Encased in Antimicrobial Gels

A 200 µl mixture of 1% molten agarose or bacteriological agar and 0.1% Triton X-100 in H_2O was cast in a 1.9 cm² well of a 24-well plate. An Acticoat strip was placed on the surface on the gel along with 20 µl of 1 mg/ml fusidic acid or bac8c. A further 200 µl of agarose or agar and Triton X-100 was added to the well and the gel was allowed to set. A 10 ml pipette with a tip that had been cut to create an opening with a diameter of approximately 1.9 cm² was used to aspirate the Acticoat gels from the wells and place them on inoculated pig skin (Figure 3.5). Bacterial survival and transference on the antimicrobial gels after 24h was measured as described above (section 3.15.2).

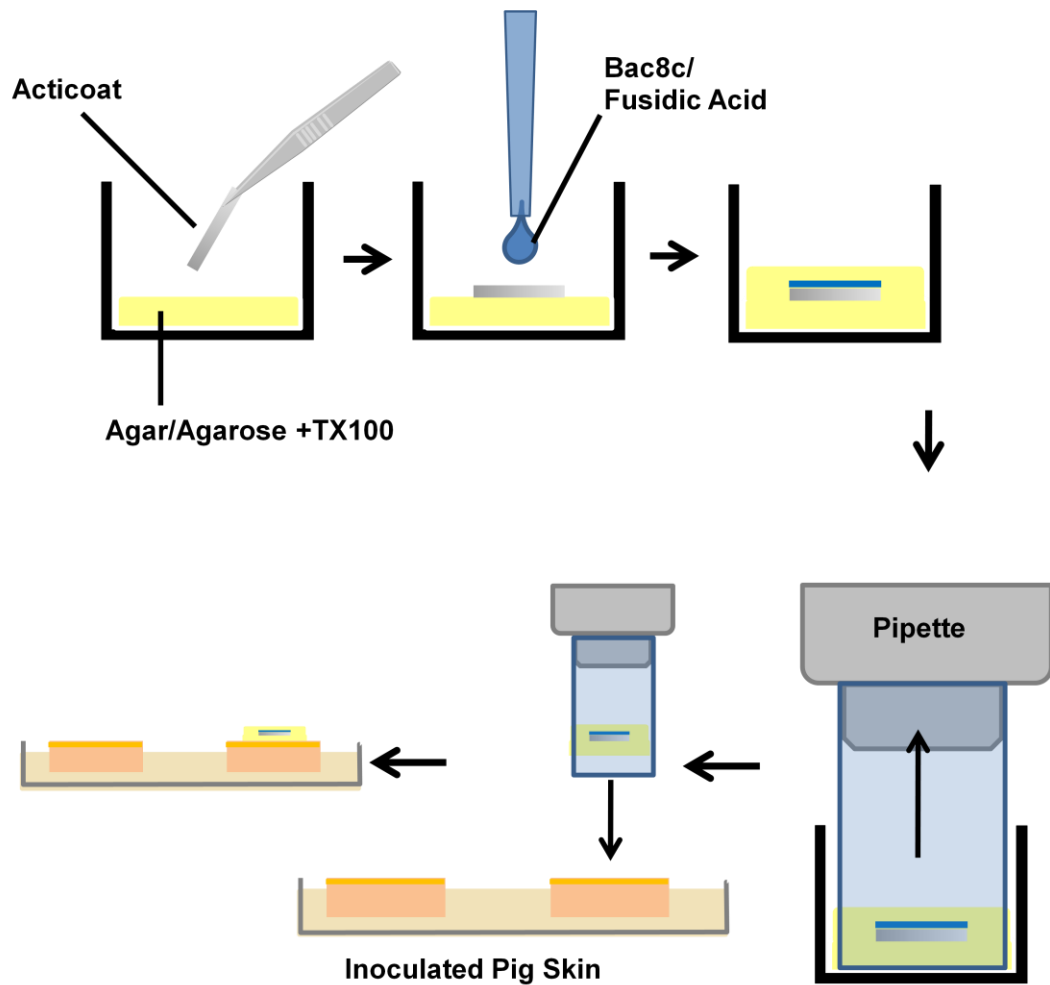


Figure 3.5 Diagram of the process of casting Acticoat in agar or agarose gels with Triton X-100 and antibacterial agents. Once the gels were set, the negative and positive pressure created by a semi-automatic pipette was used to transfer the gels to inoculated pig skin.

3.15.4 Measurement of Survival after Acticoat Treatment by Colony Survival

Using methods described in section 3.15.2, Acticoat was overlain on sterile pig skin inoculated with 2 μl of WBG 8287 cells at 10^5 CFU/ml. Immediately after the Acticoat was placed on pig skin, it was removed and placed in a 5 ml tube containing 1 ml of PBS. The skin was also transferred from the plate to a tube containing 1 ml of PBS. The tubes were mixed by vortexing for 30s and the PBS was spread on bactopectone agar plates (section 3.5.2). The plates were incubated for 24h and colonies were counted. Survival was expressed as a percentage of viable colonies on an untreated skin control. This was repeated for skin samples that were treated at 37°C for 30 minutes, 1h, 4h, 8h and 24h.

Matching experiments were performed with Acticoat that had been coated in wetting agents or antimicrobial agents. Pig skin was inoculated with 2 μl of WBG 8287 cells at 10^8 CFU/ml as described above. Acticoat strips were coated with 10% glycerol with and without 50 $\mu\text{g}/\text{ml}$ melfrag12-26 or bac8c. The coatings were applied using a sterile brush and survival was measured after 24h incubation at 37°C as described above. The same experiment was repeated using a coating of 1000 $\mu\text{g}/\text{ml}$ of lysozyme with and without the AMPs, and a combination of glycerol and lysozyme with and without the peptides (Figure 3.6).

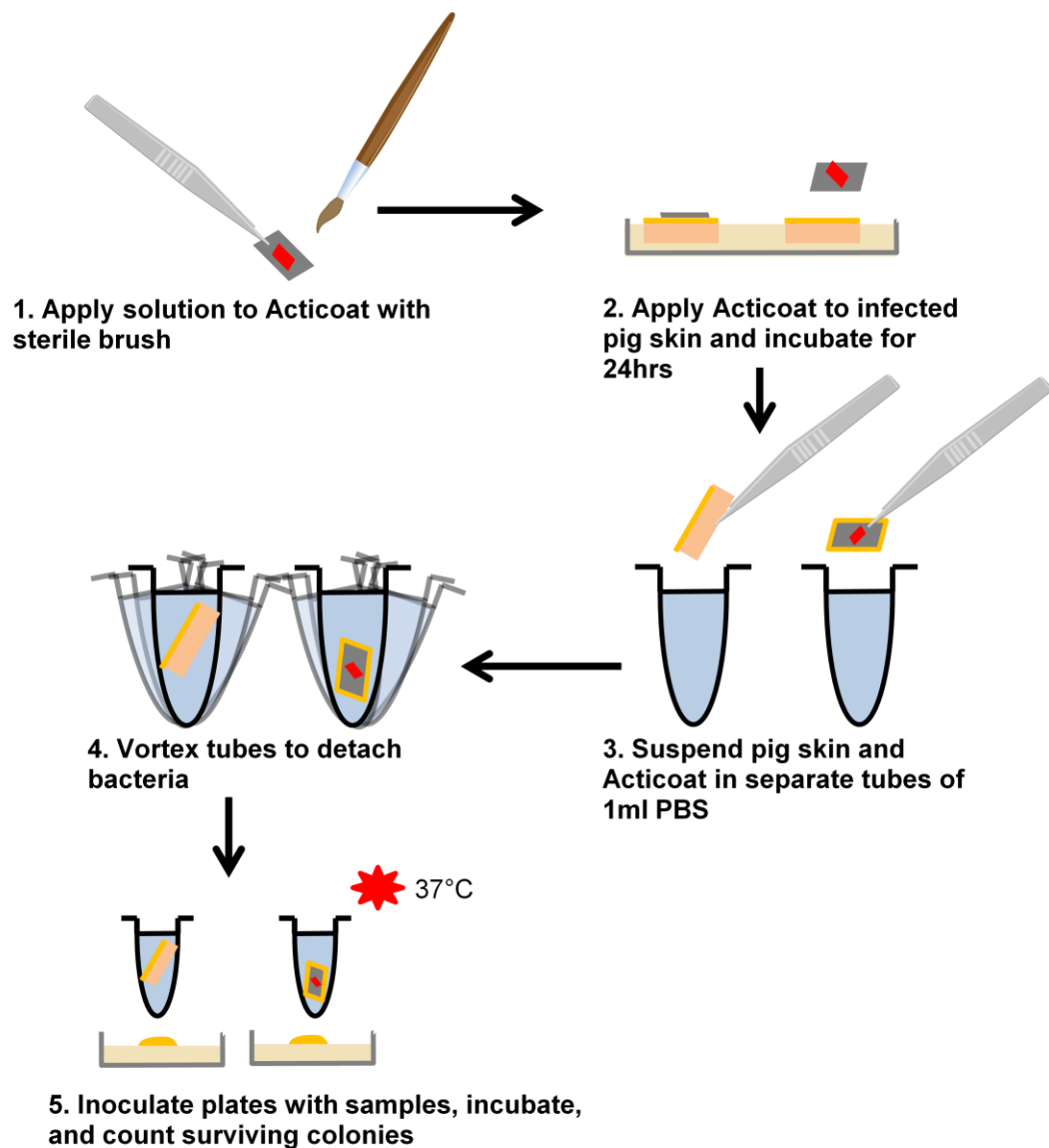


Figure 3.6 Flow diagram of the method to measure colony survival, after exposure to Acticoat, on pig skin and Acticoat strips

3.16 Contour Clamped Homogenous Field Electrophoresis

Contour clamped homogeneous field electrophoresis was performed using the protocol described by O'Brien *et al.* (310). A single colony from each strain was resuspended in 5 ml of 5 mM EDTA and centrifuged for 10 minutes at 1000 x g. This step was repeated and the cells were resuspended in EC buffer at 1.8×10^6 CFU/ml. Fifty microlitres of the cell suspension was mixed with 50 μ l of 400 μ g/ml lysostaphin and 100 μ l of 1.2% chromosomal grade agarose in TBE buffer, the solution was mixed by pipetting, and then dispensed into a 0.5 cm x 1 cm plug mould. When set, the blocks were transferred to 1.5 ml tubes, each containing 500 μ l EC buffer. The tubes were incubated at 37°C for 4h. The EC buffer was removed and replaced with 300 μ l of EST buffer and 30 μ l of 20 mg/ml proteinase K and was incubated overnight at 50°C.

The buffer and proteinase K was removed and the blocks were washed with 1 ml EDTA for thirty minutes at room temperature with gentle shaking. The EDTA solution was removed and the wash step was repeated three more times. The blocks were sliced in half using a glass cover slip to allow easy fit in the agarose wells. The half-blocks were washed with H_2O for thirty minutes. After washing, the water was replaced with 20 μ l enzyme buffer J, 40 units of SmaI restriction endonuclease and 180 μ l of H_2O and the tubes were chilled on ice for 30 minutes. The blocks were then incubated at 25°C for 3h.

After restriction enzyme digestion the blocks were removed from the tubes and placed in the wells of a 1% pulse-field-certified agarose gel in TBE, using a sterile wire loop. The gel was electrophoresed in TBE buffer at 14°C and 200V for 20h, with pulsing times of 40s using a CHEF-DR Variable Angle pulse-field electrophoresis system. After electrophoresis, the gel was stained with 0.5 μ g/ml ethidium bromide for twenty minutes and de-stained in H_2O for ten minutes. The gel was visualised and photographed using a UV transilluminator. A NCTC 8325 control block was added to the end well of each row to act as a molecular weight marker.

3.17 Statistics

Data compared across control and test bacterial populations were analysed using the T-test calculated using Microsoft Excel software.

4. Results

4.1 Effects of Extraction Buffer on *S. aureus*

The effects of the extraction buffers on MRSA are shown in figure 4.1. Buffer JEB1 showed no toxic effect against MRSA at any of the concentrations tested. Addition of 5 and 25% of JEB1 to growth media appears to increase the growth rate of cells over that of unsupplemented growth media. In contrast, buffer JEB2 inhibited the growth of cells at and above 25%, which could be attributed to the inclusion of 1mM EDTA in the buffer. From these results, JEB1 was selected as the most suitable buffer for the extraction of bio-active compounds from biological material.

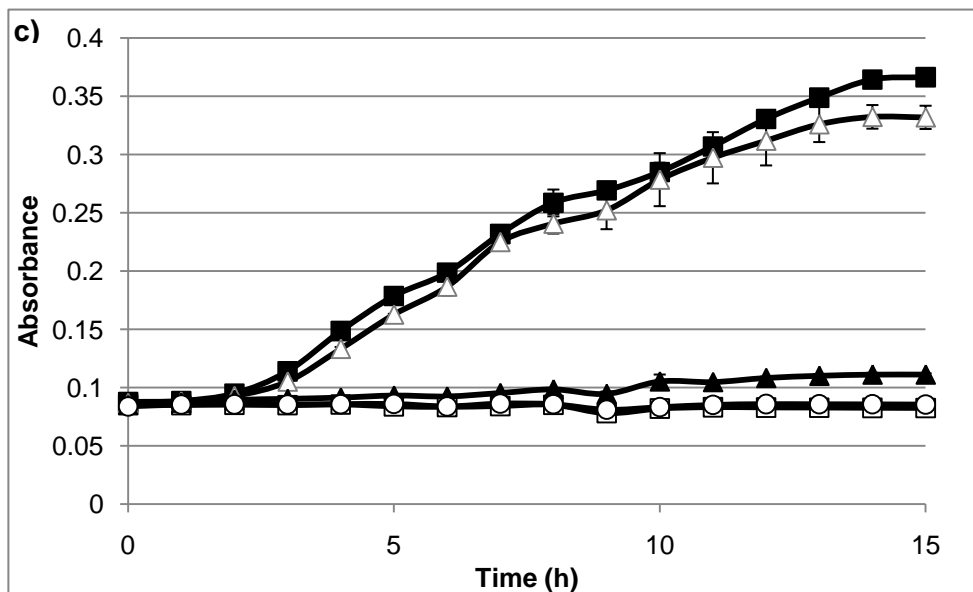
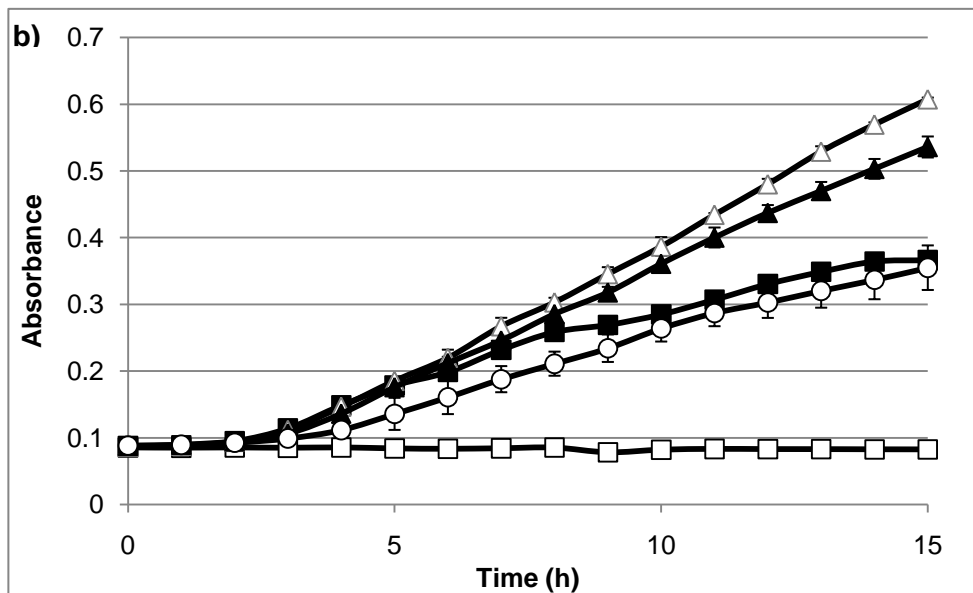
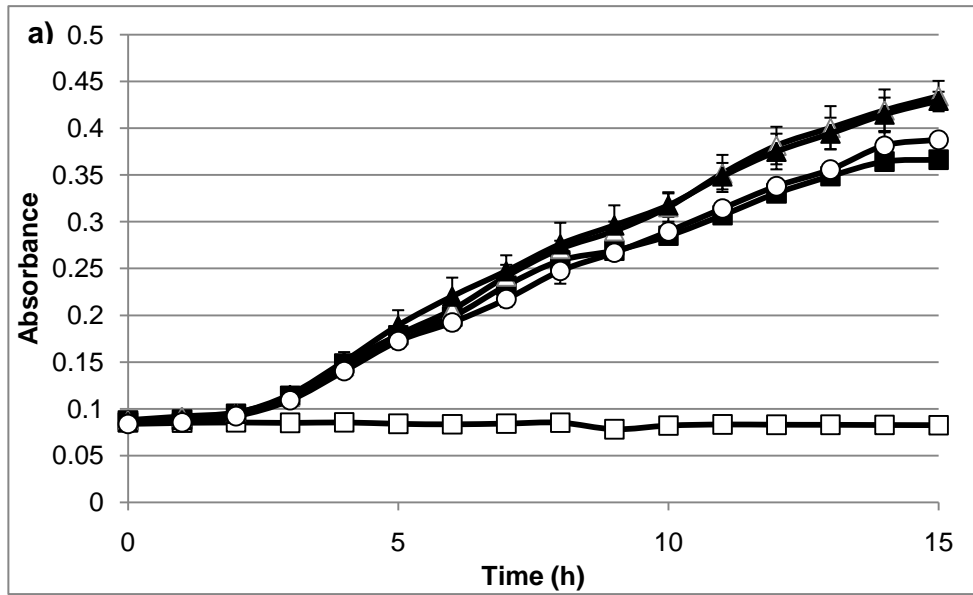


Figure 4.1 Growth curves of MRSA in media supplemented with extraction buffer. The growth rate of WBG 8287 cells in (a) 8 g/l bactopeptone supplemented with 5% (open triangles), 25% (closed triangles) and 50% (open circles) PBS, (b) JEB1 and (c) JEB2 was measured spectrophotometrically over 15h. Data represents mock inoculated (open squares) and inoculated medium (closed squares) respectively. Error bars represent standard deviation of triplicate samples.

4.1.2 Antibacterial Activity of Crude Extracts from Plant, Fungal and Invertebrate Samples

Table 4.1 shows the zone of inhibition around the well containing each extract. Most of the extracts from fruit, vegetable and fungal material as well as those from aloe vera and the green bottle fly larvae showed no inhibitory effect against MRSA. Excluding the mint leaf and aloe vera extracts, plant samples with a strong aromatic smell such as the bhut jolokia chillies, ginger root, hop flowers and garlic bulbs appeared to have a moderately strong inhibitory activity against WBG 8287 bacteria. Of the fungal samples, only the Australian honey and scarlet bracken fungus showed antibacterial activity. Extracts from the stem and cap of the fungus showed similar inhibitory activity.

Table 4.1 Antibacterial activity of biological extracts

Species	Common Name	Tissue	ZOI ^a (mm)
<i>Agaricus xanthoderma</i>	Yellow-staining mushroom	Head	0
<i>Agaricus xanthoderma</i>	Yellow-staining mushroom	Stem	0
<i>Aloe vera</i>	Aloe vera plant	Leaf	0
<i>Amanita xanthocephala</i>	Vermillion amanita	Head	0
<i>Amanita</i>	Vermillion amanita	Stem	0

<i>xanthocephala</i>			
<i>Armillaria luteobubalina</i>	Australian honey fungus	Head	5
<i>Armillaria luteobubalina</i>	Australian honey fungus	Stem	4
<i>Brassica oleracea</i>	Cabbage	Leaf	0
<i>Capscium chinese</i>	Bhut jolokia chilli	Seeds	2
<i>Capscium chinese</i>	Bhut jolokia chilli	Fruit	2
<i>Cucumis sativus</i>	Cucumber	Fruit	0
<i>Humulus lupulus</i>	Hop plant (Cascade)	Hop flower	3
<i>Humulus lupulus</i>	Hop plant (Citra)	Hop flower	2
<i>Humulus lupulus</i>	Hop plant (Chinook)	Hop flower	3
<i>Humulus lupulus</i>	Hop plant (Summer)	Hop flower	3
<i>Humulus lupulus</i>	Hop plant (Topaz)	Hop flower	3
<i>Lucilia sericata</i>	Green bottle fly	Larva	0
<i>Mentha spicata</i>	Spearmint	Leaf	0
<i>Musa acuminata</i>	Cultivated banana	Skin	0
<i>Musa acuminata</i>	Cultivated banana	Fruit	0
<i>Persea americana</i>	Avocado	Fruit	0
<i>Pisolithus tinctorius</i>	Horse dung fungus	Head	0
<i>Pisolithus tinctorius</i>	Horse dung fungus	Stem	0
<i>Pycnopus</i>	Scarlet bracken	Head	4

coccineus

<i>Solanum lycopersium</i>	Common truss tomato	Fruit	0
<i>Tulbaghia violacea</i>	Society garlic	Leaf	0
<i>Tulbaghia violacea</i>	Society garlic	Bulb	1
<i>Zingiber officinale</i>	Ginger	Root	2

^aZone of inhibition

In subsequent experiments the antibacterial activity of the extracts towards MRSA were analysed by treating WBG 8287 for 1h in growth medium with increasing concentrations of extract, after which colony survival was measured (Figure 4.2). Honey fungus and cardamom pod extracts reduced colony survival by 95% or more when cells were treated with a 1:1 mixture of extract and growth medium. At a ratio of 9:1, honey fungus, cardamom pods and juniper berries eradicated all surviving colonies. Extraction buffer controls showed that this was due to the toxicity of the extract and not the extraction buffer itself (Figure 4.1). Bhut jolokia chilli seeds and Persian lime fruit showed 75.04% and 61.36% reduction in surviving colonies respectively when used at a 9:1 ratio

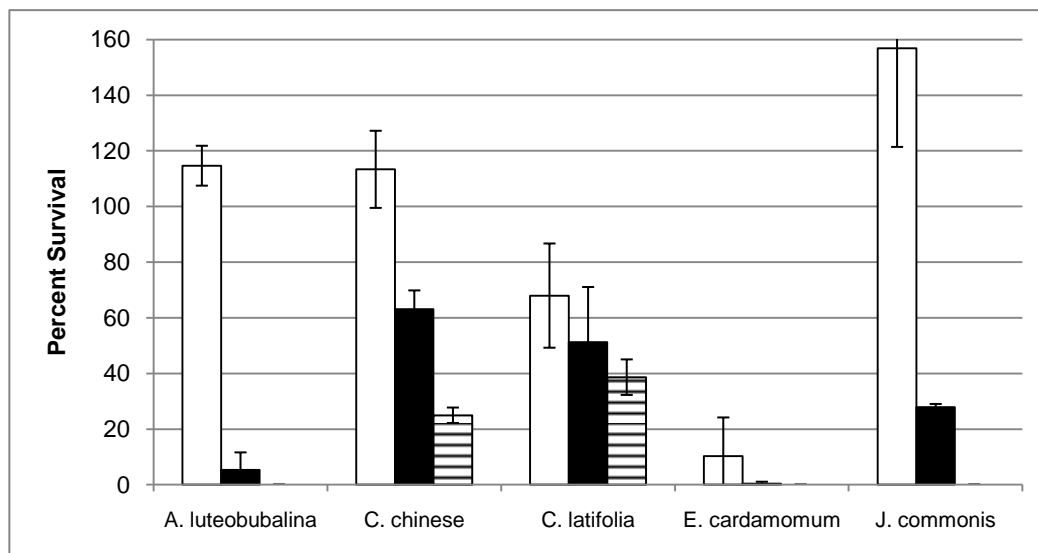


Figure 4.2 Survival of MRSA with increasing concentrations of plant and fungal extracts. *S. aureus* WBG8287 treated for 1h in bactopectone medium containing 10% (open column), 50% (closed column) and 90% (striated column) of the five most bactericidal tissue extracts. Data represent the percentage survival relative to an untreated control. Data represent the mean of triplicate tests. Error bars represent standard deviation of triplicate samples.

4.1.4 Proteinase Digest of Plant and Fungal Samples

Survival of WBG 8287 cells after treatment with proteinase digested extracts and mock digested control samples is shown in figure 4.3. Proteinase digestion of honey fungus extract had no effect on its activity. Surprisingly, proteinase digestion of Persian lime extract appeared to increase its antibacterial activity. Juniper berries digested with either proteinase showed a slight loss of inhibitory activity. Trypsin and proteinase OB digestion of the bhut jolokia chilli extract reduced its antibacterial activity by 59.5% and 34.5% respectively. Cardamom pod extracts digested with either proteinase showed complete loss of antibacterial activity.

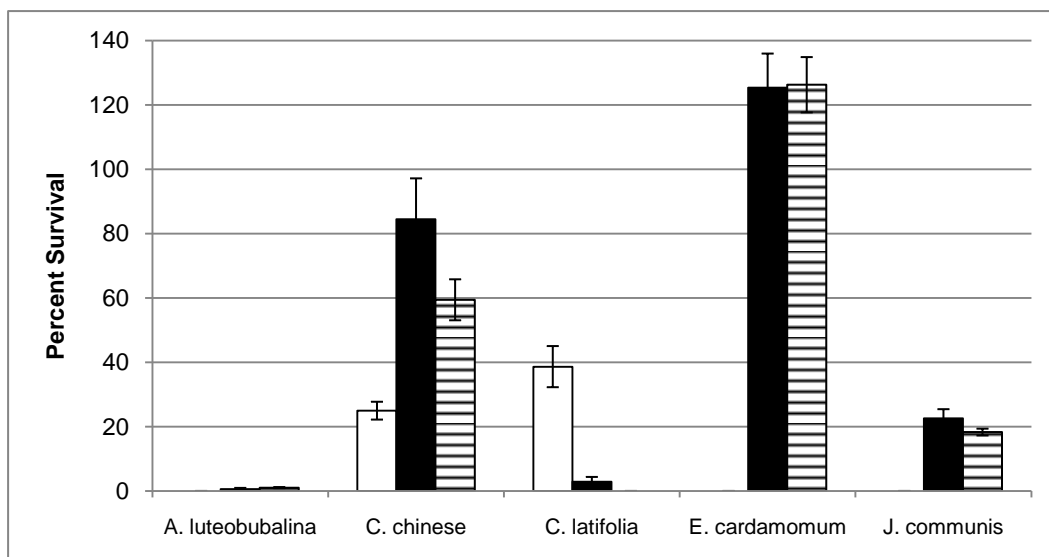


Figure 4.3 Survival of MRSA treated with protease digested plant and fungal extracts. Survival of *S. aureus* WBG 8287 treated for 1h with plant and fungal extracts (open columns). Extracts digested with trypsin (closed columns) or protease OB (striated columns). Open columns represent mock digested controls. Survival was measured as a percentage of inoculated bactopectone controls. Data represent the mean of triplicate samples. Error bars represent the standard deviation.

Table 4.2 shows the zone of inhibition of the protease digested and mock digested hop flowers on WBG 8287 inoculated plates. Most hop flowers treated with trypsin or proteinase OB showed no difference in the radius of zone of inhibition compared to the mock digested controls. Citra hop flowers showed an increase in the inhibition radius by 0.5 mm and summer hops showed an increase in radius of 1 mm when treated with trypsin and proteinase OB respectively.

Table 4.2 Zone of inhibition around proteinase treated hop extracts

<i>Humulus lupulus</i> Variety	Undigested (mm)	Trypsin (mm)	Proteinase OB (mm)
Cascade	3-4	3	3-3
Citra	2-4	2.5-3	3

Chinook	3-4	3	3
Summer	3-4	3	4
Topaz	3-4	3	3

4.2.1 Minimal Inhibitory and Minimal Lethal Concentrations of Synthetic Antimicrobial Peptides

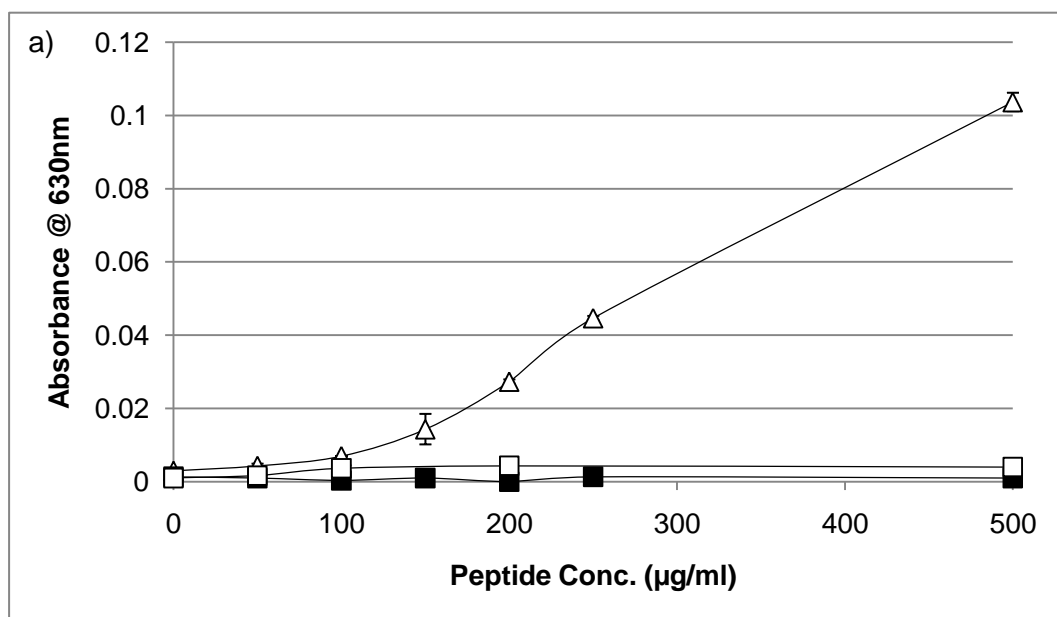
Table 4.3 shows the MIC and MLC of antibacterial peptides against *S. aureus*. In bactopectone medium, the MIC of melittin against WBG 8287 was 3 µg/ml. The MIC and MLC of the truncated analogues of melittin could not be determined because these peptides were not soluble in bactopectone medium at concentrations above 50 µg/ml (Figure 4.4a). In phosphate buffer and PBS melfrag12-26 appeared soluble at all concentrations up to 500 µg/ml. Melittin precipitated out of bactopectone medium at concentrations above 75 µg/ml (Figure 4.4b). However, the peptide appears soluble in phosphate buffer and PBS at concentrations up to 500 µg/ml. Melittin showed a greater tendency to precipitate out of bactopectone medium than its truncated analogue at concentrations below 200 µg/ml. However, at concentrations above 200 µg/ml, melfrag12-26 showed much higher absorbance in bactopectone medium compared to melittin. The minimum lethal concentration of bac8c against WBG 8287 was 7 µg/ml. But against strain W17S and Aus3 it was 80µg/ml. Peptide AP1 did not inhibit growth of WBG 8287 at 200 µg/ml. Therefore, the MIC and MLC could not be measured for this peptide.

Table 4.3 Minimal inhibitory and minimal lethal concentrations of antimicrobial peptides against MRSA

Peptide	MRSA Strain	MIC (µg/ml)	MLC (µg/ml)
	WBG 8287	3	5
Melittin	W17S	ND ^a	5
	Aus3	ND	5

	BB	ND	5
Melfrag12-24	WBG 8287	>100 ^b	>100
Melfrag12-26-L1	WBG 8287	>50	>300
Melfrag12-26	WBG 8287	ND	>130
	WBG 8287	ND	7
Bac8c	W17S	ND	80
	Aus3	ND	80
AP1	WBG 8287	>200	ND

^a Not determined ^b Minimal inhibitory or minimal lethal concentration exceeded highest concentration tested



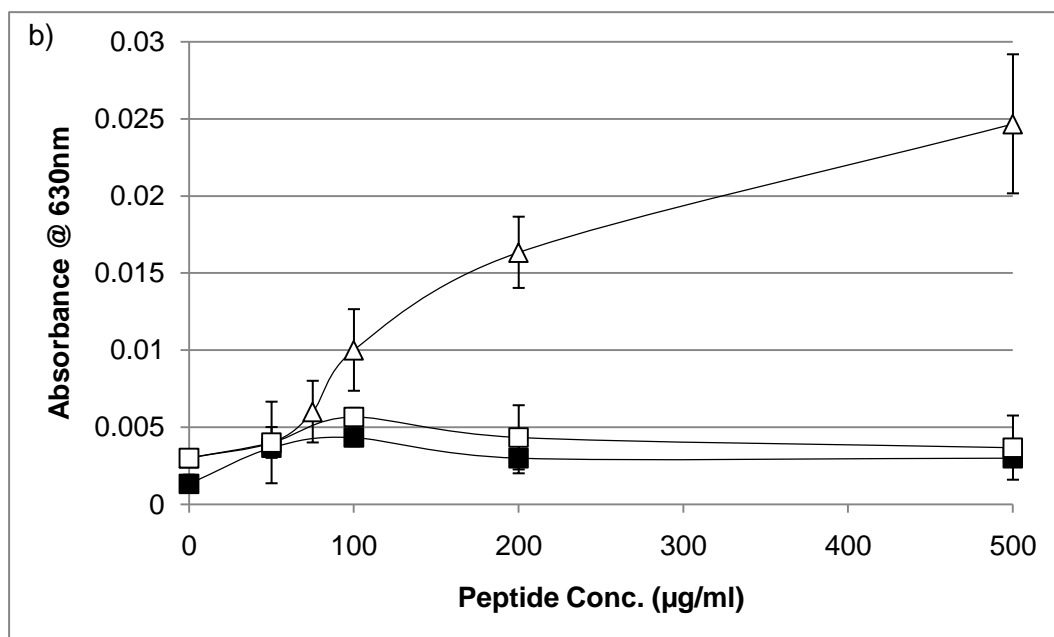


Figure 4.4 Absorbance of melittin and melfrag12-26 in different media.

Figure 4.4a shows the absorbance of increasing concentrations of melfrag12-26 in PBS (open squares) phosphate buffer (closed squares) and 8 g/l bactopectone (open triangles). Figure 4.4b shows data from an identical experiment performed with melittin. Each point is the mean of triplicate tests with error bars representing the standard deviation.

4.2.2 Minimal Lethal and Minimal Inhibitory Concentrations of Melittin, Melfrag12-26 and AP1 in Different Media

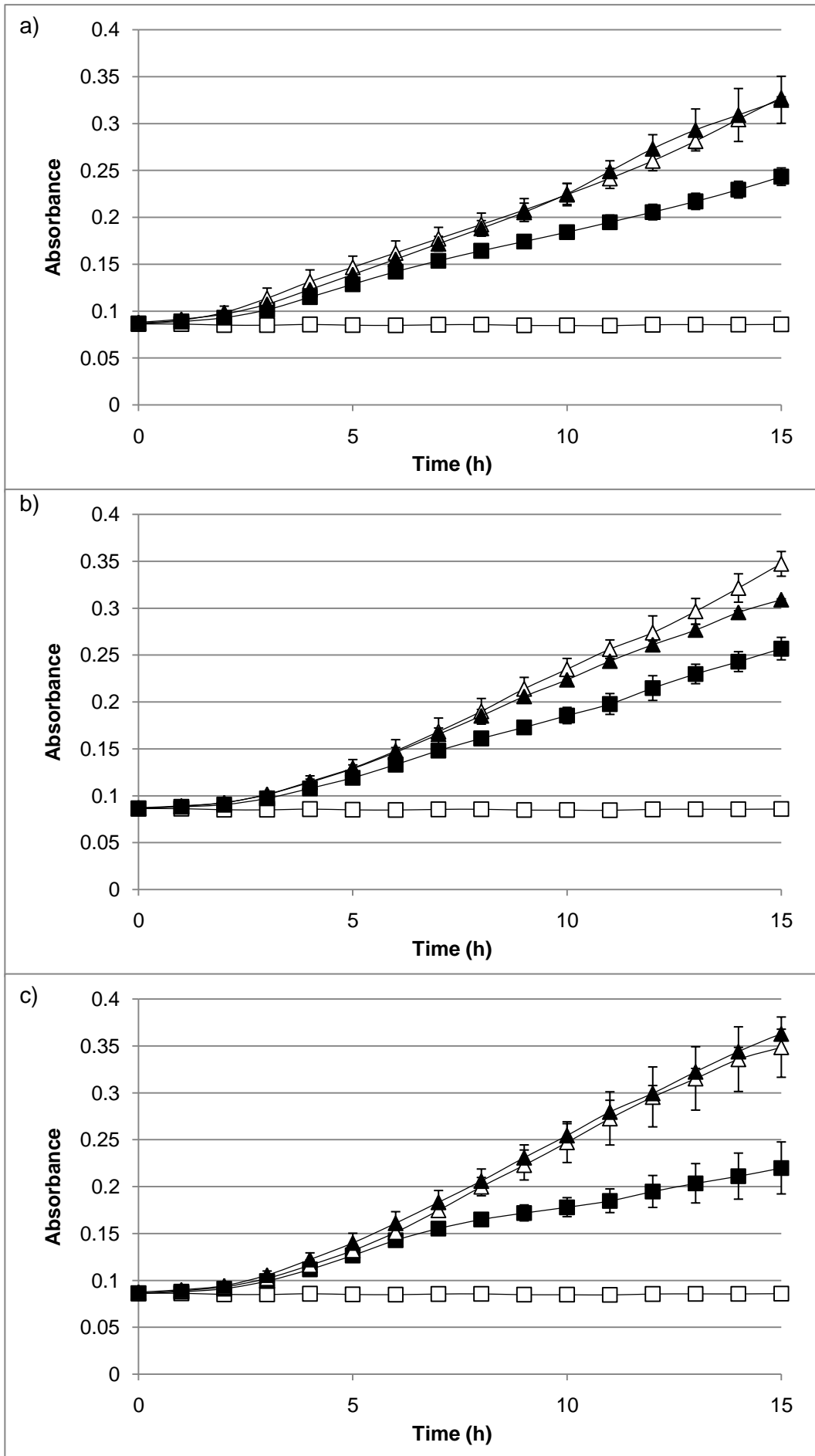
The MLC and MIC of melittin against WBG 8287, in different growth media and phosphate buffer containing increasing concentrations of NaCl, is presented in Table 4.4. Exposure to melittin in PBS showed significantly lower MLC than that measured in culture media. Tests in phosphate buffer containing different NaCl concentrations confirmed that sensitivity to melittin was inversely proportional to salt concentration. The MLC of melittin differed in the three different media and the protein concentrations of the three media correlated directly with the corresponding MLC (Table 4.4). Salt content was below 1% in all three media. In phosphate buffer, melfrag12-26 was lethal to all cells at 35 µg/ml. Figure 4.5 shows the growth curves of WBG 8287 in the presence of AP1 in bactopectone medium supplemented with MgCl₂. Addition of MgCl₂ to the medium did not increase the inhibitory activity of AP1 against WBG 8287. Cells grown in the presence of AP1 had increased growth rates over those grown without AP1. In particular, treatment with AP1 in media supplemented with MgCl₂ at 15mM

(Figure 4.5c) and 20 mM (Figure 4.5d) greatly increased the growth rate of the cells.

Table 4.4 Minimal lethal and minimal inhibitory concentrations of melittin peptides in different media

Media	Melittin MLC ($\mu\text{g/ml}$)	Melittin MIC ($\mu\text{g/ml}$)	Melfrag12-26 MLC ($\mu\text{g/ml}$)	Medium Protein Conc. ($\mu\text{g/ml}$)
Bactopeptone	5	3	ND	19.7
Lysogeny Broth	4	2	ND	17.8
Brain Heart Infusion	2	1	ND	9.1
PB	0.5	ND ^b	35	ND
PB ^a + 8 g/L NaCl	3	ND	ND	ND
PB + 15 g/L NaCl	5	ND	ND	ND

^a Phosphate buffer ^b Not determined



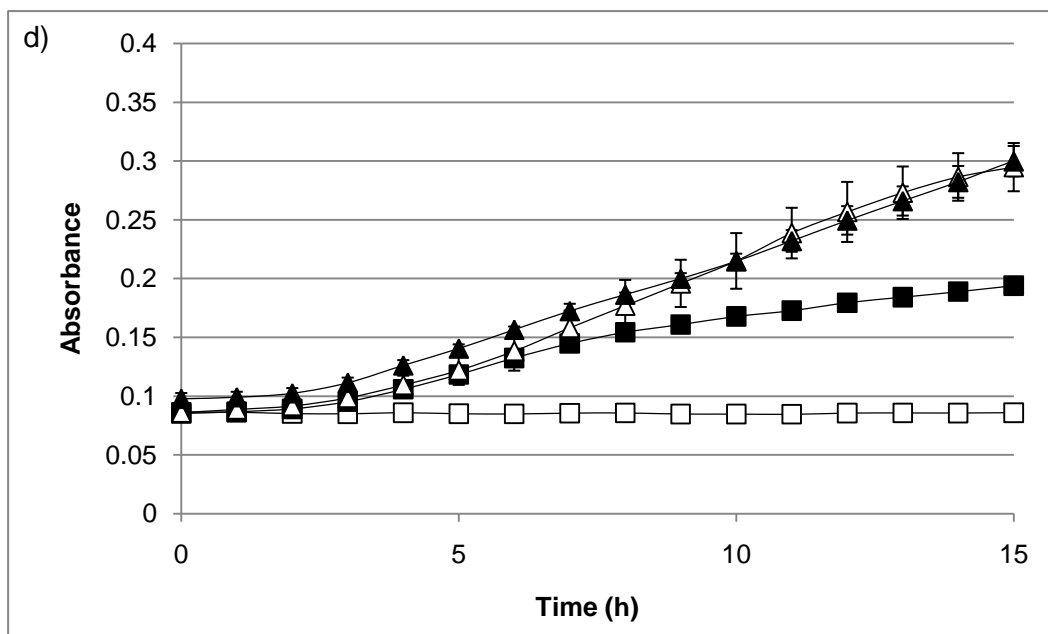


Figure 4.5 Growth curves of MRSA treated with AP1 in media supplemented with MgCl₂. WBG 8287 cells were exposed to 0 µg/ml (closed squares) 50 µg/ml (open triangles) and 100 µg/ml (closed triangles) of AP1 in 8 g/l bactopectone (a) and bactopectone supplemented with 10 mM MgCl₂ (b), 15 mM MgCl₂ (c) and 20 mM MgCl₂ (d) for 15h. Mock inoculated samples of each medium (open squares) were negative controls. Each data point represents the mean of three tests. Error bars represent the standard deviation of triplicate samples.

4.2.3 Effect of Incubation Temperature on Melittin Susceptibility.

Survival of bacteria after exposure to melittin was inversely related to the temperature during exposure (Figure 4.6). At 0.1 µg/ml melittin, survival was 97% at 5°C, but was reduced to 35% at 45°C. At 0.5 µg/ml melittin, 66% survival at 5°C was reduced to 5% survival at 45°C.

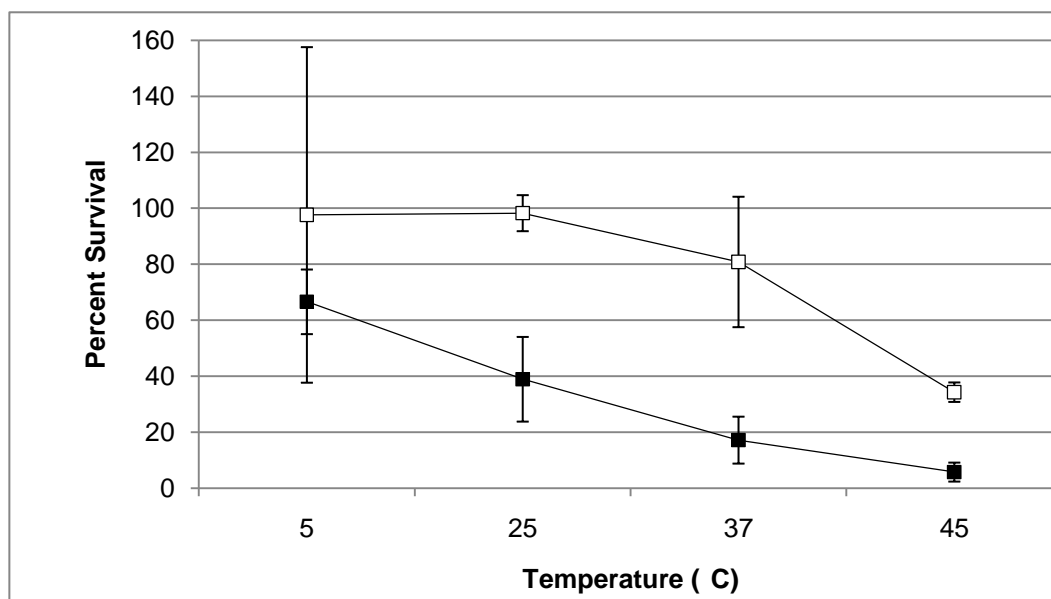


Figure 4.6 The effect of incubation temperature on the bactericidal activity of melittin. WBG 8287 exposed for 1h in 0.1 µg/ml (open squares) or 0.5 µg/ml (closed squares) of melittin in PBS, at different incubation temperatures. Colony survival was expressed as the percentage of surviving bacterial colonies compared with an untreated control. Data represent the mean of six replicates and error bars indicate standard deviation.

4.3.1 Development of Resistance to Melittin and Bac8c

All strains grown in sub-inhibitory concentrations of the AMPs were able to tolerate higher concentrations of the peptides than their wild-type counterparts. The four *S. aureus* strains sub-cultured in increasing concentrations of the peptide every 24h had comparable MLCs in bactopectone to that of cells which were sub-cultured every 12h. In these experiments the MLC of melittin against WBG 8287, W17S, Aus3 and BB was increased by 34, 30, 32 and 30 fold respectively after fourteen subcultures (Table 4.5). However, when melittin resistant WBG 8287 cells were treated with melittin in phosphate buffer the MLC dropped from 170 µg/ml to 3 µg/ml. The addition of NaCl at 8 and 15 g/l to phosphate buffer increased the MLC of melittin against resistant cells to 5 and 7 µg/ml respectively (Table 4.5). However, even at 15 g/l NaCl, the MLC of melittin against resistance cells was 7 µg/ml, which is similar to the MLC of 5 µg/ml of melittin against wild-type cells treated in bactopectone. After seven days sub-culture in the presence of bac8c, WBG 8287 could survive in concentrations of the peptide 24-fold higher than that tolerated by the wild-type population. With the

same level of exposure to bac8c as WBG 8287, the MLC of bac8c against strains W17S and Aus3 increased to 170 µg/ml and 150 µg/ml respectively (Table 4.5). However, due to the high MLC of bac8c required to kill wild-type strains of W17S and Aus3, this only corresponded to a MLC increase of 2.1 and 1.9-fold respectively. The anionic peptide AP1 did not affect the growth of melittin resistant WBG 8287 at concentrations up to 150 µg/ml (Figure 4.7). In fact, the growth curves show that, over 10h, cells treated with 150 µg/ml AP1 have almost identical growth rates as untreated bacteria.

Table 4.5 Minimum lethal concentration of melittin and bac8c against MRSA with induced resistance to the peptides in different media

MRSA Strain	Culture Medium	Melittin MLC (µg/ml)	Bac8c MLC (µg/ml)
	Bactopeptone	170	160
WBG 8287	PB	3	ND ^a
	PB ^a + 8 g/l NaCl	5	ND
	PB + 15 g/l NaCl	7	ND
W17S	Bactopeptone	150	170
Aus3	Bactopeptone	160	150
BB	Bactopeptone	150	ND

^a Phosphate buffer ^b Not determined

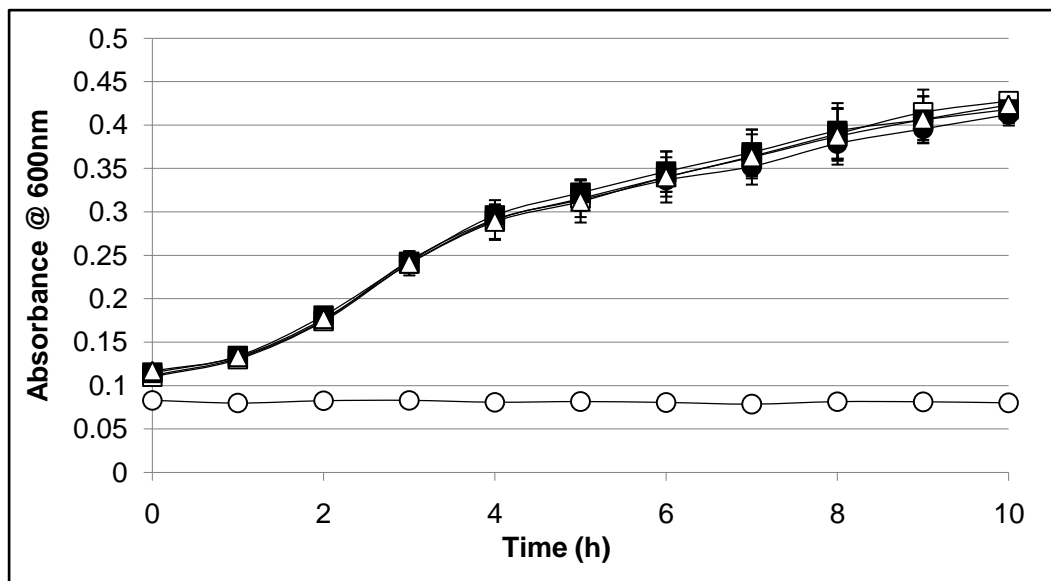


Figure 4.7 Growth of melittin resistant bacteria with AP1. Melittin resistant WBG 8287 was grown in 8 g/l bactopectone with 50 µg/ml (open squares), 100 µg/ml (closed squares) and 150 µg/ml (open triangles) of AP1. Growth was measured over 10h using a spectrophotometric plate reader. A mock inoculated (open circle) and a zero-AP1 control (closed circles) was run with the test samples. Each data point is the average of six replicates and error bars represent the standard deviation.

4.3.2 Investigation of Outer Cell Surface Damage After Antimicrobial Peptide Treatment

Wild-type and melittin resistant bacteria were exposed to 10 µg/ml of melittin and then examined by SEM. This concentration of melittin was chosen for its known toxicity against wild-type cells so that if melittin was causing surface damage this could be observed. Prior to melittin exposure wild-type cells had a ruffled surface with no visible signs of morphological damage (Figure 4.8a,b). The development of melittin resistance caused no discernable changes in the external appearance of resistant bacteria (Figure 4.8e,f). After exposure to 10 µg/ml of melittin for 1h, wild-type strains showed major structural changes (Figure 4.8c,d) ranging from protrusions or “blebs” on the cell wall to major disintegration of cellular structure. Under the same conditions, WBG 8287 cells made resistant to melittin showed occasional surface blebs but none displayed major loss of structural integrity (Figure 4.8g,h).

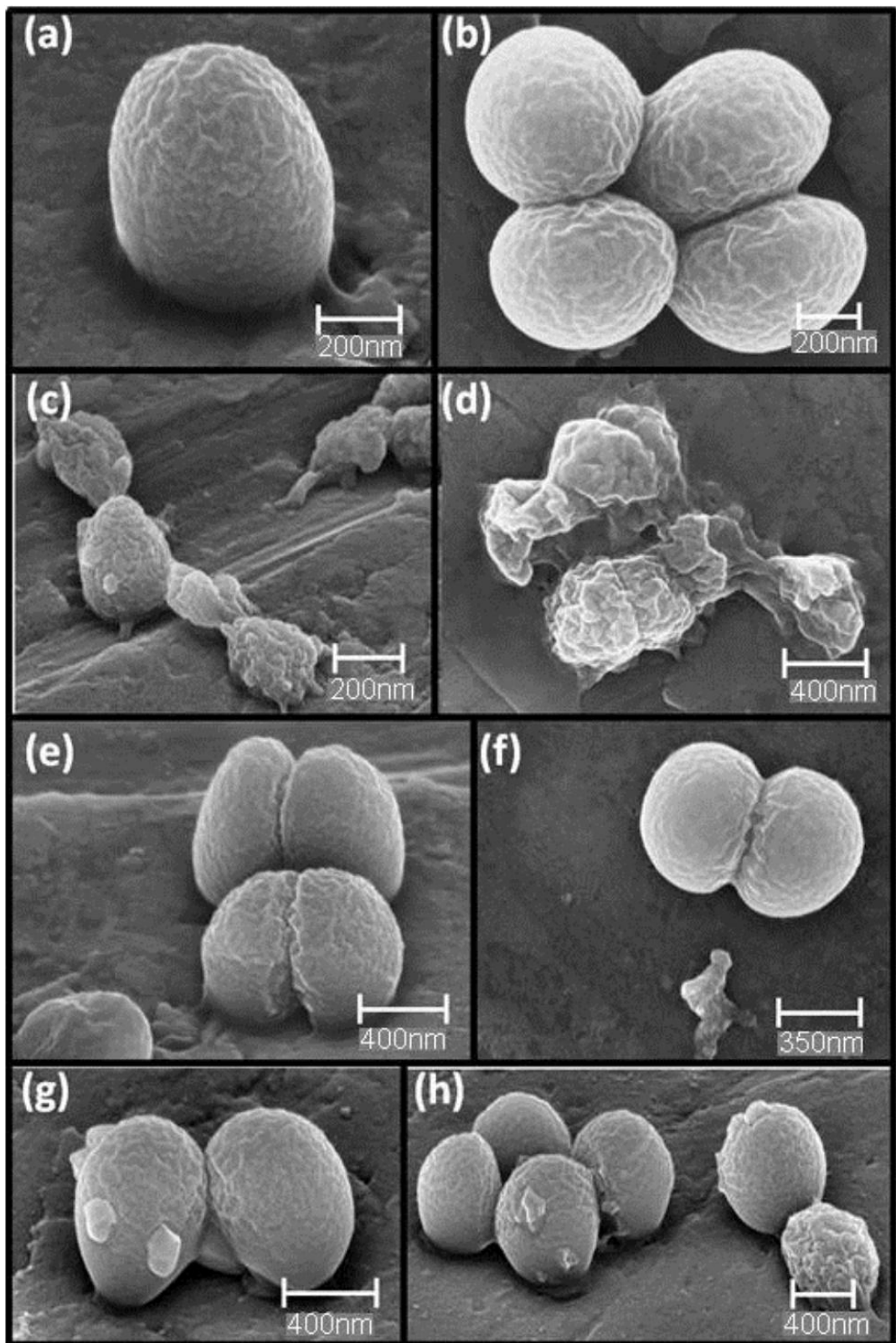


Figure 4.8 Scanning electron microscopy images of wild-type and melittin resistant MRSA. (a, b) untreated wild-type and (e, f) untreated melittin-resistant WBG 8287 cells in PBS, (c, d) wild-type bacteria treated with 10 $\mu\text{g/ml}$ melittin and (g, h) melittin resistant WBG 8287 cells treated with 10 $\mu\text{g/ml}$ melittin in PBS.

4.3.3 Binding Affinity of Eosin Dye and Bacterisense 641 to Wild-type and Melittin Resistant Cells

Melittin resistant bacteria treated with eosin dye showed significantly higher absorbance at 490nm than wild-type cells (Figure 4.9: $P < 0.005$). To test if this difference was due to a change in electrostatic potential, wild-type and resistant cells were treated with the cationic fluorescent probe, Bacterisense 645, and the fluorescent intensity was measured using flow cytometry (Figure 4.10). Binding of Bacterisense 645 to the surface of melittin-resistant MRSA was 5.3-fold lower than that of the parental population ($P < 0.001$: Table 4.6). Consistent with previous findings (311, 312), the increased tolerance to melittin was attributable at least partly to a reduction in negative-charge density on the outer bacterial surface.

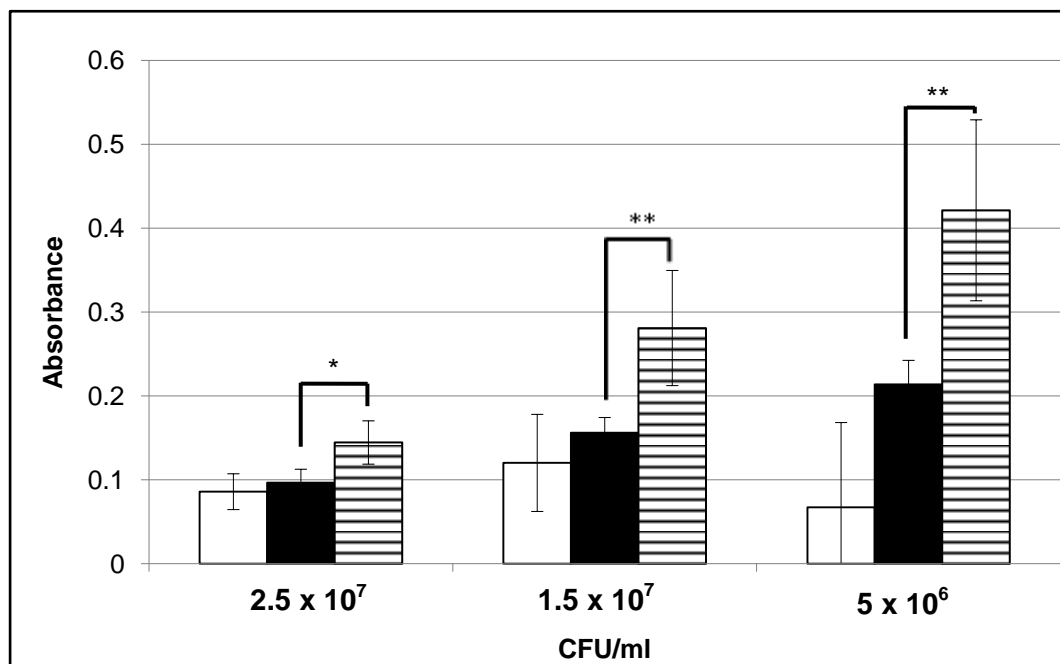


Figure 4.9 Absorbance of wild-type and melittin resistant cells stained with eosin. WBG 8287 cells naïve (closed columns) and resistant (striated columns) to melittin were diluted to 2.5×10^7 , 1.5×10^7 , and 5×10^6 CFU/ml and stained with eosin. Each suspension was analysed spectrophotometrically at 490nm. Data represents the mean of nine tests and error bars show standard deviation. Statistical significance was determined using the T-test. (*) $P \leq 0.01$, (**) $P \leq 0.005$.

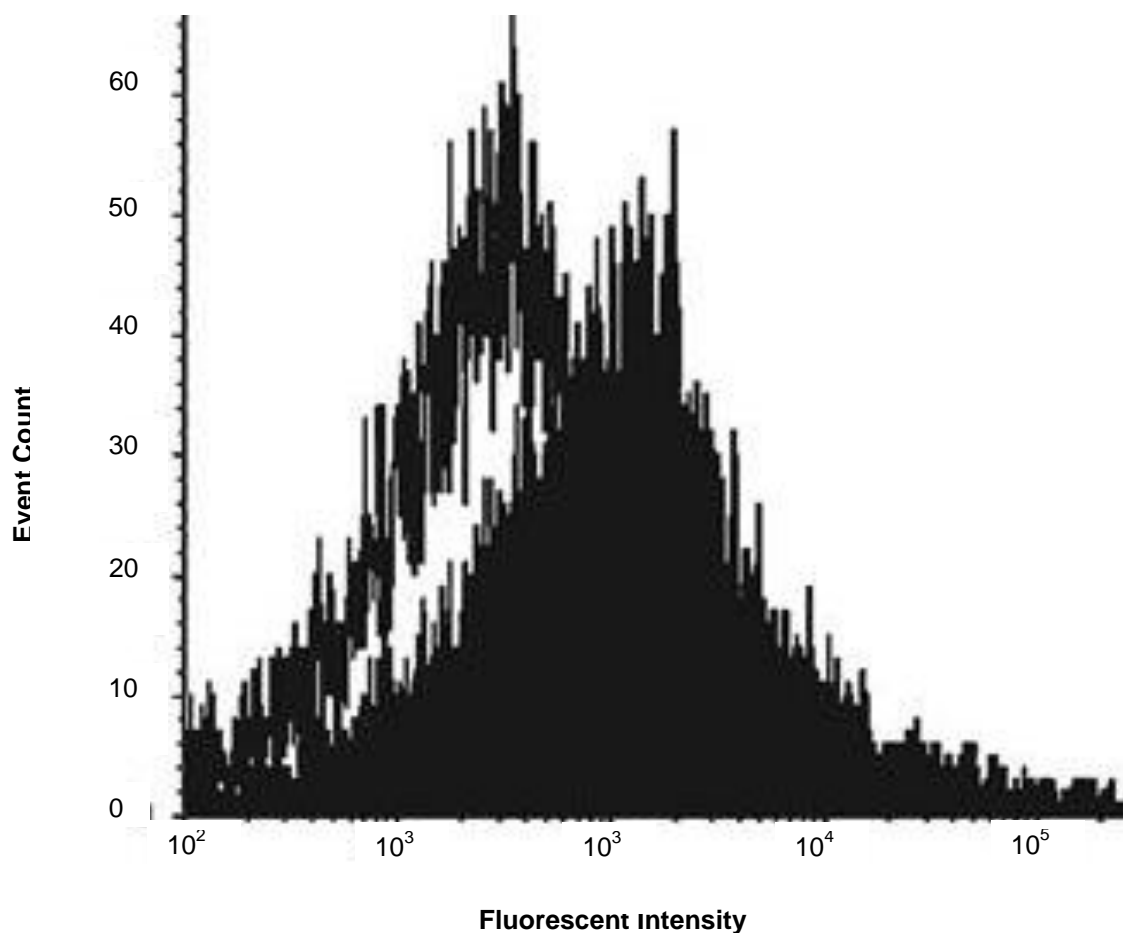


Figure 4.10 Flow cytometry of Bacterisense 645 bound to melittin naïve and resistant *S. aureus* WBG 8287. Closed plot: parental strain, open plot area: melittin resistant strain. Data show the mean of nine measurements for each cell type.

Table 4.6 Mean fluorescent intensity of bacterisense bound to wild-type and melittin resistant cells

Strain	Mean Fluorescent Intensity	Standard Deviation	P Value
WBG 8287			
Wild-type	34099	3952	P ≤ 0.001
Resistant	6445	2489	

4.3.4 Rapid Loss and Gain of Resistance in Populations of MRSA

Melittin resistant WBG 8287 cells were cultured for seven days in bactopectone medium without melittin. A control culture of the same population was grown in parallel with 12 $\mu\text{g/ml}$ of melittin. The results are summarised in table 4.7. The melittin resistant population of WBG 8287 showed 100% survival when exposed to 3 $\mu\text{g/ml}$ melittin in bactopectone medium. However, after growth for 24h in bactopectone medium without melittin, the same treatment resulted in only 46% survival (Figure 4.11: $P < 0.001$). A further six daily subcultures without melittin reduced the MLC to 30 $\mu\text{g/ml}$ and decreased the MIC to 6 $\mu\text{g/ml}$ (Figure 4.12). However, if this reversion population was cultured in media with 12 $\mu\text{g/ml}$ of melittin for 24h, the MLC of melittin against the surviving bacteria is increased to 100 $\mu\text{g/ml}$: i.e. 58.8% of the MLC of the most resistant population. In an identical experiment performed with bac8c resistant WBG 8287, the MLC was reduced from 160 $\mu\text{g/ml}$ to 110 $\mu\text{g/ml}$ after seven days culture without the peptide (Table 4.7).

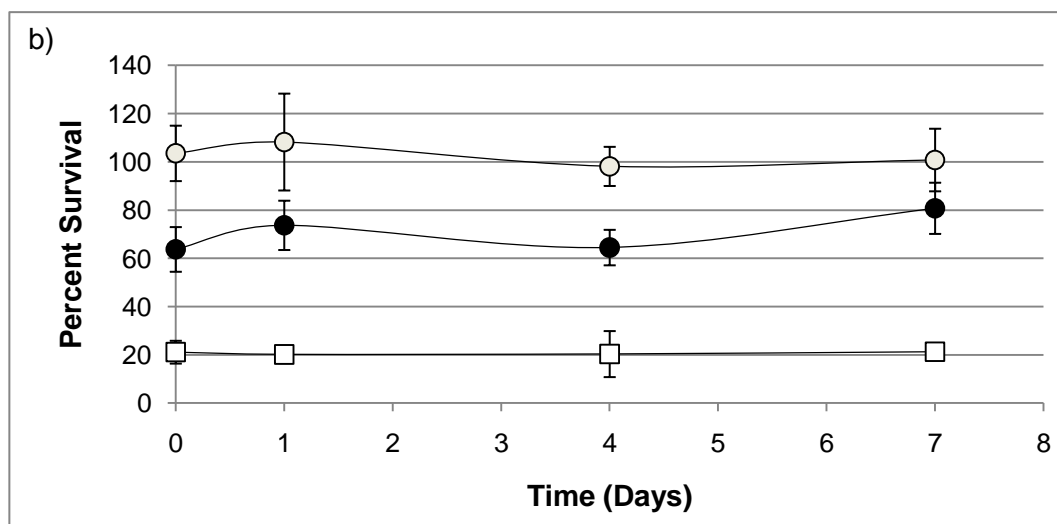
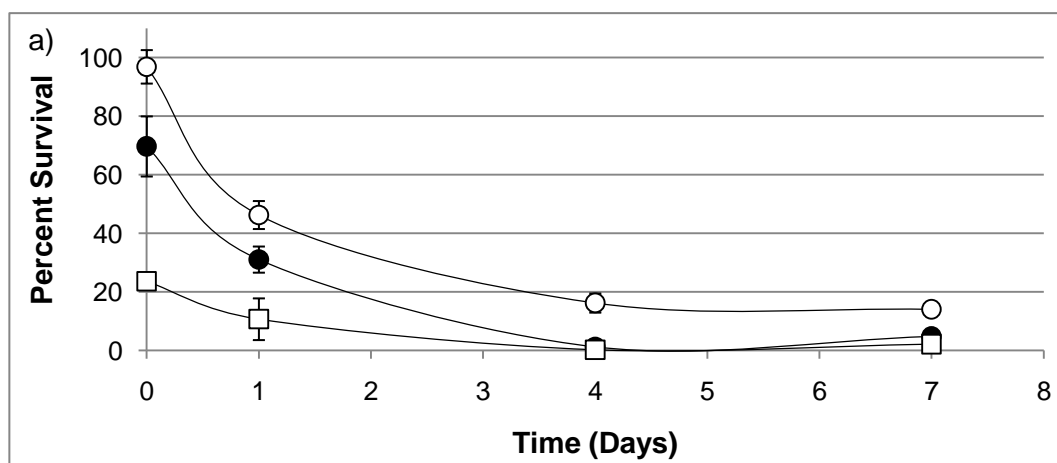


Figure 4.11 Survival of melittin-resistant bacteria cultured with and without melittin for seven days. (a) Survival of WBG 8287 cultured without melittin and exposed to 3 $\mu\text{g/ml}$ (open circles), 6 $\mu\text{g/ml}$ (closed circles) and 12 $\mu\text{g/ml}$ (open squares) of melittin on days 0, 1, 4 and 7 respectively. Survival is expressed as a percentage of untreated controls. (b) Results of a similar experiment on a resistant population that was maintained in media containing 12 $\mu\text{g/ml}$ melittin. Data are from two experiments with 3 replicates per experiment and Error bars represent the standard deviation.

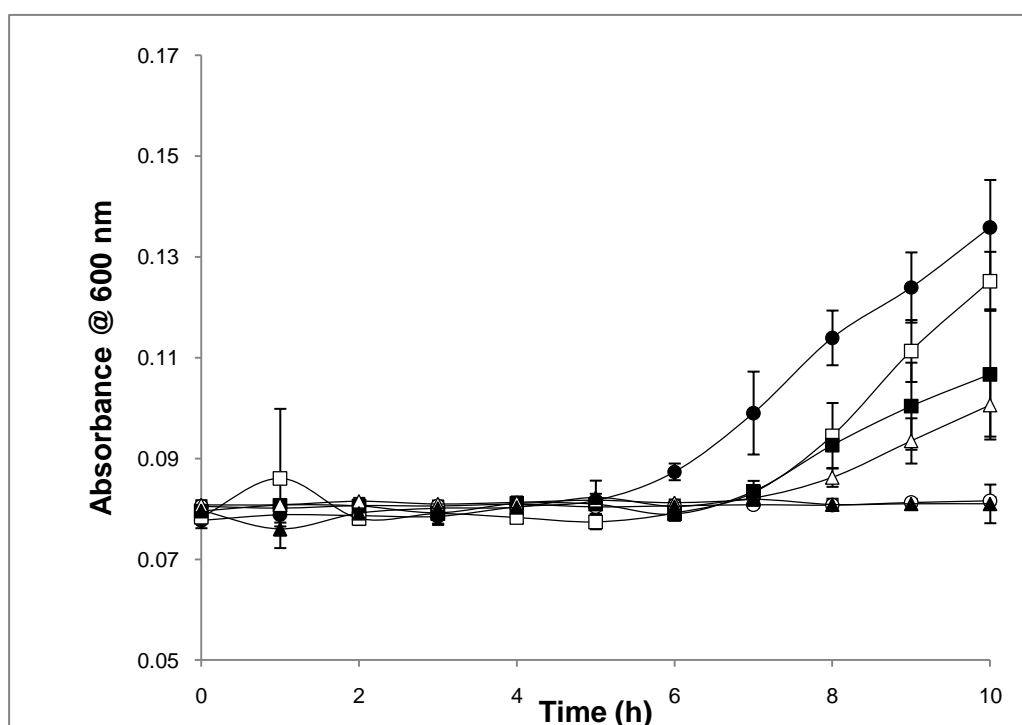


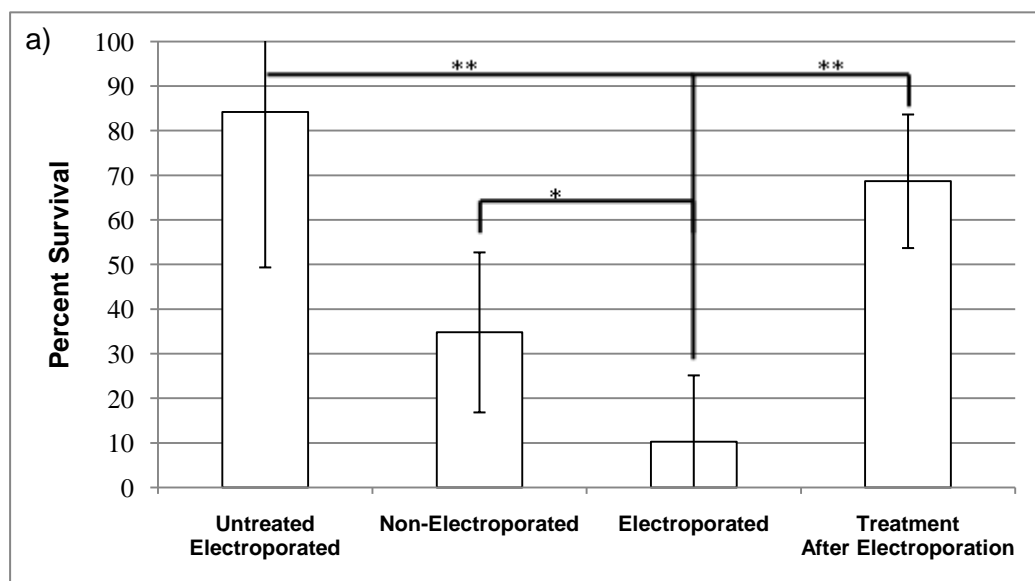
Figure 4.12 Growth of melittin-resistance reversion WBG 8287 in the presence of melittin. Growth in the presence of melittin at 3 $\mu\text{g/ml}$ (open squares), 4 $\mu\text{g/ml}$ (closed squares), 5 $\mu\text{g/ml}$ (open triangles), and 6 $\mu\text{g/ml}$ (closed triangles), control culture without melittin (closed circles) and mock inoculated control (open circles) over 10h. Data represent the mean of triplicate samples and error bars show standard deviation.

Table 4.7 Minimum lethal concentrations of antimicrobial peptides for resistant WBG 8287 and reversion/recovery populations

Population	MLC $\mu\text{g/ml}$
Melittin Resistant	170
Melittin Reversion	30
Melittin Recovery	100
Bac8c Resistant	160
Bac8c Reversion	110

4.4.1 Transmembrane Transport of Antimicrobial Peptides into MRSA

Antimicrobial peptides were transported into MRSA strain WBG 8287 by electroporation to test for intracellular inhibitory activity. All cells electroporated with the peptides had a significantly reduced survival rate compared to the controls (Figure 4.13a-c: $P < 0.05$). Electroporation with melittin and melfrag12-26 reduced the number of surviving colonies by 24.56% and 79.01% respectively compared with mock electroporated controls. The results for control populations electroporated with bac8c varied sufficiently that the difference between test and control populations reached only borderline significance ($P = 0.053$).



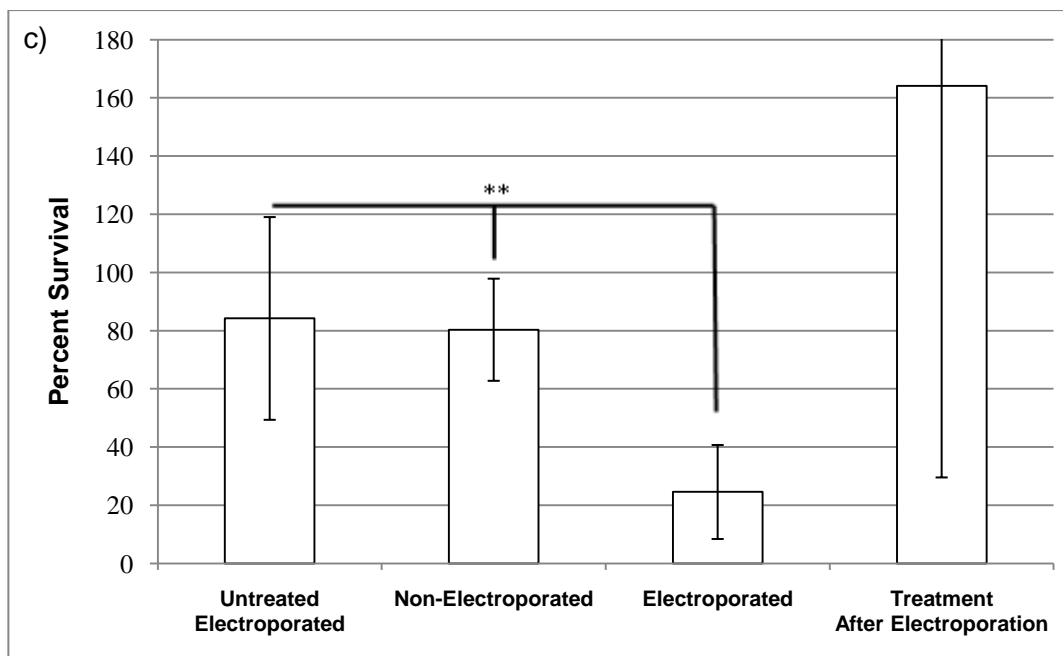
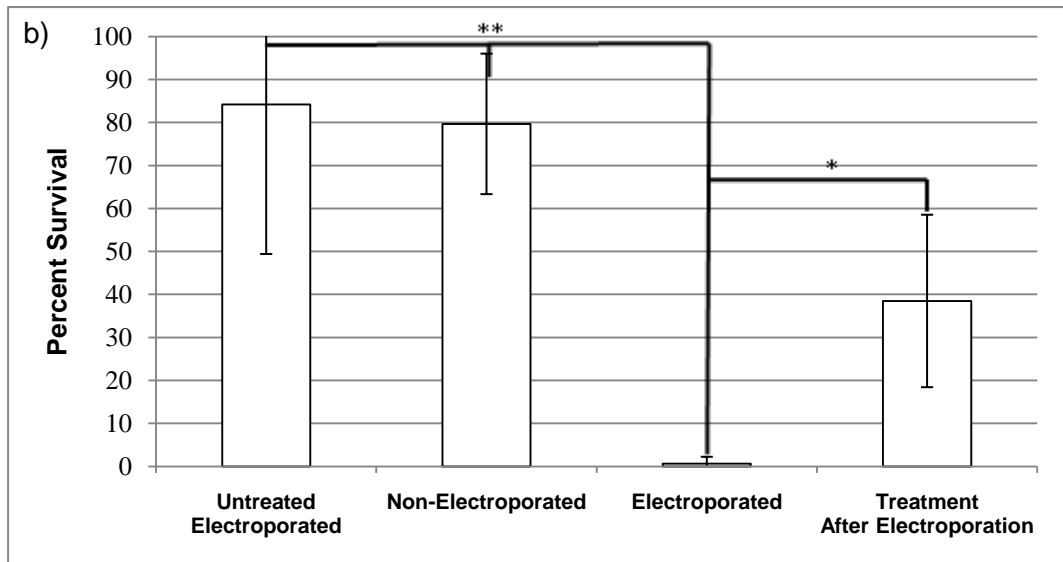


Figure 4.13 Electroporation of MRSA with antimicrobial Peptides.

(a) Electrocompetent WBG 8287 cells treated with 2.5 µg/ml melittin, (b) 50 µg/ml melifrag12-26 or (c) 2 µg/ml bac8c and then immediately electroporated. Colony survival was measured and expressed as a percentage of surviving colonies relative to an untreated, unelectroporated control. Data represent the mean of six samples and error bars represent the standard deviation. The significance of the difference between each sample and the control samples was calculated using the Pairwise t-test. (*) $P \leq 0.05$. (**) $P \leq 0.001$.

4.4.2 Effects of Triton X-100 on the Toxicity of Antimicrobial Peptides

Six populations of WBG 8287 bacteria were treated with melfrag12-26 in bactopectone medium with a range of concentrations of the detergent Triton X-100. Cells cultured in the presence of Triton X-100 had the same survival rates as the controls without Triton X-100 (Figure 4.14). Most samples treated with melfrag12-26 and cultured with Triton X-100 showed significantly reduced survival, compared to the controls. Increased concentrations of Triton X-100 correlated with decreased survival of cells exposed to the peptide (Figure 4.14). The exception to this trend was cells grown with melfrag12-26 plus 0.05% Triton X-100 which did not appear to be affected by the peptide. The MLC for cells treated with melittin, melfrag12-26 and bac8c in bactopectone containing 0.1% and 0.005% Triton X-100 is presented in table 4.8. The MLC of melittin decreased by 30% and 45% when cells were treated with 0.1% and 0.005% Triton X-100 respectively. In bactopectone, 130 µg/ml of melfrag12-26 reduced the surviving population of WBG 8287 cells to 5% of the controls. When the peptide was administered in bactopectone supplemented with 0.005% or 0.1% Triton X-100 the MLC was 80 µg/ml and 25 µg/ml respectively. In 0.005% Triton X-100, the MLC of bac8c was 4.7 µg/ml and in 0.1% Triton X-100, the MLC was reduced to 2.6 µg/ml.

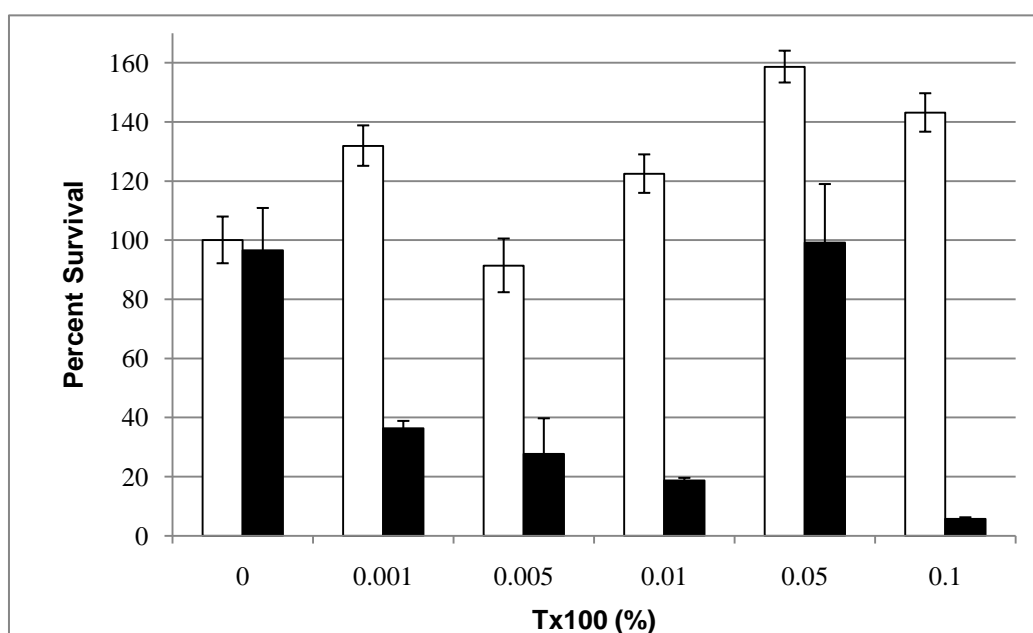


Figure 4.14 Exposure of MRSA to melfrag12-26 plus Triton X-100. WBG 8287 cells exposed for 1h to 50 µg/ml melfrag12-26 in medium supplemented with Triton X-100 (Closed bars) or cultured in Triton X-100 supplemented medium

without melFrag12-26 (open bars). Survival was calculated relative to untreated controls cultured in bactopectone without Triton X-100. Data represent the mean of three samples and error bars show the standard deviation.

Table 4.8 Minimum lethal concentration of antimicrobial peptides against WBG 8287 in bactopectone supplemented with Triton X-100

Peptide	BP ^a (µg/ml)	BP + 0.005% Tx100 ^b (µg/ml)	BP + 0.1% Tx100 (µg/ml)
Melittin	5	3.5 ± 0.55	2.75 ± 0.27
MelFrag12-26	>130	80 ± 0	25 ± 0
Bac8c	7	4.67 ± 0.52	2.58 ± 0.49

^a 8g/l bactopectone ^b Triton X-100

4.4.3 Haemolytic Potential of Triton X-100 in Combination with Antimicrobial Peptides

The pertinence of Triton X-100 on the haemolytic potential of the AMPs was investigated using human red blood cells. In PBS, 5 µg/ml of melittin was the only sample that showed any substantial increase in absorbance from erythrocyte lysis, compared to the untreated control (Figure 4.15a). Likewise, in PBS supplemented with 0.005% Triton X-100, only erythrocytes treated with melittin showed significant lysis compared to the untreated control (Figure 4.15b). There was little change in the absorbance of samples containing untreated cells or cells in PBS supplemented with 0.005% Triton X-100 alone.

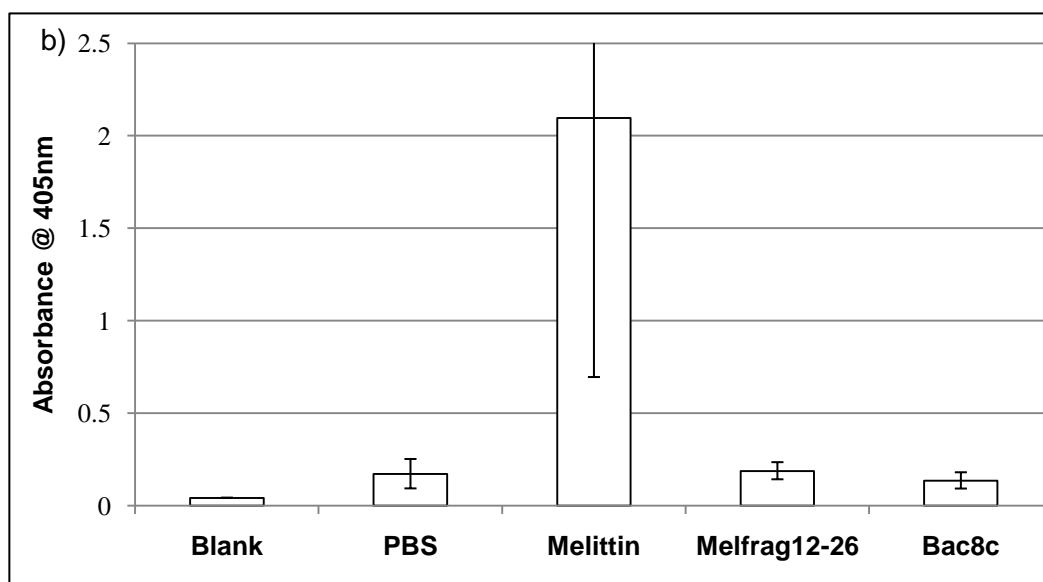
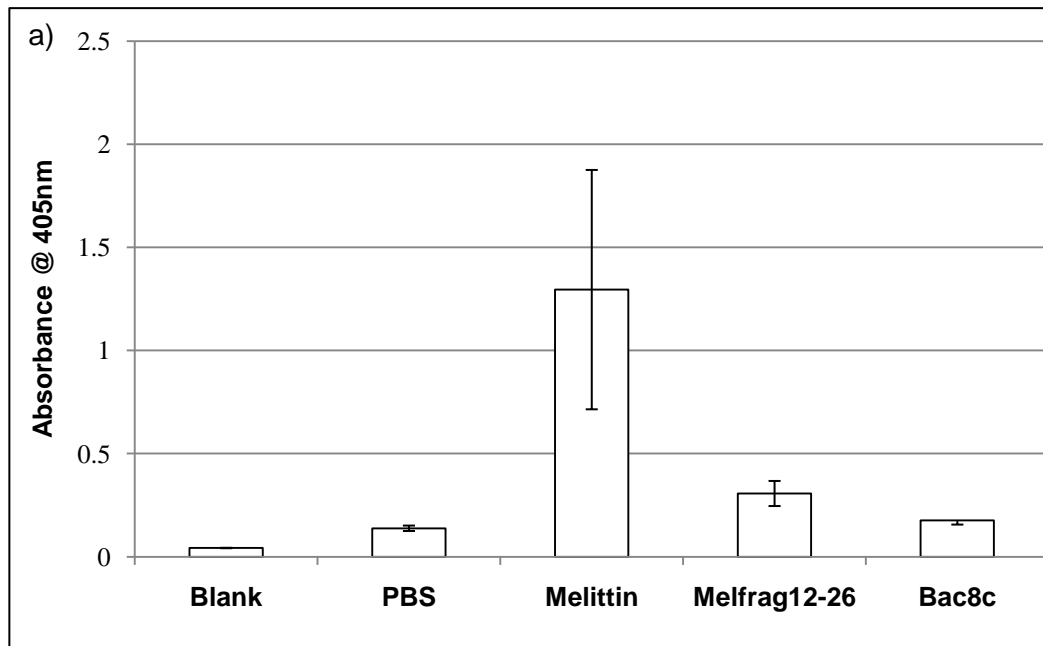
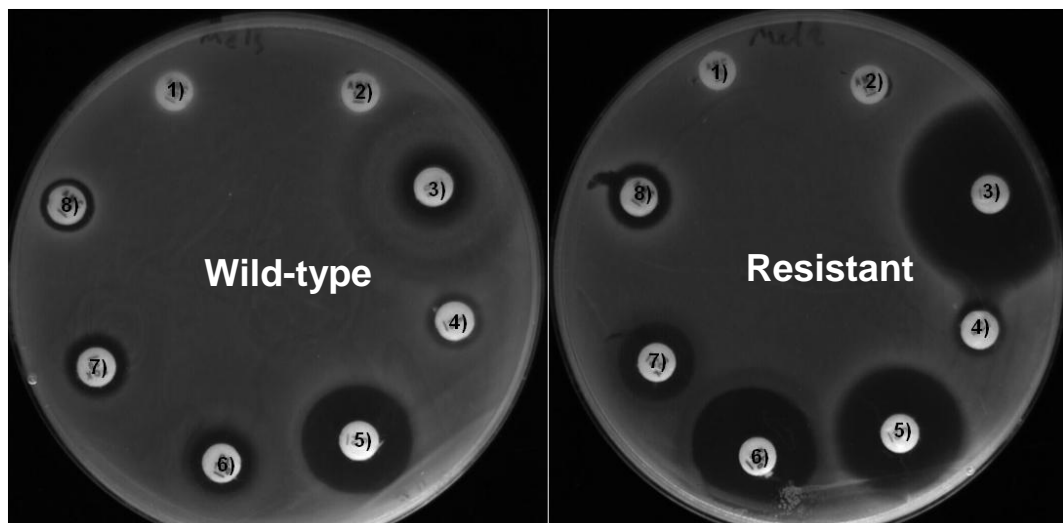


Figure 4.15 Haemolysis assay of antibacterial peptides with Triton X-100. (a) Red blood cells were exposed to 5 $\mu\text{g/ml}$ of melittin, 130 $\mu\text{g/ml}$ of melfrag12-26 or 6 $\mu\text{g/ml}$ of bac8c in PBS. (b) Cells were treated with 2.5 $\mu\text{g/ml}$ of melittin, 80 $\mu\text{g/ml}$ of melfrag12-26 and 4 $\mu\text{g/ml}$ of bac8c in PBS supplemented with 0.005% Triton X-100. After incubation the samples were centrifuged and the absorbance of the supernatant was measured. The blank sample consisted of PBS with no cells. The PBS column represents absorbance of the supernatant from cells that were mock treated in PBS. Data represent the mean of three samples and error bars represent the standard deviation.

4.5.1 Antibiotic Sensitivity of Wild-type and Antimicrobial Peptide Resistant MRSA

The sensitivity of melittin-resistant and wild-type WBG 8287 to 27 different antibiotics is shown in figure 4.16. The zone of inhibition radius for each antibiotic is presented in table 4.9. Melittin resistance did not increase the tolerance of the populations to any antibiotic tested. On the contrary, resistance to melittin appeared to sensitize the cells to many of the antibiotics tested, by varying degrees. The zones of inhibition created by cephalalexin, fusidic acid, imipenem and tetracycline were greater for melittin-resistant cells than for wild-type cells, by 5 mm or more. However, wild-type cells exposed to fusidic acid appeared to have a halo of partial clearing around the discs. This area was approximately the same size as the zone of inhibition seen for melittin-resistant cells around fusidic acid discs. This may indicate that the wild-type culture consists of a mixed population of fusidic acid sensitive and resistant cells.



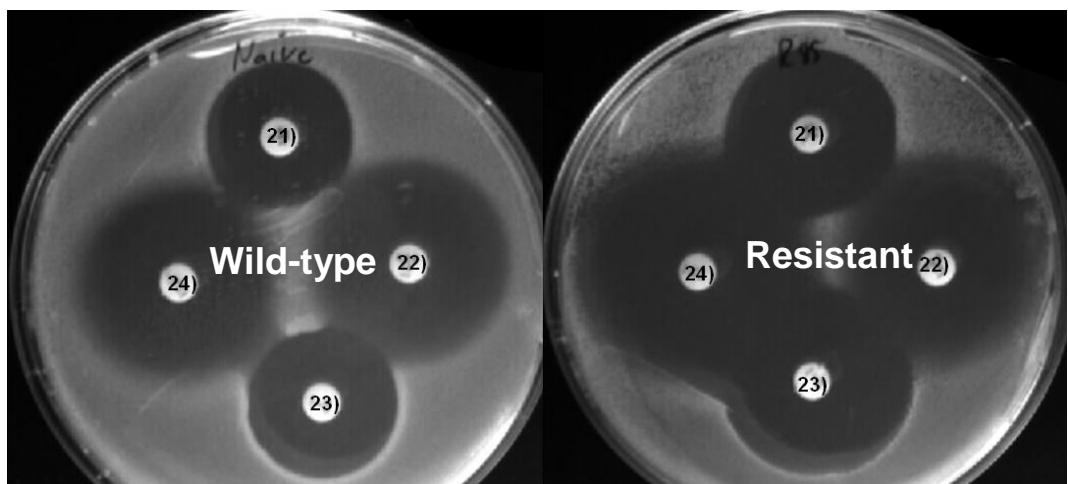
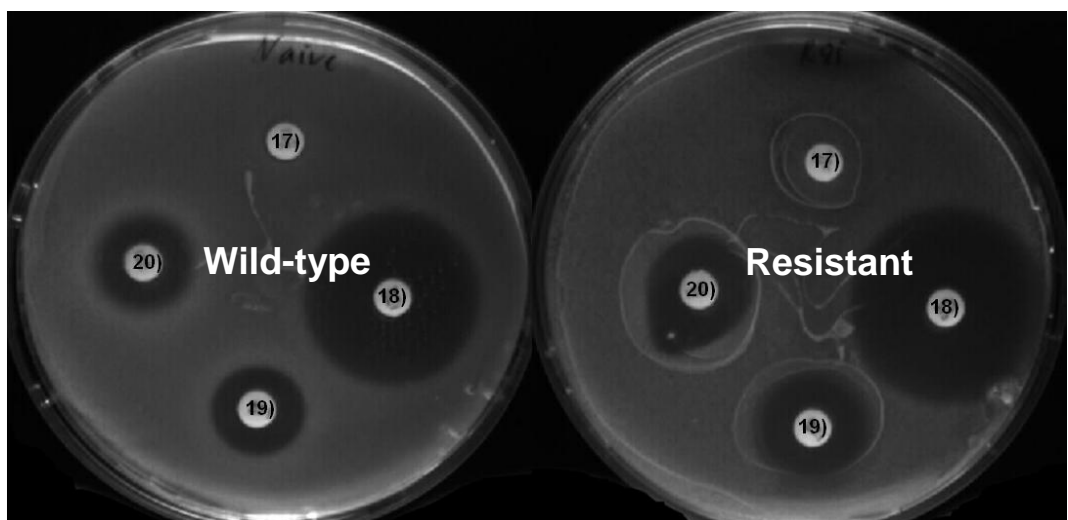
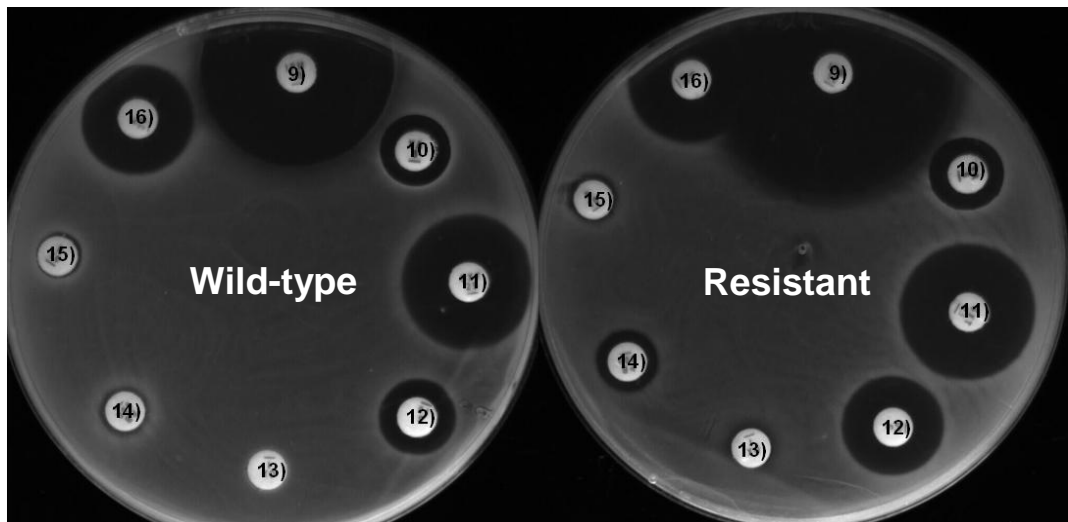


Figure 4.16 Antibiotic sensitivity of MRSA. Melittin-naïve and resistant *S. aureus* WBG 8287 was grown on bactopectone agar plates on which 27 different antibiotic discs were placed. The number on each disc corresponds to the antibiotic listed in table 4.9.

Table 4.9 Antibiotic sensitivity of melittin sensitive and resistant MRSA

Number	Antibiotic	Conc.(µg)	Wild-type ZOI ^a (mm)	Stdev (mm)	Resistant ZOI (mm)	Stdev (mm)
2	Ampicillin	10	0	NA ^b	0	NA
1	Amoxicillin	30	0	NA	0	NA
8	Augmentin	60	2	NA	2	NA
17	Cefaclor	30	0	0	0.3	0
7	Cefoxitin	10	1.5	NA	3	NA
6	Cephalexin	100	3.7	NA	9	NA
18	Ciprofloxacin	2.5	9.3	1.21	11.2	0.7
5	Chloramphenicol	10	2.5	NA	2	NA
4	Erythromycin	15	0.5	NA	1	NA
3	Fusidic Acid	10	3.1	1.75	18.6	6.36
19	Gentamicin	10	4.7	0.6	5	0
9	Imipenem	10	15.5	2.4	23	5.66
16	Kanamycin	30	6.5	NA	7	NA
20	Nalidixic acid	30	3.5	1	7.3	1.4
21	Nitrofurantoin	5	9.3	0.6	10.3	0
22	Norfloxacin	10	11.3	1.2	14.3	0
13	Oxacillin	1	0	0	0	0
15	Penicillin	9.91	1.5	0.7	1	0

14	Polymyxin B	35.7	3	1.4	3.4	0.7
23	Rifampicin	1	9	0	12.3	0
12	Streptomycin	10	4	NA	4	NA
11	Sulfamethoxazole	25	8.5	NA	8	NA
24	Tetracycline	10	12.3	0.5	16.3	0
25	Ticarcillin	75	1.8	1.5	3	0
26	Tobramycin	10	3.5	1	5.7	0
27	Trimethoprim	5	2.3	1.5	0.7	1.4
10	Vancomycin	5	3.5	2.12	3	0

^aZone of inhibition

^bNot applicable as samples tested in duplicate

4.5.2 Fusidic Acid Resistance Loss in Antimicrobial Peptide Resistant MRSA

As wild-type WBG 8287 cells already appeared sensitive to fusidic acid, before the MLC of fusidic acid was determined, all strains were grown with sub-lethal doses of fusidic acid to increase their resistance to the antibiotic. There was a large degree of variation in the MLC of fusidic acid between strains (Table 4.10). Contrary to previous findings, resistance to melittin did not affect the sensitivity of WBG 8287 or BB strains to fusidic acid. In contrast, strains W17S and Aus3 showed a significant decrease in the MLC of fusidic acid as they became more tolerant to melittin (Table 4.10). At 250 µg/ml, the highest concentration of fusidic acid tested, the growth of W17S was greatly inhibited. However, the bacteria were still able to survive at this concentration. The MRSA strains that were made resistant to bac8c also appeared to be more resistant to fusidic acid (Table 4.11). The MLC of fusidic acid for WBG 8287 and Aus3 increased by 70.8 µg/ml and 17.8 µg/ml respectively over that of bac8c naïve cells. Bac8c resistant W17S showed similar levels of survival in 250 µg/ml fusidic acid as that of its naïve counterpart.

Table 4.10 Minimal lethal concentration of fusidic acid against melittin naïve and resistant MRSA

Strain	Population	MLC (µg/ml)	Stdev (µg/ml)	P-Value
WBG 8287	Naïve	19.22	1.72	0.7
	Resistant	21.67	9.83	
W17S	Naïve	>250 ^a	0	<0.001
	Resistant	96.67	5	
Aus3	Naïve	101.11	3.33	<0.001
	Resistant	66.67	15.81	
BB	Naïve	156.67	18.03	1
	Resistant	156.67	18.03	

^a Minimal lethal concentration exceeds highest concentration of fusidic acid tested

Table 4.11 Minimal lethal concentration of fusidic acid against bac8c naïve and resistant MRSA

Strain	Population	MLC (µg/ml)	Stdev	P-Value
WBG 8287	Naïve	19.22	1.72	<0.001
	Resistant	90	0	
W17S	Naïve	>250 ^a	0	1
	Resistant	>250 ^a	0	
Aus3	Naïve	101.11	3.33	<0.001

Resistant

118.89

3.33

^a Minimal lethal concentration exceeds highest concentration of fusidic acid tested

4.5.3 PCR Analysis of Fusidic Acid Resistance Genes

Amplification of the fusidic acid resistance genes FusB and FusC was attempted in WBG 8287, W17S, Aus3 and BB strains that were naïve or resistant to melittin (Figure 4.17). Of the four naïve strains tested, only WBG 8287 yielded an amplification product with the FusB/FusC primers (Figure 4.17: lanes 1 and 10). The FusB/C genes were also able to be amplified from melittin-resistant WBG 8287 strains (Figure 4.17 Lanes 2 and 11). Melittin naïve Aus3 appeared to carry both FusB and FusC genes (Figure 4.17: lanes 5 and 14). However, these genes could not be amplified with the primers tested, from melittin resistant Aus3 bacteria (Figure 4.17: lanes 6 and 15). Amplification of FusB and FusC could not be achieved with DNA from strains W17S or BB regardless of whether they were naïve or resistant to melittin.

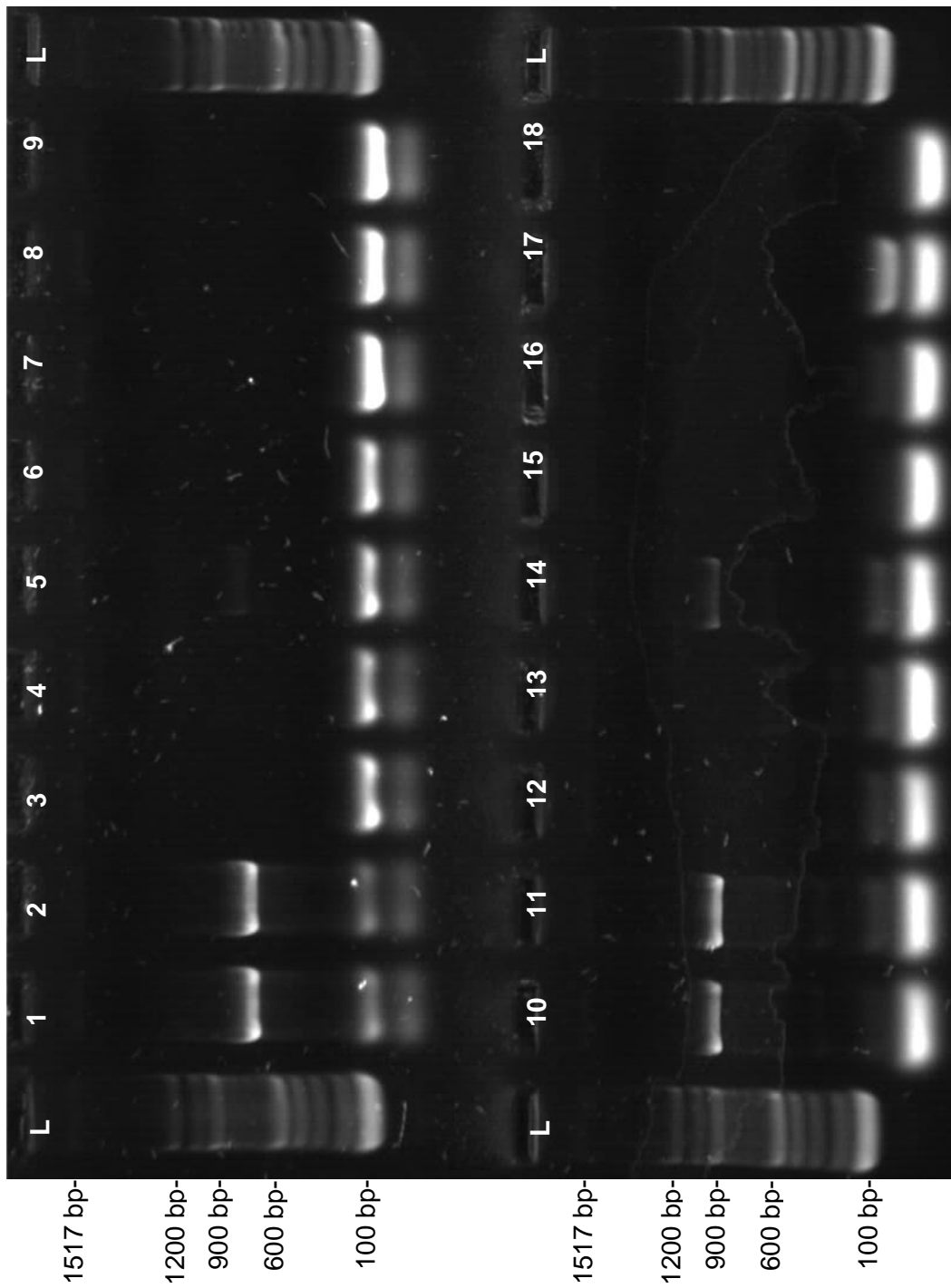


Figure 4.17 FusB (top row) and FusC (bottom row) gene sequences amplified from melittin naïve and resistant MRSA. Data-represent three separate experiments.

Lane	Sample	Lane	Sample
Lane L	Ladder	Lane 10	WBG 8287 Naïve
Lane 1	WBG 8287 Naïve	Lane 11	WBG 8287 Resistant
Lane 2	WBG 8287 Resistant	Lane 12	W17S Naïve
Lane 3	W17S Naïve	Lane 13	W17S Resistant
Lane 4	W17S Resistant	Lane 14	Aus 3 Naïve
Lane 5	Aus 3 Naïve	Lane 15	Aus 3 Resistant
Lane 6	Aus 3 Resistant	Lane 16	BB Naïve
Lane 7	BB Naïve	Lane 17	BB Resistant
Lane 8	BB Resistant	Lane 18	FusC Neg ^a
Lane 9	FusB Neg ^a		

^aReaction mix containing no bacterial DNA

Figure 4.18 shows a representative FusB/C PCR profile from four independent DNA extractions from bac8c naïve and resistant *S. aureus* strains. Both primer sets were able to amplify a product from wild-type WBG 8287 DNA. Similarly, FusB amplification was consistently observed in resistant WBG 8287 cells. However, FusC amplification was observed in only three out of the four DNA extracts for these strains. The FusB/FusC amplification profile of bac8c-naïve W17S and Aus3 cells conflicted with previous findings in this study. In some cases, the ability to amplify the resistance genes varied between colonies of the same population. FusB amplification was achieved from all wild-type W17S DNA extracts but was only achieved from three out of the four extracts of resistant cell DNA. In two of the four DNA extracts from wild-type and resistant Aus3 bacteria, FusB amplification was observed whereas FusC amplification was not achieved for either population.

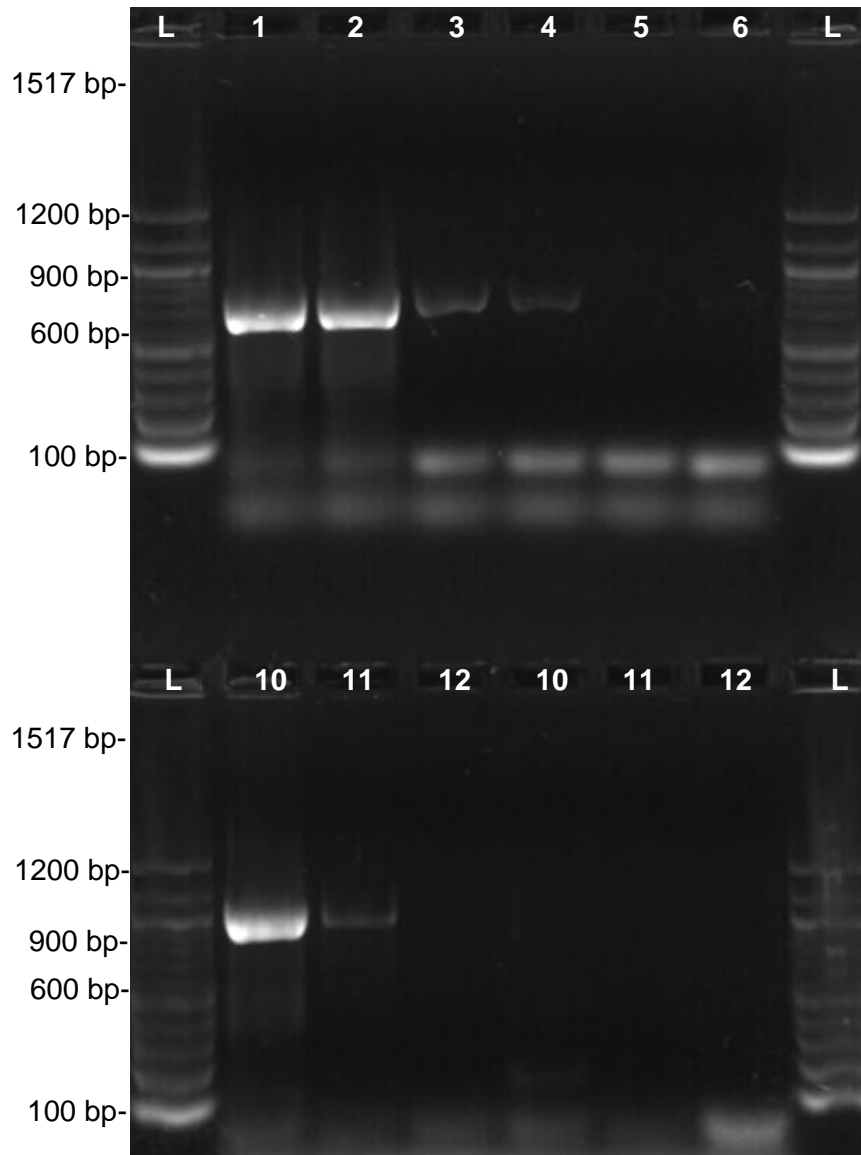


Figure 4.18 FusB (top row) and FusC (bottom row) gene sequences amplified from bac8c-naïve and resistant MRSA. Data represents four separate experiments.

Lane	Sample	Lane	Sample
Lane L	Ladder	Lane 7	WBG 8287 Naïve
Lane 1	WBG 8287 Naïve	Lane 8	WBG 8287 Resistant
Lane 2	WBG 8287 Resistant	Lane 9	W17S Naïve
Lane 3	W17S Naïve	Lane 10	W17S Resistant

Lane 4	W17S Resistant	Lane 11	Aus 3 Naïve
Lane 5	Aus 3 Naïve	Lane 12	Aus 3 Resistant
Lane 6	Aus3 Resistant		

4.6.1 Acticoat Treatment of MRSA

Figure 4.19 shows that when Acticoat was placed on MRSA-inoculated agar plates, the silver nanoparticles were able to spread through the agar and inhibit growth within 1 mm of the material. In subsequent experiments, WBG 8287 was overlain with pieces of Acticoat or filter paper (negative controls) on textured agar plates (Figure 4.20: 1a-1d). On plates with striated or creased textures (Figure 4.20: 1b, 1d) the level of inhibition from Acticoat was similar to that observed on agar with a flat surface (Figure 4.20: 1a). On blistered agar, significant growth of bacteria was observed underneath and around the Acticoat in some tests (Figure 4.20: 1c). After exposure times of 24, 48 and 72h, the Acticoat and filter paper strips were lifted from the plate and placed for ten seconds on a fresh agar plate to measure transference of surviving bacteria (Figure 4.20: 2a-2d). Acticoat from all plates showed transference of viable bacteria indicating that bacteria can survive on the surface of the gauze. Despite the observations of bacterial growth under the Acticoat, samples lifted from the blistered agar were found to transfer fewer bacteria than those lifted from a flat surface (Figure 4.20: 2c), while samples from the creased agar transferred the most (Figure 4.20:2d). It appeared that Acticoat in contact with the inoculated plate for three days generally carried more bacteria than those removed at days one and two. However, the relationship between exposure time and the extent of transference was not clearly defined.

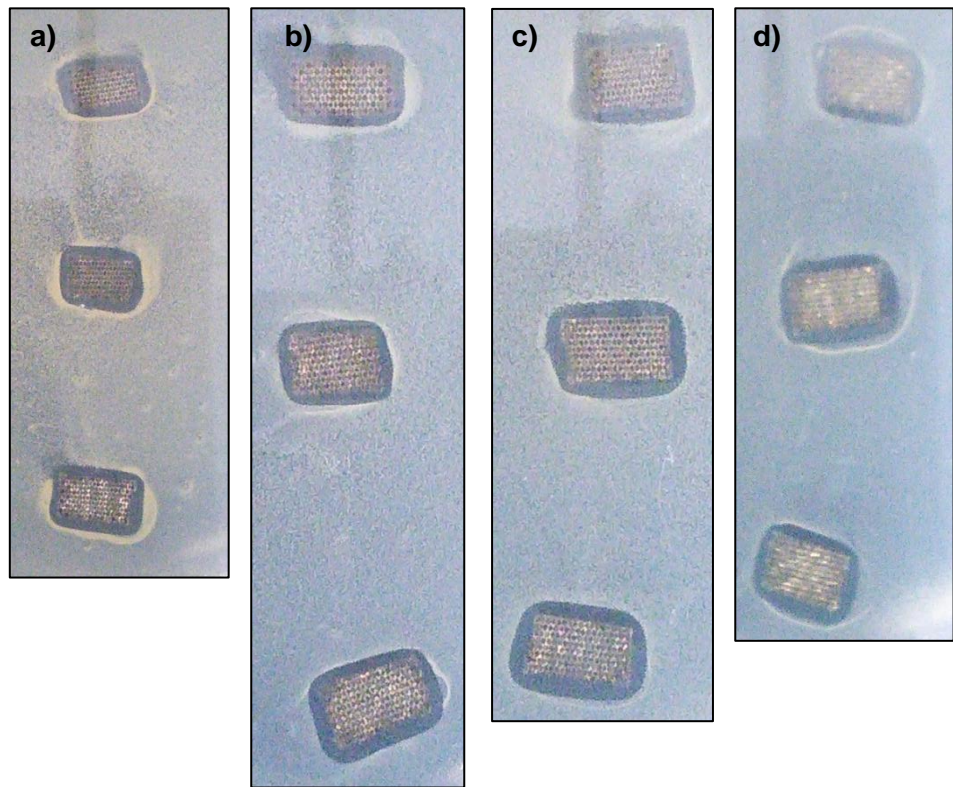
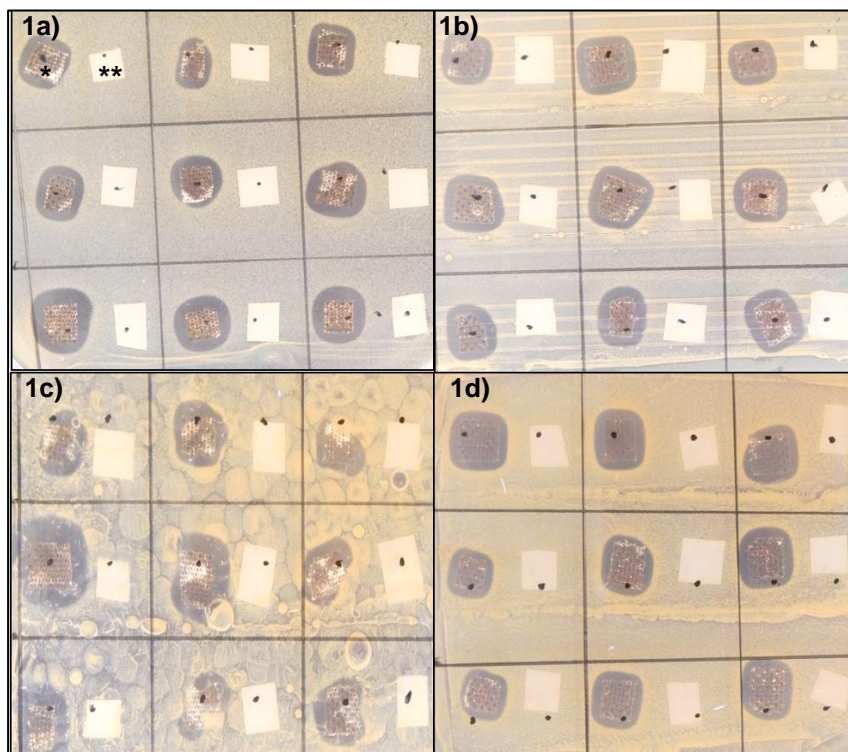


Figure 4.19 Acticoat Treatment of MRSA on agar plates. (a) Wild-type WBG 8287, (b) W17S, (c) Aus3 and (d) BB strains were overlain with acticoat on agar plates.



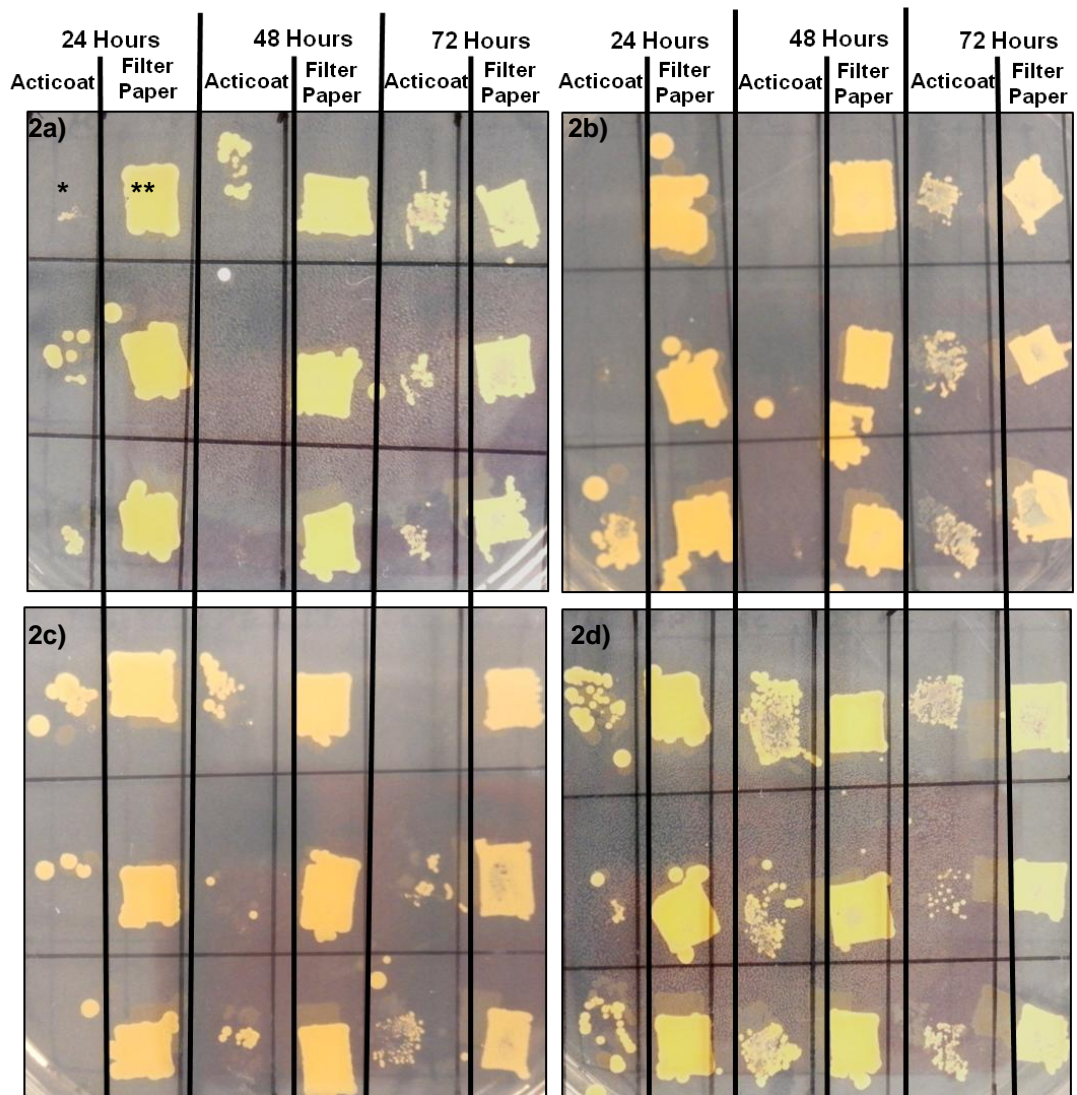
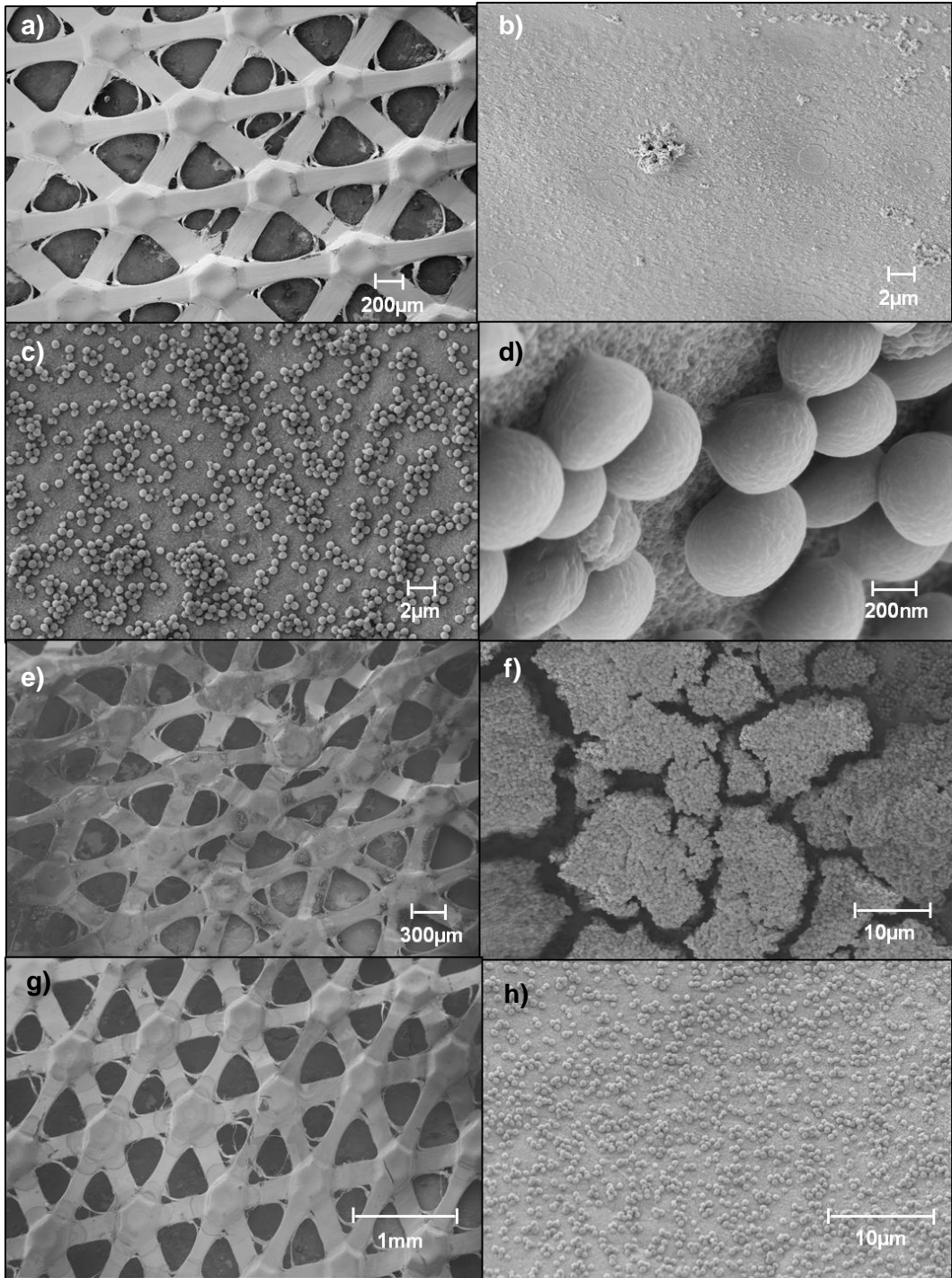


Figure 4.20 Acticoat treatment of MRSA on textured agar plates. WBG 8287 populations were spread on agar plates with (a) flat, (b) striated, (c) blistered and (d) creased surfaces. Strips of Acticoat (*) and sterile filter paper (**) were placed onto the inoculated surface for 24, 48 and 72h (columns left to right respectively: 1a-d). After incubation the strips were lifted carefully from the plate and placed briefly onto fresh agar plates which were incubated for 24h (2a-d). Columns show triplicates of each test sample.

4.6.2 Scanning Electron Microscopy Analysis of Acticoat and Attached Bacteria

Wild-type WBG 8287 were over-lain with Acticoat on agar plates with either flat or striated surfaces for 24, 48 and 72 hours (Figure 4.21: c-d, g-h and k-l and e-f, i-j

and m-n respectively). The Acticoat surface was seen under the microscope to consist of a network of raised nodes each connected to five other nodes by a lattice of connecting arms (Figure 4.21: a, e, g and m). When viewed under SEM, the Acticoat surface has a rough or grainy texture with irregular dimples spaced across its surface (Figure 4.21: b). Over the three days of exposure, bacterial clusters, 1-5 cells in height, accumulated on the arms of Acticoat that was in contact with the cells on agar with a flat surface (Figure 4.21: c, h and l). Acticoat from both flat and striated agar surfaces showed dense clusters of cells in their nodes (Figure 4.21: k, f and j). In some cases, these bacterial protrusions could be observed from a relatively low magnification (Figure 4.21: e, i and m). When viewed at a high magnification, cells showed little or no surface damage (Figure 4.21: d, l and n). However, there was the occasional cell that showed severe disruption to its structural integrity (Figure 4.21: d). The density of cells in the bacterial clusters did not appear to be affected by the length of Acticoat exposure time. Likewise, the cells from all samples showed undamaged surfaces indicating that up to three days exposure did not affect the health of the cells.



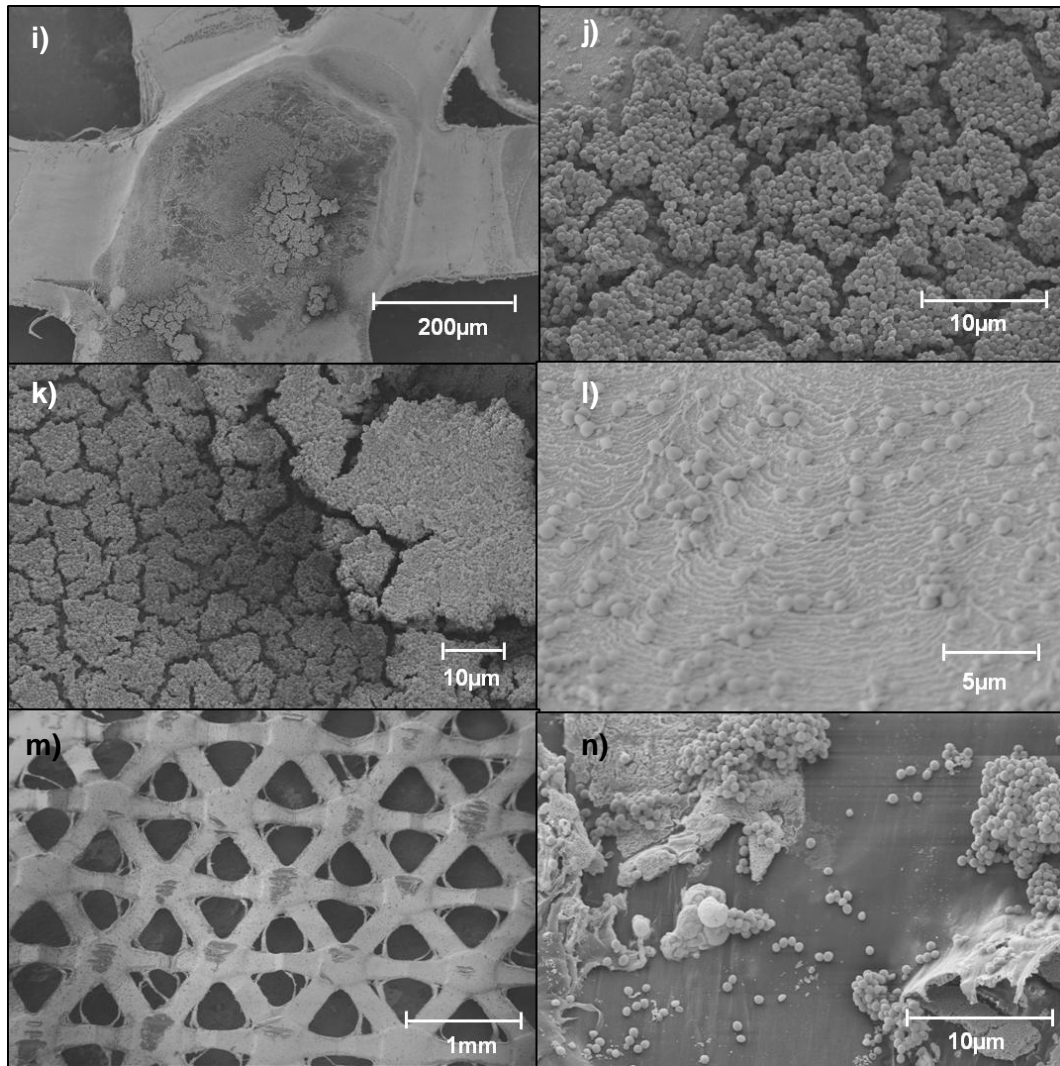


Figure 4.21 Scanning electron microscopy images of Acticoat from overlay experiments with MRSA. WBG 8287 on bactopectone agar plates with a flat texture were treated with Acticoat strips for 24 (c, d), 48 (g, h) and 72h (k, l). In a parallel experiment, cells were treated on striated agar plates for 24 (e, f), 48 (i, j) and 72h (m, n). After incubation the strips were prepared and viewed using SEM. Acticoat strips that had not been exposed to bacteria were used as a negative control (a, b)

4.6.3 Exposure of MRSA to Acticoat on Pig Skin; Transfer of Viable Bacteria from Acticoat Surface

Bacteria were grown on pig skin and covered with Acticoat to measure the efficacy of the gauze when treating cells *in situ*. Figure 4.22 shows that viable bacteria were transferred from the surface of the gauze when the strips were blotted onto fresh agar. The number of *S. aureus* WBG 8287 cells transferred

from pig skin to culture plates by Acticoat (Figure 4.22a) was similar to that from filter paper controls (Figure 4.22b) Transfer from pig skin was visibly greater than was from agar plates. Acticoat that was encased in agar or agarose to form an antimicrobial disc (Figure 3.5), also showed transference of bacteria from inoculated pig skin to fresh agar plates (Table 4.12). However, in most of these samples there appeared to be a rectangular region underneath the location of the Acticoat that showed no transference of bacteria. It appears that the gel casings may improve the activity of Acticoat, but the diffusion of silver at bactericidal concentrations is limited to regions that are directly inferior to the gauze strips. Experiments in which antimicrobial agents were added to the agar or agarose with the Acticoat did not increase the antibacterial activity of Acticoat over those made with just HpH_2O .

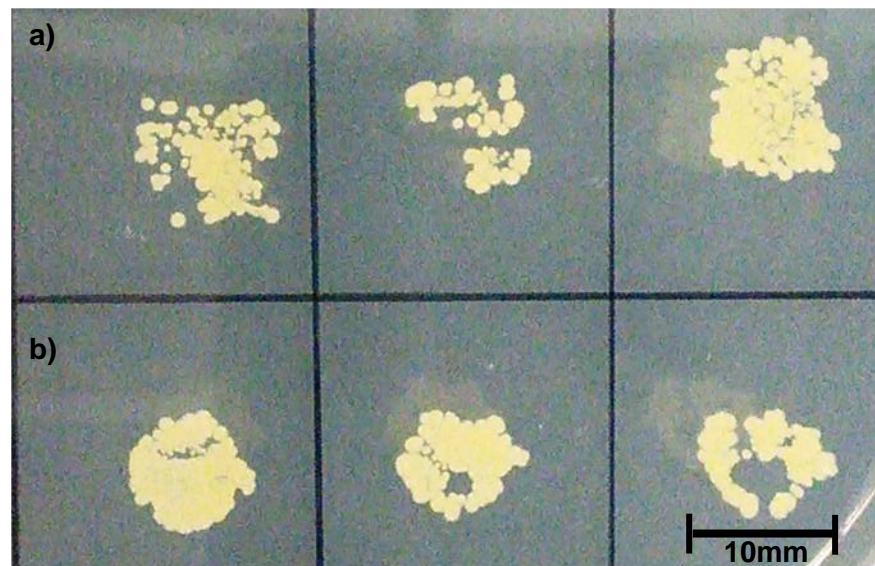



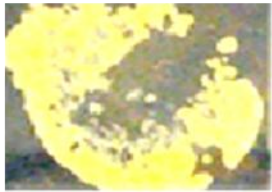

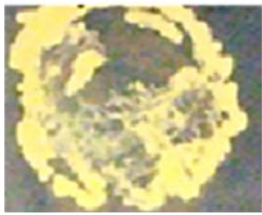





Figure 4.22 Transference of MRSA from Acticoat after 24h on MRSA inoculated Pig Skin. The epidermal layer of pig skin was infected with WBG 8287 and immediately treated with Acticoat or sterile filter paper strips. After 24 hrs incubation, the Acticoat (a) and filter paper (b) strips were blotted onto a fresh agar plate to measure transference of bacteria

Table 4.12 Transference of MRSA from Acticoat encased in gels

	Agarose	Agar
HpH2O		
Bac8c (100 µg/ml)		ND
Bac8c (1000 µg/ml)		
Fusidic Acid (100 µg/ml)		
Fusidic Acid (1000 µg/ml)		

4.6.4 Exposure of MRSA to Acticoat on Pig Skin; Bacterial Survival Beneath and on the Surface of Acticoat

The percentage of bacteria surviving on pig skin and on the Acticoat was measured over 24h of treatment and is shown in Figure 4.23. Despite a large

degree of variation in the results among independent tests, it appeared that growth of cells was largely inhibited for the first 8h after placement of Acticoat. However, after 24h the cells growing underneath the Acticoat appeared to outnumber those growing on the pig skin controls which were inoculated but had no Acticoat overlay by a factor of 2.3. In contrast, the number of surviving cells on the Acticoat surface after 24h corresponded to 11.9% of that of the controls.

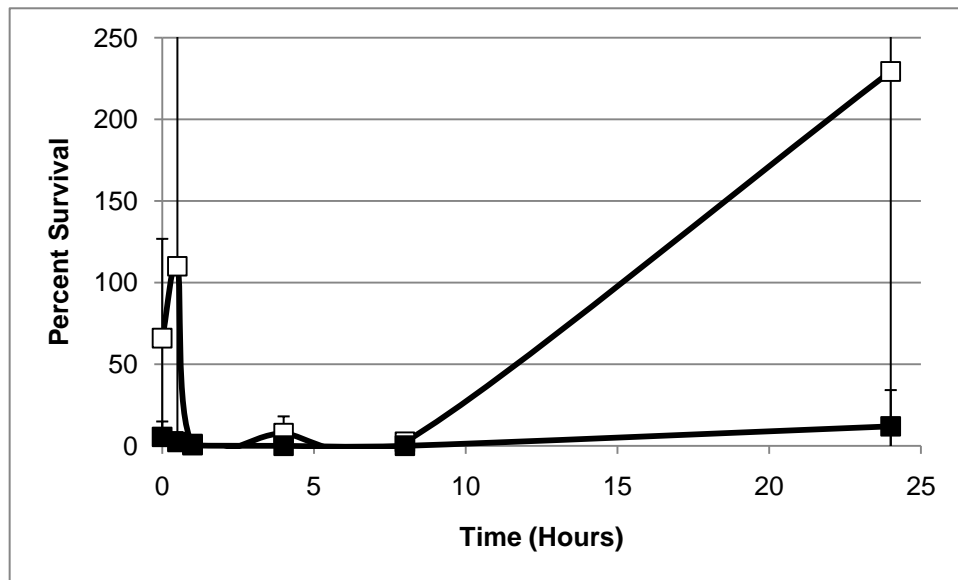


Figure 4.23 Survival of MRSA on pig skin and Acticoat over time. Survival on Acticoat treated pig skin (open squares) and the Acticoat strips (closed squares) is expressed as a percentage of cells relative to cells grown on pig skin without an Acticoat overlay. Each data point is the mean of twelve replicates over four separate experiments. Error bars show standard deviation.

In subsequent experiments, the wetting agents glycerol (Figure 4.24a) and Tween-20 (Figure 4.24b) were applied to the Acticoat prior to application to MRSA. Exposure of the bacteria to Acticoat plus glycerol or Tween-20 resulted in a 100% mortality rate of cells on the surface of the gauze. Application of 1% glycerol to the Acticoat strips reduced bacterial survival on the pig skin samples to 38.3% of the HpH₂O-coated controls and increasing concentrations reduced survival to 0% (Figure 4.24a). In contrast, Tween-20 did not reduce the number of surviving bacteria on the pig skin samples when applied at concentrations of 10% or 20% and reduced survival only to 45% when applied at 100%.

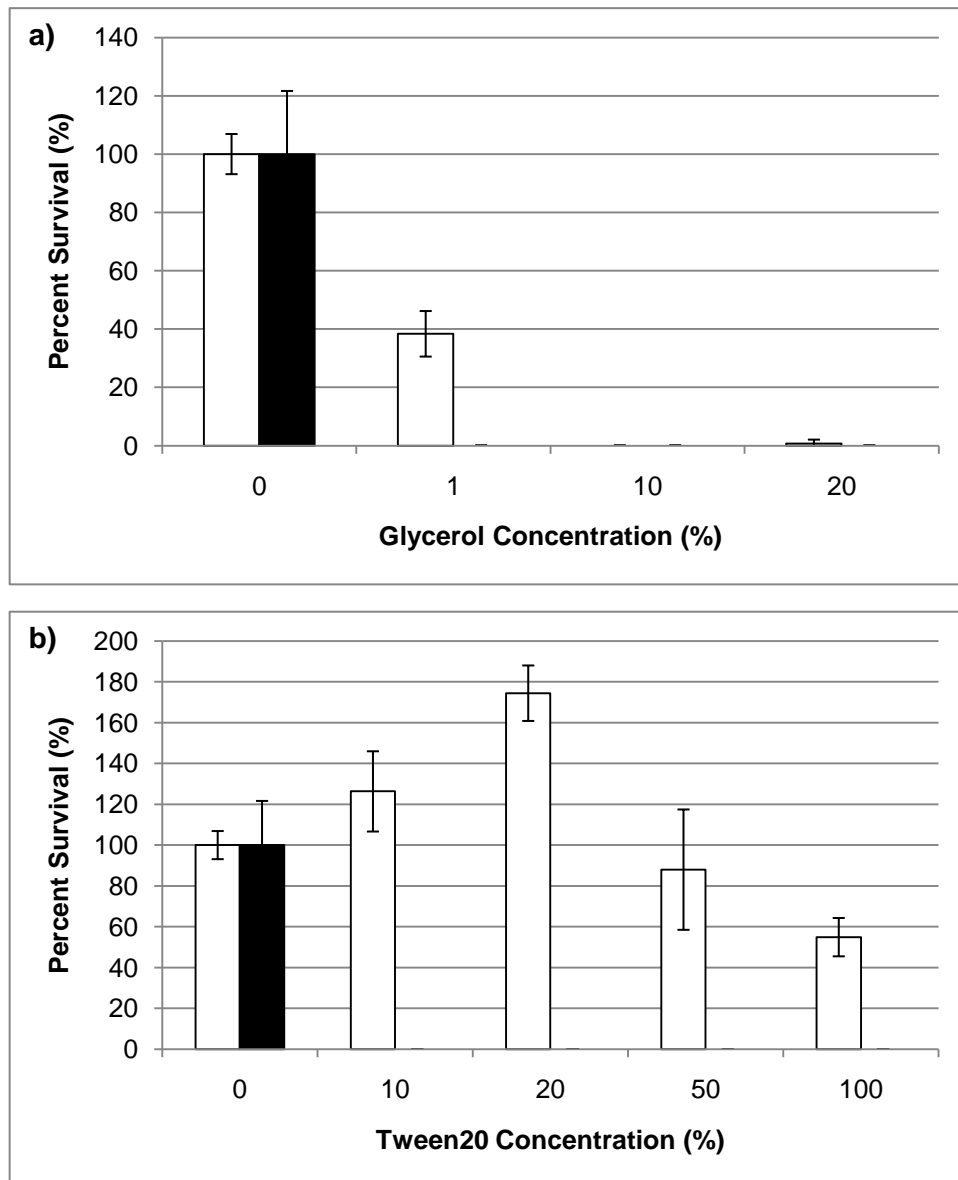
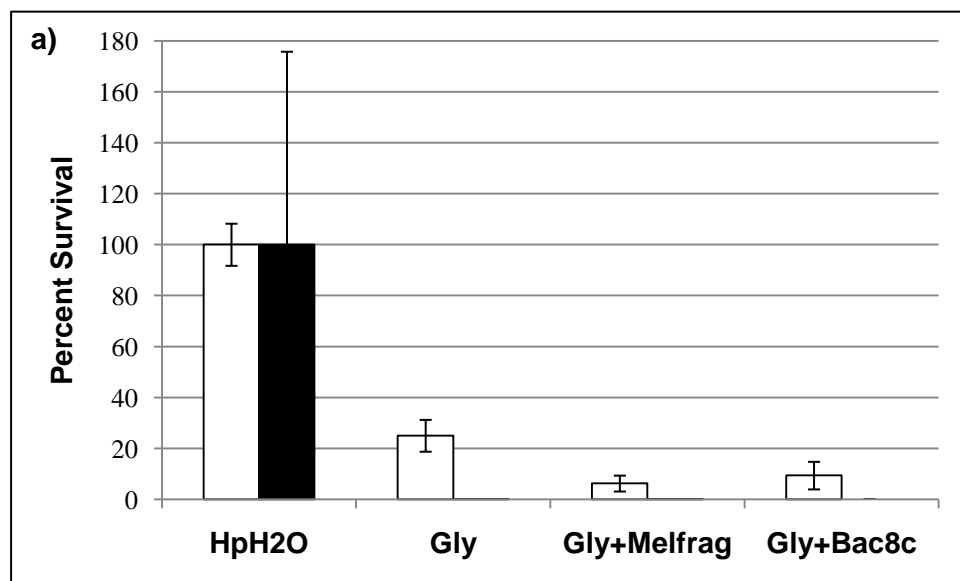


Figure 4.24 Survival of *S. aureus* WBG 8287 after exposure to Acticoat coated with wetting agents. Survival of WBG 8287 after 24h exposure to Acticoat, wet with a range of (a) glycerol or (b) Tween-20 concentrations prior to placing on *S. aureus* inoculated-pig skin. Survival of cells on the skin underneath the Acticoat (open columns), and on the Acticoat itself (closed columns). Data represent the percentage of surviving bacteria relative to those treated with Acticoat alone. Values are the mean of data from triplicate samples. Error bars show standard deviation between samples.

Finally, the antibacterial effectiveness of applying glycerol, lysozyme and melfrag12-26 or bac8c to Acticoat, was tested on pig skin inoculated with 2 μ l of WBG 8287 cells at 10^8 CFU/ml. Glycerol alone and in combination with AMPs was able to inhibit all growth on the Acticoat (Figure 4.25a). On pig skin, glycerol was more effective at reducing survival of bacteria when used in combination

with the AMPs. However, complete inhibition of growth was not observed in any of the tests. Lysozyme was not able to eradicate all bacteria from the Acticoat either alone or when used in combination with either peptide (Figure 4.25b). When used alone, lysozyme reduced the number of viable bacteria on the Acticoat surface to 62.5% of the untreated control. When used in combination with melfrag12-26, bacterial survival was reduced to 18.75% and when combined with bac8c bacterial survival was reduced to 0.03% (Figure 4.25b). Acticoat coated with a combination of lysozyme and bac8c or melfrag12-26 did not appear to be any more effective than coating Acticoat with only lysozyme at reducing bacterial numbers on pig skin.

The greatest reduction in bacterial survival was observed when Acticoat was coated with a combination of glycerol, lysozyme and bac8c or melfrag12-26 (Figure 4.25c). Acticoat coated with glycerol, lysozyme and melfrag12-26 was able to reduce bacterial survival on pig skin and Acticoat by 98.96% and 99.99% respectively (Figure 4.25c). No growth was observed on pig skin or Acticoat when the gauze was coated with glycerol, lysozyme and bac8c (Figure 4.25c).



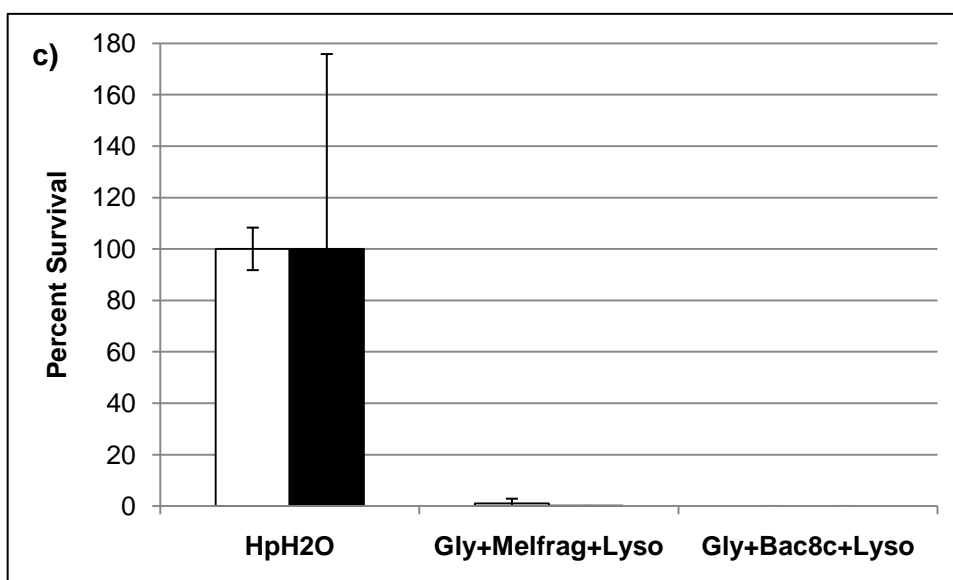
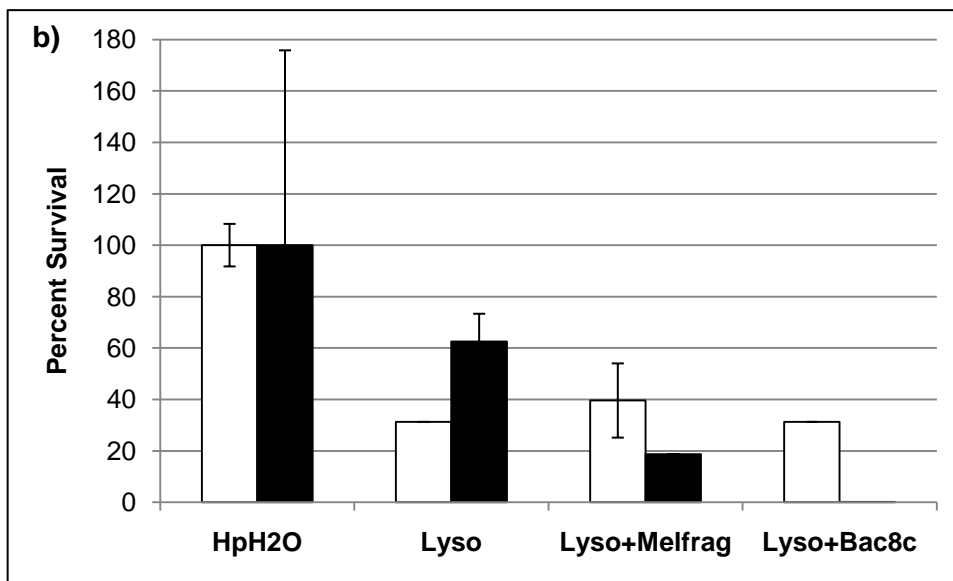


Figure 4.25 Survival of MRSA on pig skin after treatment with Acticoat plus wetting agents and antimicrobial agents. WBG 8287 was overlain with Acticoat which was coated with a mixture of (a) glycerol with AMPs, (b) lysozyme with AMPs (b) and (c) a combination of glycerol and lysozyme with AMPs. Survival of cells (open columns) on the skin and (closed columns) on the Acticoat after 24 h. Data represent the mean percentage survival of triplicate samples relative to untreated control samples. Error bars represent the standard deviation.

The absorbance of supernatant from erythrocytes treated with a combination of glycerol, lysozyme and melfrag12-26 or bac8c is shown in table 4.13. Samples treated with a combination of glycerol, lysozyme and melfrag12-26 had a slightly

higher absorbance than that of glycerol and lysozyme. Substituting melfrag12-26 for bac8c further increased the haemolytic potential; however, this was only 23% higher than that of the negative control.

Table 4.13 Haemolytic potential of acticoat coatings

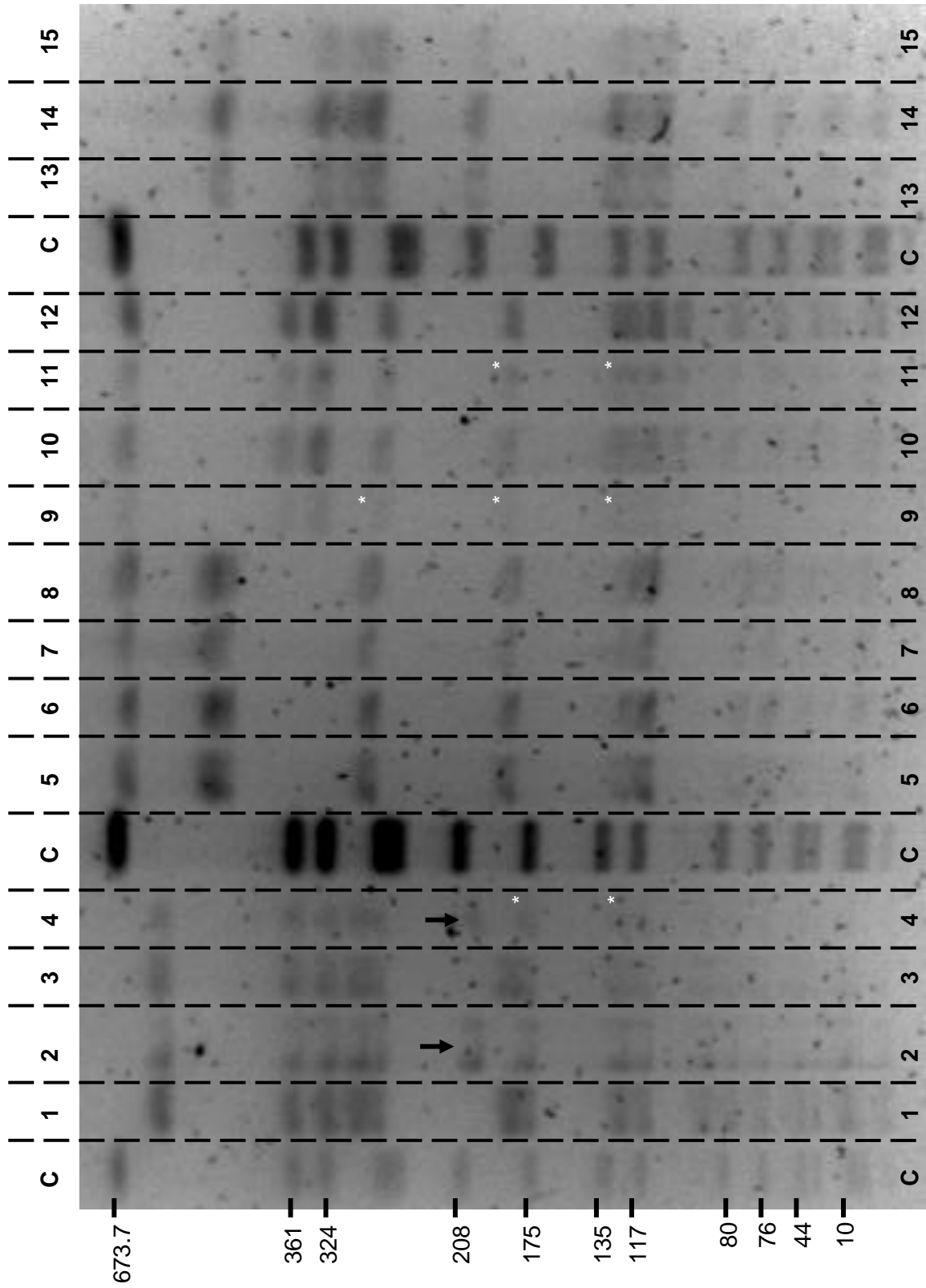
Test	Absorbance^a (405nm)	Absorbance Stdev	% Lysis
Negative Control	0.042	0.001	0
1% Triton X-100	2.71	0.21	100
Glycerol+Lysozyme	0.197	0.114	4.7
Glycerol+Lyszyme+Melfrag12-26	0.261	0.141	6.2
Glycerol+Lyszyme+Bac8c	0.97	0.064	23

^aAbsorbance of supernatant after sedimenting cells and debris

4.7.1 Contour Clamped Homogenous Field Electrophoresis

The genetic profile of each strain used in these experiments was generated at the completion of this project by digesting chromosomal DNA with restriction enzyme SmaI and exposing the DNA fragments to pulsed field electrophoresis (Figure 4.26). WBG 8287 was the only strain that showed any genetic variation between populations exposed to melittin, bac8c and fusidic acid (Lanes 1-4). Fusidic acid resistant strains of WBG 8287 appeared to increase the size of the 6th largest fragment from around 180 kilobases to 208 kilobases as shown by the arrows (Lane 2). Fusidic acid resistant strains that were exposed to melittin lost this apparent insertion in the 6th band while cells exposed to bac8c did not (Lanes 3 and 4 respectively). The other samples showed no difference in fragment size. However, at the quantities of DNA present, the samples showed only moderate signal intensity. Therefore it is difficult to determine whether there may have been variation between the shorter bands. Lanes 4, 9 and 11 show samples with bands of very low signal intensity. These bands were able to be distinguished in

the original photo but became less clear in the imported images. The questionable bands have been marked with an asterisk (*).



Lane 1	WBG 8287: Melittin Naive FD ^a Naive	Lane 9	Aus3: Melittin Naive FD Naive
Lane 2	WBG 8287: Melittin Naive FD Resistant	Lane 10	Aus3: Melittin Naive FD Resistant
Lane 3	WBG 8287: Melittin Resistant FD Resistant	Lane 11	Aus3 Melittin Resistant FD Resistant
Lane 4	WBG 8287: Bac8c Resistant FD Resistant	Lane 12	Aus3: Bac8c Resistant FD Resistant
Lane 5	W17S: Melittin Naive FD Naive	Lane 13	BB: Melittin Naive FD Naive
Lane 6	W17S: Melittin Naive FD Resistant	Lane 14	BB: Melittin Naive FD Resistant
Lane 7	W17S: Melittin Resistant FD Resistant	Lane 15	BB: Melittin Resistant FD Resistant
Lane 8	W17S: Bac8c Resistant FD Resistant	Lane C	NCTC 8325

^a Fusidic acid

Figure 4.26 Contour-clamped homogenous electric field electrophoresis of Genomic DNA from *Staphylococcus aureus* strains. Smal digested DNA from *S. aureus* strains separated by pulse field electrophoresis for 21h. Numbers on the bottom border correspond to the size of the DNA fragment in kilobases. Arrows in lanes two and three indicate a band approximately 200 kilobases in size that is absent in the fusidic acid naive or melittin resistant variants of WBG 8287. Lanes 4, 9 and 11 show very faint bands marked with a white asterisk (*). Lane C represents the CHEF profile of an NCTC 8325 control strain which produces bands of well established sizes (313).

5. Discussion

The adaptability of pathogens to inherent and technological defence systems of humans is as remarkable as it is dire. Sophisticated immune systems have evolved to allow plants and animals to inhabit a world populated with invasive microorganisms. However, it seems that for every defensive pathway identified in multicellular organisms there is already a resistance strategy developed in pathogens. This presents a problem when designing therapeutic agents based on antimicrobial material found in nature. If a pathogen has been exposed to an antimicrobial agent over a long period of time, chances are it has developed resistance mechanisms that allow it to tolerate that compound as well as others that have similar biocidal *modus operandi*. However, the effectiveness of some antimicrobial compounds suggests that they have been fine tuned by evolution to remain biocidal against a broad spectrum of microbial species. Misuse of these compounds in medicine and agriculture has allowed a rapid dissemination of resistance throughout the microbial community and may have even compromised the future development of antimicrobial drugs.

Antimicrobial peptides have been proposed as the solution to the declining effectiveness of antibiotics (314). Their rapid, broad spectrum and non-specific mechanism of action is thought to overcome previously established antibiotic resistance strategies as well as limiting the risk of resistance to the peptides developing. In practice it is evident that some peptides are able to treat bacterial infections without resistance developing, while other studies show that bacteria can rapidly develop resistance if exposed to sub-lethal doses of the peptides (169-173). The therapeutic applications of AMPs are also limited by their potential antigenic characteristics. Immunological clearance of the peptides reduces their therapeutic effectiveness inside the body and may induce harmful reactions in the patient. In the short-term however, if the potential for resistance development can be restricted, it seems that AMPs may prove an effective alternative or adjunct to antibiotics for treating topical infections.

This study aimed to expand our understanding of AMP resistance in MRSA and to examine ways the therapeutic potential of the peptides could be improved. Initially, the antibacterial activity of a variety of plant and invertebrate samples against MRSA was examined. The effectiveness of four different peptides against MRSA under different conditions was investigated. In addition, the potential for resistance against these peptides to develop in MRSA, the stability of this

resistance in a population over time and explanations behind these resistance mechanisms were explored. Subsequent experiments investigated strategies to counteract resistance. Finally, the effectiveness of AMPs in combination with topical therapeutics was explored.

5.1 Screening of Biological Tissue for Anti-MRSA Activity

The dearth of emerging antimicrobial agents could be addressed by investigating antimicrobial compounds and molecules present in nature and improving on these by synthetic modifications. These sorts of studies have the benefit of potentially identifying compounds with novel mechanisms of action that are rare in nature and as such, have not already encouraged a selective bias for resistant populations. In this study, a rapid extraction technique for bioactive compounds from a broad spectrum of common biological material was developed. It was anticipated that screening of these samples would allow the identification of new sources and variants of AMPs.

5.1.1 Buffer Development for Bio-active Compound Extraction

Two solutions were made for extracting antibacterial compounds from homogenised samples: JEB1 and JEB2. Both buffers had NaCl concentrations that were slightly lower than that of physiological salt levels. This was intended to increase the solubility of the extracted proteins and to prevent them aggregating through hydrophobic interactions. Glycerol was added to increase the stability of the peptide and prevent aggregation. Likewise, a low concentration of DMSO was added to each solution to act as a cosolvent and increase the stability of the proteins by organising and compacting water molecules onto the surface of the protein. This protective effect comes from the high affinity of DMSO for water but low preference for binding to the surface of protein molecules. The water molecules of the DMSO-water complex strongly bind to the surface of the protein due to preferential hydration while the DMSO molecules are displaced from the immediate surface of the protein. This helps organise and compact the water molecules on the surface of the protein molecule and preserves the protein in its native state (315, 316). Finally, EDTA was added to buffer JEB2 to prevent metal induced oxidation of the peptides and inhibit the activity of ion-dependent

proteases. Both buffers were adjusted to pH 7.8 to increase the stability of extracted proteins in solution.

These buffers were designed to be non-toxic to MRSA and maintain the extracted proteins in their biologically-active state so that the extracts could be tested without removing the buffer. The toxicity of EDTA at the concentrations used in JEB2 made it unsuitable as an extraction buffer for testing biocidal potential. Buffer JEB1 was non-toxic and allowed the extraction of active antibacterial molecules from biological sources. This study describes a quick and simple extraction procedure which was successfully used to identify antibacterial activity in multiple biological samples. It is likely that some of the compounds used in the buffer such as NaCl and glycerol could have interfered with the extracted molecules and masked their antibacterial activity. Therefore, the concentrations of the components of the buffer could be further optimised to increase the sensitivity of assays in which the buffer is used.

5.1.2 Identification of Biological Tissue with Antibacterial Activity

At a time where medicine is under threat of moving to a post-antibiotic era, the search for new antimicrobial agents could be advanced by studying established medicinal databases. Traditional medicine represents collective knowledge of the effectiveness of different treatments based on experiences, creeds and theories that are indigenous to a culture (317) Research into the validity of such treatments, using evidence based approaches, has so far, been lacking. However, the broadly documented customary uses and effectiveness of herbs, animal products and minerals provides a substantial collection of data that may fast track the search and discovery of new bioactive compounds.

Based on the results presented in this study it appears that plant samples with more aromatic qualities, such as hops, juniper berries and cardamom seeds more likely to have bactericidal activity than samples in which the aroma is less noticeable. Generally, the volatile compounds which give the plant its “aroma” are extracted in the essential oil of the plant. Numerous studies have identified antibacterial molecules in the essential oils of plants (318-322). However, antibacterial peptides have not been identified in plant oil extracts. The experiments presented in this report focused on the antibacterial activity of whole crude aqueous extracts of plant material, not just the essential oils. This enabled

the use of a non-toxic extraction buffer containing minimal organic solvents, which could be used to test for antibacterial activity without influencing the results. Fourteen of the thirty-one extracts showed antibacterial activity. Of those, *C. chinese*, *E. Cardamomum*, and *J. Communis* appeared to have bactericidal activity related to proteinase susceptible components.

Surprisingly, extracts from the larvae of *Lucillia sericata* showed no inhibitory activity against MRSA. Decomposing material in which *L. sericata* larvae feed prior to pupation, exposes the larvae to a myriad of bacterial, fungal and viral species (323). For this reason, it was hypothesised that for the larvae to persist in these environments they would need a significant repertoire of antimicrobial defences. Despite the results from this study, it has been shown before that secretions from these necrophages have antiseptic activity (324). However, it also appears that ingested microorganisms readily colonise the gut of the larva (323, 324). The persistence of these microbes continues through to adulthood, making the flies transmission vectors for pathogens and may even be the reason for the short life spans of the insects (323). It seems then that the pathogenic bacteria colonising the flies have already developed strategies to overcome the chemical defences of these insects. Therefore, carrion scavenging insects, especially those with relatively short life-spans, may not prove to be a reservoir of effective antimicrobial agents.

All Hop flower extracts showed potent inhibition of MRSA that was independent of protein-based antimicrobial agents. Historically, hops have not been a common ingredient used in medicine, although their ability to prevent the spoilage of beverages has been employed since the middle ages (325, 326). *Humulus lupulus* produces three phytochemicals which have previously been shown to have antibacterial activity against Gram-positive species: humulone (α -acid), lupulone (β -acid) and xanthohumol (326). It is believed that the hop acids transfer hydrogen ions and divalent cations across the bacterial membrane, which undermines the transmembrane proton gradient and disrupts proton motive force-dependent nutrient trafficking. This causes membrane leakage and impedes respiration and normal metabolism in affected bacteria (327). Resistance to hop compounds in bacteria is of major concern to the brewing industry as the antibiotic effects of hops are a major protectant against beer spoilage (325-328). It is not clear how bacteria resist hop acids, although it does appear that resistance may be dependent on a combination of mechanisms (327, 329, 330). Behr *et al.* (330) have shown that resistance correlates with an

increased lipoteichoic acid content in the cell wall. An increase in the anionic charge on the surface of hop-resistant bacteria could increase the affinity of cationic AMPs for the cells and enhance their antibacterial activity. Additionally, some AMPs have enhanced antimicrobial activity at pH levels similar to that of beer. It has been shown that resistance to hop acids relates to the capacity of a microorganism to produce the foul-tasting metabolites that spoil beer (331). Therefore, if hop resistant strains continue to impact the brewing sector, further research into AMPs as sterilization agents in beer could prove cost effective for the whole industry. Adding AMPs to the grains in the mashing stage of brewing could reduce the chances of bacterial colonisation of the wort. Bacteria which survive peptide exposure could be more susceptible to heat-induced destruction in the subsequent boiling stage. If the peptides are harmful to humans, the boiling stage would likely denature the peptides and inhibit their toxic activity. Additionally, creating transgenic brewing yeast strains which produce antibacterial peptides could eliminate the costs of producing the peptides while protecting the beer throughout the brewing process.

Two out of the five fungal extracts showed antibacterial activity: scarlet bracket fungus (*Pycnoporus coccineus*) and Australian honey fungus (*Armillaria luteobubalina*). Although, the origin of the antibacterial activity of *P. coccineus* was not explored in this project, it has been documented that *Pycnoporus* species produce phenoxazinone-based antibiotics (332). Protease treatment of the *A. luteobubalina* extracts did not inhibit its antibacterial activity. *Armillaria luteobubalina* is parasitic to trees but is non-toxic to humans if cooked, and may contain non-protein based antibacterial compounds that could be safe for antibacterial therapy (333, 334). *Armillaria luteobubalina* has a strong bitter taste if eaten raw so it is generally blanched before consumption. This heat treatment may inactivate antimicrobial compounds which could explain why its potential medicinal qualities have not yet been identified. Further investigation of the antibacterial qualities of Australian honey fungus appears warranted

Extracts from *C. chinese* seeds and fruit had strong inhibitory activity against MRSA. Traditionally, these chillies have been used to alleviate joint pain, asthma attacks, toothache and, oddly enough, gastro-intestinal discomfort (335). The alkaloid capsaicin constitutes 3-5% of bhut jolokia chillies and has been shown to have antimicrobial activity against a broad spectrum of bacteria including *S. aureus* (335, 336). In other studies, *C. annuum* has been found to contain two peptides of 15 and 22 amino acids in length with antifungal activity (337, 338). In

this project, the antibacterial activity of bhut jolokia extracts was greatly inhibited, but not completely lost, after protease treatment. This suggests that the chillies contain a bactericidal compound of proteinaceous origin. Collectively it is likely that the antibacterial activity of capsaicin in *C. chinese* is augmented by one or more peptides that could be related to antifungal peptides previously discovered.

Of the other bactericidal plant extracts, only juniper berries and cardamom seeds showed reduction in their antibacterial activity after protease treatment. Lime extract activity increased after protease digestion. This may indicate that Persian limes synthesise proteins that can be cleaved into smaller fragments that have greater antibacterial activity than the full-length protein. The antibacterial activity of cardamom seeds was completely lost after treatment with either Trypsin or proteinase K. This suggests that cardamom seeds contain bactericidal peptides with basic amino acids. In contrast, the antibacterial activity of juniper berry extract was inhibited by approximately 80% after exposure to either protease. In a study by Pepljnjak *et al.* (320) it was found that essential oil extracts from *J. communis* had potent broad spectrum fungicidal and bactericidal activity. The main compounds in juniper berry oils are the terpenes: α -pinene, β -pinene and sabinene all of which have been shown to possess antimicrobial activity (318, 319). Therefore, as with the bhut jolokia chillies, these results expand on previous research to suggest that juniper berries contain antibacterial compounds that are both proteinaceous and non-proteinaceous in origin. Further characterisation of plant and fungal extract fractions was planned using mass spectrometry. Unfortunately time constraints made this impractical.

It seems logical that drugs designed from novel compounds could be more effective at treating multi-drug resistant pathogens than modified versions of established antimicrobial agents. However, since the 1930s the rate at which new therapeutic agents are introduced into clinical practice has remained stable or declined, while the use of derivatives of already established drugs has increased (339). The difficulty of getting therapeutic approval for a compound with an original molecular structure, compared to a drug that is modelled off a previously approved therapeutic, may be influencing antimicrobial drug development. Despite this, a recent report has shown that 73% of antimicrobial agents currently in clinical development are compounds that have not been designed from patented drugs, which suggests this trend may be reversing (289).

5.2 Antibacterial Peptide Activity is Dependent on the Treatment Environment

The reduced antibacterial activity of AMPs in different culture media was correlated with increased protein concentration of the medium. Survival was greatly reduced when bacteria were exposed to melittin or melfrag12-26 in phosphate buffer solutions. Previous studies have shown that NaCl can inhibit the activity of cationic antimicrobial peptides (340, 341) and these results confirmed this. The reduced bactericidal activity seen in growth media, compared to phosphate buffer, could be partly attributable to the salt concentration in those media. However, the differences observed between media with similar salt content, but differing protein content, suggest that media proteins may play a dominant role in reducing melittin toxicity. This could be attributable to the sequestering of melittin through binding to anionic media peptides and also to the reduction of melittin binding sites on the bacterial cell wall by competitive binding from cationic media peptides.

It was also observed that MRSA is more sensitive to melittin at higher temperatures. It has been well established that bacteria can increase the amount of unsaturated fatty acids in their membrane to retain fluidity at lower temperatures (342). However, this is unlikely to be the cause of enhanced tolerance to melittin at lower temperatures, because membranes with a lower concentration of unsaturated fatty acids have previously been shown to be more susceptible to the membranolytic, α -helical AMP: warnericin RK, which has similar physiochemical properties to melittin (343, 344). Reinés *et al.* showed that *Yersinia enterocolitica* was more sensitive to the AMP polymyxin B at higher incubation temperatures (186). It was believed this was largely due to the down-regulation of activity in certain two-component regulatory systems, which allow the cells to respond to changes in temperature, osmolarity and pH (186, 345). Similar regulatory systems have been identified in *S. aureus* (345) and their down-regulation may contribute to the increased melittin-susceptibility at higher temperatures, observed in this study.

The results in this study show that the presence of exogenous proteins, high salt concentrations and low temperatures reduce the bactericidal efficacy of melittin. These apparently protective effects suggest that the efficacy of cationic peptides for topical treatment of clinical infections could be enhanced by thorough pre-treatment of the infected area to remove inhibitory molecules. For example,

debridement of the wound with a warm, low ionic strength buffer may increase the effectiveness of the treatment. Additionally, multiple reports (268, 346, 347) have shown that substituting tryptophan and histidine residues with the bulky aromatic amino acids β -naphthylalanine and L-(4,4'-phenyl)alanine increased the biocidal activity of antimicrobial peptides against fungal and bacterial species in media containing high levels of salt. Where control of salt levels is impractical, this modification might significantly increase the efficacy of peptide treatment for clinical infections.

In contrast to an earlier report (348), the melittin sub-fragments melfrag12-26 and melfrag12-26-L1 showed low toxicity to the *S. aureus* strains used in this study, except in phosphate buffer, and the susceptibility levels observed more closely match those reported by Subbalakshmi *et al.* (349). Likewise, results from experiments with the shorter melittin fragment, melfrag12-24, conflicted with previous findings (296) with this peptide showing no antibacterial activity against *S. aureus*. In nutrient media, melfrag12-26 showed a solubility limit of approximately 100 $\mu\text{g/ml}$ and its relatively low efficacy in culture media prevented the measurement of a minimum lethal concentration. Melittin also aggregated and precipitated at concentrations above 100 $\mu\text{g/ml}$ in nutrient media. The tendency for these peptides to aggregate at higher concentrations in nutrient media might partly explain the higher MLC observed in those media, compared with PBS. Melittin is a convenient model peptide, but even topical therapeutic use would be better suited by a peptide of similar antibacterial activity without the haemolytic function. For this reason the antibacterial activity of melfrag12-26, which lacks haemolytic activity was tested in this study. However, its much reduced activity, under the conditions used in this study, suggests that it will not serve that purpose.

Experiments using the anionic peptide AP1 showed the peptide had no inhibitory activity against MRSA. Supplementing nutrient media with MgCl_2 had no effect on peptide bactericidal activity. It was believed that the divalent magnesium ions might form a bridge between the anionic peptide and the membrane phospholipids, thereby increasing the likelihood of the hydrophobic components of the peptides interacting with the membrane surface (115, 350). On the contrary, treating bacteria with MgCl_2 and AP1 increased the turbidity of the culture. This may indicate that a combination of AP1 and MgCl_2 increased the growth rate of *S. aureus*. Alternatively, MgCl_2 could cause AP1 to precipitate out of solution which would increase the absorbance of the medium. Surprisingly,

despite its opposite net charge, AP1 showed no antibacterial activity against melittin resistant MRSA. The results from other experiments in this study showed that melittin resistant bacteria have a lower cationic charge than wild-type bacteria. Therefore, it was expected that there would be less electrostatic repulsion between AP1 and the membrane of melittin resistant bacteria, resulting in the peptide being more toxic to these cells. The results from these experiments support the hypothesis that MRSA develops other resistance strategies that may be unrelated to membrane charge shielding. These resistance mechanisms may have evolved to protect bacteria that have masked their anionic membrane charges, in response to cationic peptide exposure, against anionic antimicrobial agents. The concentrations of AP1 used in this study were far below that of Dennison *et al.* (292) who originally reported the antibacterial properties of the peptide. It was believed that, under the conditions used in this study, AP1 would be effective at inhibiting the growth of MRSA at low concentrations. Although this was not observed, the potential resistance mechanism identified in melittin-resistant MRSA suggests that these bacteria may become susceptible to AP1 at higher concentrations.

5.3 Rapid Gain and Loss of Antimicrobial Peptide Resistance in MRSA

Despite what is suggested in the literature (93, 96, 97), it is unlikely that pathogenic microbes would fail to develop tolerance to AMP-based therapeutic agents, if they were used inappropriately. In fact, the capability of some bacteria to invade, colonise and cause disease in animals suggests that resistance to these mammalian antibacterial peptides is already well established in nature. This study aimed to characterise the capability of selective pressure from AMP treatment, to generate a resistant population of MRSA. To complement this, environmental factors that might enhance the protective effects of the developed resistance, the likely cause of resistance and whether resistance decreased the fitness of the bacteria to other antimicrobial agents was also investigated. It was anticipated that the results from these experiments could assist in developing strategies to mitigate the protective effect of these AMP-resistance mechanisms.

Results presented here show that resistance to two AMPs with potent bactericidal activity can be readily induced in different strains of MRSA when they are exposed to sub-lethal concentrations. These findings, along with similar

studies (169, 171, 351), conflict with previous reports which assumed resistance against AMPs was unlikely to develop in bacteria with short term exposure (352, 172, 173). The rapidity with which resistance is initiated and enhanced suggests that resistance mechanisms pre-exist within populations of MRSA. Further experiments showed that high levels of melittin resistance, developed during exposure to sub-lethal concentrations, declined rapidly but not completely in the absence of the peptide. This finding is inconsistent with at least one previous study (351) which reported resistance to pexiganan in *Pseudomonas fluorescens* and *E. coli*, remained stable over four days when under no selective pressure. Scanning electron microscopy revealed that the resistant cells were markedly less damaged by exposure to melittin than the parental bacteria, as predicted from the enhanced survival. Nevertheless, protruding membrane vesicles, or “blebs” were visible on some resistant cells, indicating that melittin was able to bind and form transmembrane pores to some extent. Within the parental population, a very small proportion of cells appeared less seriously damaged by melittin than the majority, possibly with the potential to recover. This raises the question of whether a resistant population that is able to survive exposure to high concentrations of melittin might arise quickly by favouring growth from a specific sub-set of cells within the population.

This study also showed that growing resistant bacteria without AMP selection pressures (reversion bacteria), decreases the MLC of the peptides substantially. The minimum lethal concentration, as defined by this study, was the lowest concentration of the peptides required to kill off 99.99% of bacteria. A survival rate of 0.01% means that one in 10,000 bacteria can survive peptide exposure. When the MLC of melittin against the melittin-reversion bacteria was measured, the original inoculum contained 2×10^5 CFU. This meant that a maximum of twenty cells could possibly have survived at the MLC. Therefore, the sensitivity of the assays measuring the MLC may not have been high enough to identify a resistant sub-population that persists in reversion bacterial populations. This may explain why the MLC of melittin to reversion bacteria exposed to a sublethal dose of the peptides for 24h increased to approximately 59% of the MLC of the parental resistant population. If a resistant sub-population persists in the reversion population, exposure to the peptides could allow these cells to emerge as the dominant phenotype in the culture.

In any case, the rapid loss of resistance suggests that maintaining resistance to melittin has a physiological cost that may correlate with increased metabolic

demand. Some antibiotic resistant bacteria have slower generation rates or are unable to grow on nutrient depleted media. This has been suggested to be the cost of metabolic resources from developing antibiotic resistance (353). This is not universal between species or even strains of the same species as multiple studies have shown some antibiotic resistant bacteria have increased growth rates over their parental population in certain media (353-356). Additionally, two separate studies have shown that antibiotic resistant bacteria persisted in patients 1-4 years after treatment (357, 358). This casts doubt on whether the metabolic cost of antibiotic resistance must eventually cause a reversion of the population to its wild-type state if selection pressures are removed.

In contrast to these observations with antibiotics, this study has shown that resistance to melittin is substantially reduced in a population after seven days when melittin is removed. It seems that AMPs have multiple intracellular and extracellular bacterial targets (95, 102, 359). Therefore, for bacteria to become resistant to AMPs they may have to mutate multiple aspects of their physiology and metabolism and this could place multiple demands on bacterial energy reserves. A similar dynamic is observed with fluoroquinolone resistance in *E. coli* requiring the acquisition of multiple resistance genes (360). Marcusson *et al.* (360) have shown that an inverse relationship exists between the growth rates of the cells and the accumulation of multiple fluoroquinolone resistant mutations. This relationship does not always hold true, as a triple mutant with a reduced growth rate had a growth rate that was similar to wild-type cells while retaining strong antibiotic resistance if a compensatory fourth mutation was acquired (360). The rapid loss of resistance to AMPs when peptide selection is removed may be because the metabolic cost of increasing pre-existing metabolic processes to increase the tolerance of the bacteria to the peptides allows wild-type cells, which do not have these energy demanding phenotypes, to outcompete and dominate the population.

The reduced affinity of a cationic fluorophore, Bacterisense 645, for melittin-resistant bacteria was consistent with a reduction in the net negative charge on the bacterial surface. This finding was also supported by the increased affinity of the anionic dye eosin to melittin resistant bacteria. A previous study has demonstrated a correlation between zeta potential and sensitivity to the cationic peptide, LL37, across 54 *S. aureus* strains (361). Multiple peptide resistance factor-mediated resistance of *S. aureus* against AMPs coincides with modulated peptide interactions with the bacterial membrane. It has been suggested that the

decreased affinity of cationic peptides for the membrane results from aminoacylation of negatively charged phospholipid head-groups with cationic amino acids, mediated by the *mprF* (311). The fluorophore-binding experiments in the current study confirmed the reduction in negative charge on the outer surface of the resistant strain tested, apparently induced by exposure to sub-lethal doses of melittin. The ability to measure this in culture may be useful in future studies of *mprF*.

Pulsed-field electrophoresis revealed that, for most strains, melittin resistance is not demonstrably dependent on the gain or loss of measurable amounts of DNA. Likewise, fusidic acid or bac8c resistance did not change the genetic profile of most strains. In WBG 8287, fusidic acid resistance increased the size of the sixth largest DNA fragment by about twenty kilobases: demonstrating that a large amount of genetic material has been acquired or duplicated in this population. Curiously, melittin resistance seemed to cause this additional DNA to be lost, while bac8c had no effect. However, loss of this DNA did not affect the cells capacity to tolerate fusidic acid, which suggests that this alteration is not essential for fusidic acid resistance. In addition, after fusidic acid exposure, WBG 8287 still seemed to be the most sensitive to the antibiotic. This suggests that any large DNA sequences this population has acquired are less effective at counteracting fusidic acid than the smaller mutations that seem to have substantially increased resistance to the antibiotic in the other strains. It may be interesting in future experiments to investigate whether the additional DNA in WBG 8287 has homology with any other antibiotic resistance genes and why melittin resistance might be incompatible with the pathways and metabolites with which this genetic material is associated.

The reduction of melittin toxicity by salts and proteins analogous to those in bodily tissues (341), together with the bacterial capability for quite rapid reduction of negative charge on their outer cell wall, could explain why endogenous AMPs of eukaryotes may not prevent the spread of infection once it is established. It has been suggested that the therapeutic use of antibacterial peptides could encourage the development of resistance among target organisms, which could stimulate cross-resistance towards endogenous antibacterial defences (282). These factors, coupled with the natural immune response to foreign peptides, may prevent the effective use of AMPs for the treatment of systemic infections. However, the construction of modified or substituted peptide forms might overcome these limitations. Perhaps more importantly, the MLC of melittin

against resistant bacteria decreased 57-fold when the cells were exposed to melittin in phosphate buffer. Therefore, resistance to AMPs may be circumvented in a clinical setting by debridement of a wound prior to treatment and rotating periods of treatment with different therapeutic agents to maximise the antibacterial efficiency of the peptide, which may benefit from any resulting loss of resistance.

5.4 Antimicrobial Peptides may have Potent Intracellular Activity

Until recently, it was believed that the main target of AMPs was the cell membrane. It is now becoming evident that the antimicrobial function of transmembrane pore formation may be more important in allowing the peptides access to intracellular organelles and metabolic processes than in directly killing the cell through cytoplasmic leakage. Multiple studies have shown that bacteria treated with low concentrations of AMPs do not show signs of pore formation, but still show inhibition of metabolic processes (128, 143). For AMPs to form pores in bacterial membranes *in vivo*, a sizeable concentration of the peptides needs to be synthesised and transported to the infected region. Therefore, from a biological perspective, it makes sense that AMPs affect intracellular processes in microbes by diffusing across the membrane rather than forming pores by grouping *en masse* to localised regions of the membrane.

In this study, it was shown that a peptide with no bactericidal activity in bactopectone medium, melfrag12-26 can significantly inhibit the growth of bacteria if transported into the cell by electroporation. Melittin, and an unrelated peptide, bac8c, also showed increased bactericidal activity when transported inside the cell. It could be suggested the increased antibacterial activity resulted from electroporation-induced weakening of the cell membrane. However, bacteria exposed to the peptides immediately after electroporation produced no significant reduction in survival compared to untreated controls. From these results it appears that the eleven amino acids missing from melfrag12-26 are required to form pores in bacterial membranes but are not essential for intracellular toxicity. As other studies have suggested, the leucine zipper motif of melittin and similar peptides promotes dimerization and secondary structure formation (280, 362). This may explain why melfrag12-26, which has lost the leucine zipper motif, is less haemolytic. It also suggests that without the zipper motif, melfrag12-26 is unable to aggregate in solution into complexes that are capable of membrane pore-formation. However, the intracellularly active portion of melittin still appears

to be present in melfrag12-26 and, once inside the cell, it is bactericidal at relatively low concentrations.

5.5 Additive and Synergistic Action between Antimicrobial Peptides and Surfactants

Detergents are amphiphilic surfactants which have a hydrophobic tail and a hydrophilic head (363). These molecules solubilise lipid membranes by inserting into and partitioning lipid bilayers. This reduces the surface tension between the membrane lipids and creates negative curvature on the membrane surface. This curvature perturbation thins the hydrophobic portion of the membrane and increases the tendency of the membrane to disassociate as micelles (364). The types of lipids in the lipid bilayer differs between bacterial and mammalian cells (102). In eukaryotic membranes, cholesterol and sphingolipids preferentially interact with each other through hydrogen bonding of the ceramide of sphingomyelin and the 3-hydroxyl group of cholesterol, or by binding of sphingolipid acyl chains to the steroid ring of cholesterol (365, 366). These interactions cause the lipids, in regions saturated with sphingolipids, to become tightly packed together and reduces their fluidity, increasing their resistance to detergent-mediated disorganisation (366, 367). Cholesterol is absent from prokaryotic membranes and most bacterial species do not produce sphingolipids (367). Therefore, bacterial membranes may be more likely than those of eukaryotic cells to become disordered and vulnerable to the pore-forming action of AMPs. This vulnerability may be exploited to make the bacteria more susceptible to the peptides by adding a surfactant to weaken the intra-molecular forces between the phospholipids.

Patel *et al.* (368) have shown that the permeabilizing potential of three different lipo-peptide antibiotics towards artificial membrane vesicles was increased in the presence of a non-ionic detergent. In another report, the beta-lactam antibiotic oxacillin was shown to be more effective against MRSA in the presence of Triton X-100 (369). They also showed that Triton X-100 did not affect the penicillin binding proteins of the cell or the susceptibility of the cell wall to lytic enzymes (370). Triton X-100 may not alter oxacillin resistance levels in the cells as the authors suggest (369, 370). Rather, oxacillin-induced inhibition of peptidoglycan synthesis may increase the sensitivity of the cells to membrane destabilization by Triton X-100.

In the present study, investigations into the sensitivity of MRSA to AMPs plus surfactants showed that the antibacterial activity of melfrag12-26 increased substantially with increasing concentrations of Triton X-100. The antibacterial activity of melittin and bac8c was also slightly increased in the presence of Triton X-100. The increased bactericidal activity of the AMP in the presence of Triton X-100 suggests that the detergent permeabilizes the membrane, allowing the peptides rapid access to intracellular targets. Alternatively, the disassociation forces of both the peptide and detergent could weaken the intermolecular forces between phospholipid molecules to de-stabilise the membrane. The specific mechanism behind the increased antibacterial activity of the peptides in the presence of Triton X-100 or when transported intracellularly with electroporation was not investigated. However, bacteria that were electroporated with melittin or melfrag12-26 generally had a survival rate that was approximately 40-60% lower than those which were electroporated and then treated with the peptides. If electroporation was weakening membrane organisation it would be expected that these two samples would have the same survival rates. Therefore, these experiments suggest that internalisation of the peptides using electroporation or detergent-mediated permeabilization is likely the cause of cell death rather than lysis. The relatively small increase in bactericidal activity when melittin and bac8c were used with Triton X-100 could indicate that the dissociation forces exerted on the phospholipids by these peptides may be strong enough that addition of another permeabilising agent has only a marginal additive effect on the peptides activity.

The higher concentrations of Triton X-100 alone proved to be highly haemolytic. It seems then that the addition of detergents may reduce the selectivity of AMPs, which essentially negates one of the most promising aspects of their use as therapeutics. However, at 0.005% Triton X-100, melfrag12-26 and bac8c had no haemolytic activity while still showing augmented bactericidal activity. Cholesterol molecules in eukaryotic membranes decrease fluidity by filling spaces between phospholipids and limiting the movement of membrane fatty acyl chains (371, 372). It has been shown that artificial membranes devoid of cholesterol are far more susceptible to AMP-induced lysis (373, 374). Bacterial membranes are devoid of cholesterol and it has been shown that membranes lacking these molecules are more susceptible to the perturbations of Triton X-100 (375). This could explain why low concentrations of Triton X-100 were able to improve the bactericidal activity of these peptides without affecting their haemolytic potential.

The use of Triton X-100 and AMPs in combination could allow the peptides to be used at concentrations that are non-haemolytic, non-cytotoxic and non-immunogenic.

5.5 Antimicrobial Peptide Resistance Influences Tolerance to Antibiotics

Initial experiments showed that the sensitivity of melittin resistant WBG 8287 to a variety of antibiotics increased in comparison with melittin-naive populations. Fusidic acid was chosen for more comprehensive investigations because it was considered possible that the negatively charged antibiotic might have greater affinity for melittin resistant bacteria. Fusidic acid inhibits bacterial protein synthesis by preventing the release of elongation factor G (EF-G) from the ribosome, thereby halting the translocation of mRNA and tRNA along the ribosome (376). The wild-type MRSA strains were highly sensitive to fusidic acid even before melittin exposure. Therefore, prior to melittin resistance experiments all bacterial strains were made tolerant to fusidic acid by exposing the bacteria to sub-lethal concentrations of the antibiotic. The resulting fusidic acid resistance was not uniform across strains, with some showing MLCs below 20 µg/ml while others showed MLCs over 250 µg/ml. Contrary to previous results, melittin resistance did not affect fusidic acid resistance in WBG 8287 and BB bacteria exposed to fusidic acid. However, in strains W17S and Aus3, fusidic acid resistance was reduced significantly as melittin resistance increased. This suggested that while some mechanisms of antibiotic resistance may be negatively affected by the development of AMP resistance, others remain effective even as the cell alters its structure and metabolism to resist toxic peptides.

Synergism between certain antibiotics and AMPs has been demonstrated before (377, 378). Nuding *et al.* (378) suggested that synergism is due to membrane active peptides compromising the outer barrier of the cell and allowing antibiotics access to internal targets. If this is the case, combinational therapy may improve antibiotic effectiveness against bacteria which alter their membrane permeability to resist the drugs. However, this strategy is unlikely to improve treatment against bacteria that employ multiple antibiotic resistance mechanisms such as efflux pumps, modification of drug active sites and proteolytic degradation. In this study it was shown that synergism between antibiotics and AMPs is not restricted to

concurrent use of both antimicrobial agents. Ultimately, a sequential treatment may be more effective than using both antimicrobial agents simultaneously as it seems, in some MRSA strains, AMP resistance reduces or eliminates the proportion of antibiotic resistant bacteria in a population. Importantly, MRSA may be able to rapidly develop resistance to AMPs, but this resistance may be a liability to the cell when treated with another antimicrobial agent.

To gain a better understanding of how melittin resistance could be affecting bacterial strategies to counteract fusidic acid, the genetic profiles of the “Fus” resistance genes in each population were analyzed. To date, two main groups of genetic determinants that inhibit the action of fusidic acid have been identified: FusA and FusB (379, 380). FusA-mediated resistance arises from mutations in the coding region, denoted the FusA region, for elongation factor G. This modifies the structure of the EF-G proteins, which reduces the affinity of fusidic acid for the translocase-ribosome complex (379, 380). FusA resistance was the first fusidic acid resistance mechanism identified. Since its discovery multiple new mutations in the FusA gene have been discovered in the same strains, leading to the hypothesis that continued use of the antibiotic is selecting for bacteria in already resistant populations that are even more difficult to treat (380).

The FusB family collectively represent the most commonly distributed fusidic acid resistance genes in MSSA (379, 381). It is likely that this broad distribution is due to the genes being incorporated into multiple plasmids, including the penicillinase and cadmium resistance plasmid (313). Although FusB was first identified in mobile elements, FusB and a FusB-like gene: FusC, have now been found in the chromosome of some *S. aureus* isolates (382, 383). The FusB genes produce proteins which bind to and protect EF-G by preventing fusidic acid locking the translocase to the ribosome (384). FusB/C may also interact with the EF-G-ribosome complex and allow it to disassociate after fusidic acid has locked the two together (379, 380). The FusB protein consists of two domains: an N-terminal region with a hydrophobic core and a cationic C-terminal with five α -helical and β -sheet structures and a unique zinc²⁺ binding region (381). A separate study has shown that the FusC protein has a very similar structure to the FusB protein (385). Cationic AMPs may have a strong affinity for the anionic and hydrophobic N-terminal regions of FusB/C. Binding of melittin to these proteins may alter the function of FusB/C so that the proteins interfere with the normal functions of EF-G causing a reduction in protein synthesis. If this were the case, it could explain why bacteria which produce FusB/C proteins might disappear from the population

if they were constantly exposed to melittin. Therefore, to investigate potential reasons for the increased sensitivity to fusidic acid in melittin-resistant populations, the presence of the FusB/C genes in wild-type and AMP-resistant bacteria was examined.

PCR analysis showed variation in the dissemination of the resistance genes among melittin naive strains. Curiously, both FusB and FusC were amplified from WBG 8287. It is possible that the two peptides work synergistically to counteract antibiotic resistance. However, it seems redundant for a cell to produce two peptides of such similar structure (385) which perform the same roles. Melittin resistance in fusidic acid resistant WBG 8287 did not affect the sensitivity of the cells to the antibiotic. As might be predicted, melittin exposure did not remove the Fus genes from the WBG 8287 population. Fusidic acid resistance in Aus3 cells may be, at least in part, determined by FusB since loss of this gene in melittin-resistant cells coincides with increased susceptibility to the antibiotic. This suggests that melittin exposure can select for cells without the FusB genotype. Whether this is because melittin and FusB proteins form a toxic complex or because the metabolic demand to resist AMPs is so great that other non-essential metabolite sequences are lost requires further investigation. FusB and FusC genes were not amplifiable from W17S or BB DNA. This suggests that fusidic acid resistance in these strains is modulated by other genes or that they have FusB/C genes that could not be amplified with the primers used in this study. If W17S does not contain the FusB/C genes it suggests that melittin exposure and the development of resistance is detrimental to metabolic pathways, in particular, those related to antibiotic resistance.

Unfortunately, it is difficult to make any solid conclusions due to the conflicting results observed between the bacteria made resistant to bac8c or melittin. Resistance to fusidic acid in MRSA exposed to bac8c seemed to increase over the naïve population. Spindler *et al.* (386) have shown that resistance to bac8c in *E. coli* does not affect sensitivity to antibiotics that affect protein synthesis (386). However, their research suggested that bac8c resistance correlated with a reduced metabolic rate. A reduced metabolism would be expected to decrease the demand for protein turnover and could explain why bac8c-resistant bacteria were able to tolerate higher concentrations of fusidic acid. Genetically, the FusB/C profiles differed among colonies of the same population. This variation was seen in both the bac8c naïve and resistant bacteria. As such, it suggests that the FusB/C genes may only be present within some bacteria from a strain

and places doubt on the relevance of the findings of this study in regards to the gene being apparently lost in melittin-resistant Aus3 bacteria.

5.6 Silver Nanoparticle-Loaded Gauze is not comprehensively Bactericidal

A multitude of studies have challenged the effectiveness of an AMP- based therapeutic drug that can be used inside the body (258, 387, 388). However, the rapid, potent, selective, and at the same time, broad spectrum activity of these peptides make them attractive candidates for topical treatments of bacterial infections. Silver ions also have broad spectrum antimicrobial activity resulting in its continued use in medicine for the past 1300 years (76). It has been demonstrated that silver has wound healing properties and may also protect cells against viral infection (85). For these reasons, medical gauzes have been developed with silver nanoparticles incorporated into the weave to treat wounds at risk of bacterial infection and scarring. The nanoparticle silver-loaded gauze, Acticoat, is commonly used prophylactically to protect patients with significant burns against bacterial infections prior to eschar excision and tissue grafting. Multiple studies have demonstrated that Acticoat is able to decrease the bacterial load in the wound and reduce patient discomfort (73). However, a recent consensus from medical practitioners has concluded that the wound healing ability of silver-loaded bandages is negligible (72). Animal studies have also shown that Acticoat and similar bandages, may inhibit bacterial growth, but are not effective at eliminating pathogenic bacteria from topical wounds (71, 73, 389). Research in this study aimed to evaluate the efficacy of Acticoat against *S. aureus* and investigate additives that might improve the bactericidal efficacy of the gauze. A porcine skin model was used to test the effectiveness of Acticoat against MRSA infection. In subsequent experiments, the bactericidal activity of the gauze was augmented by applying a surface-coating of various non-toxic materials in combination with AMPs.

On agar medium the silver nanoparticles appeared to diffuse through the medium and inhibit the growth of cells around the Acticoat. Textured agar medium did not appear to change the inhibitory effectiveness of the dressing. The exception to this was cells treated on agar with a blistered texture from which bacterial growth could be seen under the Acticoat. It seemed that the blistered texture had gaps in the contact between Acticoat and the agar medium which protected the cells from

the inhibitory effect of silver. In the clinic, if the Acticoat bandage does not have close contact with the wound, a barrier of air between the dressing and the skin could prevent the effective diffusion of silver across the surface of the wound. Guidelines provided to medical personnel by the West Australian Department of Health suggest that nanocrystalline bandages in combination with a moisturizing agent should be applied to burns if the wound is dry and free of exudate (390). Moistening the Acticoat allows a sustained release of silver into the wound. A moist wound may also allow the silver to spread into infected areas which are not in direct contact with the dressing. When Acticoat is applied directly to the skin without a moisturizing agent, antiseptic or analgesic cream, wound exudate may provide a conduit medium for the silver particles to treat a broader area of the infected wound. However, anionic compounds in the fluid may also interfere with the therapeutic activity of silver nanoparticles.

The inhibitory effect of Acticoat seen in the initial experiments did not correspond to its reported bactericidal activity. Although the bacterial population was much reduced by Acticoat compared to that observed with filter paper controls, survival of MRSA on the dressing was evident when it was spotted onto fresh agar plates. Over the course of three days, electron microscopy consistently showed cells with healthy morphologies distributed across the Acticoat network. Although it is difficult to accurately quantify the amount of bacteria across a 1 cm^2 surface when an electron microscope reduces the field of view to $100 \text{ }\mu\text{m}^2$ or less, a general scan of the Acticoat strips showed that growth of the cells was not substantially increased or decreased as with extended exposure time. This could suggest that after the bacteria have adapted to their initial exposure to the silver-loaded gauze, the growth rates of the bacterial cells is exponentially increased to build up numbers and develop biofilm colonies. This was supported by the observation that, after 24h, the number of bacterial colonies from pig skin underneath Acticoat was approximately 2.25 fold that of bacteria that were grown on pig skin without exposure to Acticoat. Once the bacteria are protected from the silver by biofilm shielding, the metabolic demands for maintaining biofilm integrity and other resistance mechanisms may reduce the growth rate of the cells. On the nodes of the Acticoat, columns of what appears to be biofilm-encapsulated bacteria, protruding from the surface of the dressing, were evident. The difference in cell growth and biofilm secretion observed between the nodes and “arms” of the Acticoat may be due to a difference in silver density between the regions. The raised nodes may also have had more surface contact with the

agar and therefore could have provided the cells attached to the dressing a more moist and nutrient rich environment. The raised areas of Acticoat seem a flawed design because, on a nanoscale, this greatly increases the distance between the network of the dressing and the pathogens in the wound, which decreases the surface area of the wound that is in direct contact with Acticoat. However, Acticoat treatment of a moist wound may not require complete contact, as a wetting agent may provide a conduit between the gauze and the wound.

5.7 Acticoat Bactericidal Activity can be Augmented with other Non-toxic Molecules

Acticoat was measurably less effective against MRSA on pig skin than on agar medium. Importantly, the pig skin model more closely mimics an infected wound. However, the semi-liquid medium of agar appears to allow greater mobilization and activation of the silver particles. In an attempt to improve the dispersion of the silver from the dressing on the pig skin, strips of Acticoat were wetted with 1% w/v agar or agarose gels. The results showed that the gels allowed the silver to kill bacterial cells directly beneath the Acticoat. However, bactericidal activity did not extend beyond the area of the gauze strip. Addition of bac8c to the gel-dressing complex did not improve the area of its bactericidal effectiveness. The anionic polymers of agropectin in the agar could be sequestering the cationic peptide, preventing its diffusion out of the gel. However, bac8c also proved to be ineffective in the agarose gel which contained no charged molecules. This suggests that polysaccharide-based gels may not be suitable delivery vectors for small antibacterial peptides. Curiously, fusidic acid also failed to increase the inhibitory effect of Acticoat when both were incorporated into the gels. This could also mean that the concentrations of antimicrobial agents used in these tests were too low to show any great reduction in bacterial cell survival.

The qualitative results from the initial experiments, using bacterial growth on semisolid media, did not give enough detail to make any strong conclusions on how to improve the antibacterial efficacy of Acticoat. Therefore, in subsequent experiments, bacterial survival was quantified on pig skin and on Acticoat. Acticoat significantly inhibited the growth of bacteria on the skin and on the dressing itself for at least eight hours. However, after 24h Acticoat was unable to suppress the growth of MRSA on pig skin. Surprisingly, when the number of bacteria surviving on the pig skin underneath Acticoat was quantified, there were

more cells on the Acticoat treated samples than on the untreated control-skin. A flaw in the methodology of this experiment was that the control skins did not have an equivalent “mock-overlay” to Acticoat such as filter paper or polyethylene strips. The increased growth rate of cells overlain with Acticoat compared to cells that were grown on pig skin without an overlay may be because the gauze prevented, or slowed, desiccation of the cells.

An international consensus for the application of silver nanoparticle-based dressings suggests leaving antimicrobial gauzes on the wound for two weeks (72). On the Smith and Nephew website it recommends leaving Acticoat and Acticoat 7 on a wound for three and seven days respectively. From the results presented in this study, it appears that after 24h the bacteria have overcome the bacteriostatic activity of the dressings and will probably have clustered into difficult to treat biofilm communities. Therefore, in the initial 12-24h of treatment, it may greatly reduce the risk of recurring infection if the dressings are frequently changed.

Encasing Acticoat in gels appeared to improve the inhibitory activity of silver, but at the cost of limiting the effectiveness of additional antimicrobial agents. Therefore, in subsequent experiments, wetting agents, which are generally benign to epithelial cells, were applied to Acticoat prior to bacterial treatment. Glycerol and Tween-20 were selected for this purpose because they are non-toxic, inexpensive, routinely used in cosmetics and therapeutics and classified as generally recognised as safe by the U.S Food and Drug Administration. In combination with Acticoat, both of these wetting agents decreased the load of surviving bacteria on the gauze and the skin. The differences observed between glycerol, Tween-20 and the HpH₂O-coated control suggest that simple wetting of the gauze is not the only factor affecting bactericidal activity. Non-ionic detergents like polysorbate have been shown to increase the fluidity of mammalian cell membranes (391). One proposed mechanism of cell death relating to silver is the formation of transmembrane pores in the cell membrane (77) and a reduction in the rigidity of the membrane from Tween-20 exposure could make the bacteria more susceptible to the destabilising forces exerted on the cell surface by silver-nanoparticles.

Glycerol appeared even more effective at reducing the survival of *S. aureus* on both the skin and dressing, even at concentrations as low as 1%. Interestingly, glycerol in combination with manganese ions, has been reported to strongly

promote the formation of biofilm in *Bacillus subtilis* (392). It could be hypothesised that biofilm formation by these bacteria is a defence reaction in response to exposure of glycerol and manganese. If the combination of nanoparticle silver and glycerol had any kind of biofilm promoting activity for MRSA, it seems that process is not sufficient to overcome the toxicity of the two molecules. It is unclear why glycerol was so effective at enhancing the antibacterial effect of Acticoat. Silver nanoparticles may be more mobile in glycerol than in water because of the greater volumetric mass density of the solvent (1.26 g/ml). In addition, at temperatures above 15°C glycerol has been shown to expand the surface area of artificial phospholipid monolayers (393). As silver appears to have antibacterial mechanisms which destabilise membranes (394, 395), interactions between glycerol and the phospholipid bilayer may weaken the bonds between phospholipids and increase the effectiveness of the membranolytic activity of silver.

The data shown above suggests that a combination of an effective wetting agent such as glycerol and a non-toxic detergent or permeabilizing agent may work synergistically to activate the silver and increase its bactericidal activity. The increased efficacy in bactericidal activity observed when Acticoat was used in combination with Tween-20 suggested that the use of Acticoat together with another extracellularly-active antibacterial compound such as lysozyme might greatly improve the effectiveness of the gauze in controlling or eliminating *S. aureus* infections. Lysozyme catalyses the degradation of peptidoglycans in the cell wall of Gram-positive bacteria (396) which compromises the defence mechanisms of the bacteria against extracellular toxic molecules. Treatment of a high concentration of bacteria with Acticoat and lysozyme was a more effective bactericide than Acticoat alone, but was less effective than a combination of Acticoat and glycerol. Addition of an AMP increased the antibacterial activity of Acticoat combined with either lysozyme or glycerol. However, a combination of all three seems to be the most effective coating for improving the antibacterial activity of Acticoat. The mechanisms of the antibacterial compounds in this mixture appear able to complement each other to overcome bacterial defence mechanisms to increase bactericidal efficacy. For example, lysozyme degradation of the cell wall removes a protective barrier which prevents access of AMPs and silver ions to the cell membrane (397). Silver will also gradually degrade cell wall components which likely augments the activity of lysozyme and allows more silver ions and AMPs to reach the cell membrane (397, 398). Silver

and AMPs have similarities in their mechanisms of action: both being membrane-active and able to target intracellular components (77, 102, 122, 143, 399). Simultaneous attack of the bacterial membrane with the two antimicrobial agents may be sufficient to overcome any repair mechanisms employed by the bacterial cell. This may result in rapid lysis of the bacteria, or could allow a concentrated dose of the peptides and silver ions to enter the cell.

5.8 The Multifunctional Nature of Antimicrobial Peptides may Increase their Therapeutic Potential

This study measured the potential of AMPs as therapeutic agents, based on the effectiveness of their antibacterial activity. However, it seems that in nature these peptides have roles that are independent of host defence against bacteria and the true value of the peptides may be in the multiple roles they can play in maintaining host homeostasis. Recent studies have shown that AMPs play critical roles in wound healing and angiogenesis (400-402). In addition, AMPs show activity against tumours and viruses, and play an important role in maintaining sperm viability and motility (94, 403-405). Therefore, the importance of these peptides is well documented and studying the multifunctional roles they play may lead to the development of treatments for a broad range of diseases that may not be related to bacterial infection.

5.8.1 Wound Repair and Vascularisation

Epithelial tissue is generally the first barrier pathogens must cross to generate infection. The physical and chemical defences of this barrier can be disrupted by injuries such as sub-dermal abrasions and burns. Such injuries lead to the localised release of multiple cytokine growth factors such as insulin-like growth factor I and transforming growth factor α , to stimulate wound healing (406, 407). These molecules also increase the expression and release of AMPs from keratinocytes and neutrophils, presumably to combat invading pathogens and assist in wound healing (407). Keratinocyte migration, an important process in the closure and healing of wounds, has been shown to increase when the AMP LL-37 interacts with the keratinocyte epidermal growth factor receptor (402). Furthermore, LL-37 increases keratinocyte and fibroblast proliferation and inhibits keratinocyte apoptosis by upregulating the expression of cyclo-oxygenase-2 (408-410). LL-37 also contributes to the recovery of the wound by initiating and

supporting angiogenic processes. Two separate studies have shown that topical application of LL-37 significantly increased neovascularisation after wounding (400, 411). Mice lacking the murine equivalent of LL-37 also showed decreased vascularisation in the wound area (411). It is believed that LL-37 activity is mediated by interactions with the G-protein coupled receptor: formyl peptide-like receptor 1, on endothelial cells (400, 411). In addition, LL-37 indirectly contributes to angiogenesis by attracting macrophages and neutrophils, which synthesise and release angiogenic stimulators to the wound (411). The defensin peptides also play important roles in the recovery of wounds and the initiation of angiogenesis. In a study by Otte *et al.* (412), human β -defensin 2 was found to increase intestinal epithelial cell migration in disrupted epithelial layers, mediated by the C-C chemokine receptor 6 and possibly other G-protein coupled receptors. The peptide also suppressed the apoptotic-activity of TNF-related apoptosis inducing ligand and increased the expression of mucin genes, the products of which form a protective barrier against chemical and mechanical damage from luminal contents (412).

Other wound healing studies have shown that α - and β -defensins stimulate migration and proliferation of keratinocytes and fibroblasts. Furthermore, α -defensins aid in the initial stages of wound closure and repair by up-regulating the expression of extracellular matrix proteins in fibroblasts (413, 414). Angiogenesis is an important step in wound repair. However, un-regulated angiogenesis can also contribute to pathologies such as tumorigenesis, and intra-ocular neovascular disorders (415). Economopoulou *et al.* (416) have demonstrated that α -defensins can inhibit pathological vascularisation of retinal endothelial cells, by obstructing the angiogenic molecule: vascular endothelial growth factor (416). In contrast, multiple studies have shown that certain β -defensins and LL-37 peptides are up-regulated in oncogenic tissue, which suggests that angiogenic and proliferatory effects of these peptides may contribute to the growth of the tumour (417-419). The expression of α -defensin is also up-regulated in tumour cells, which suggests the peptide is of net benefit to the cells, despite its anti-angiogenic activity (417). Collectively, it seems that topical wounds could benefit from both the antibacterial and wound healing ability of the peptides. However, epithelial wounding has been shown to be associated with the activation of oncogenes in follicular stem cells resulting in tumorigenesis at the wound site (420). Therefore, the tumorigenic activity of some AMPs may exacerbate the events that lead to malignant transformation of damaged tissue to

carcinoma tissue. The complex relationship between AMPs and tumour cells has led to new avenues of research, investigating the risks of the peptides as tumor-agonists and the antagonistic roles other AMPs may have against cancer.

5.8.2 Anti-Cancer Roles of Antimicrobial Peptides

In 2012, 19% of all diagnosed diseases in Australia were cancer related (421). Early diagnosis and resection of the affected organ or tissue can increase patient life expectancy. However, if the malignant cells metastasise or if neoadjuvant therapy is recommended, chemotherapy or systemic radiotherapy may be required. Despite the large amount of research and development committed to this field, current treatments are expensive, can severely decrease the quality of life of the patient, and have low success rates depending on the type and location of the malignant tissue (422-424). When a cell becomes malignant it increases its membrane concentration of anionic molecules such as phosphatidylserine, sialic acid and heparin sulphate (425). Most, but not all cancerous cells also decrease the concentration of cholesterol in their membranes, thereby increasing membrane fluidity (94, 426). As demonstrated with bacteria, anionic membranes are the key selective target for antimicrobial peptides. A membrane containing low levels of cholesterol is less rigid and may be more susceptible to peptide destabilisation. These factors make AMPs more selective towards tumour cells than normal cells and have led to the proposal of antimicrobial peptides as anticancer therapeutics (94, 427). *In vivo* and *in vitro* experiments have shown that antimicrobial peptides such as cecropin, magainin, melittin, achyplesin 1 and defensins are able to kill a variety of tumour cell types at concentrations that are benign to most eukaryotic cells (427-429). The antitumor mechanisms seem to vary between peptides and cell types. In most cases it appears that AMPs initially damage cancerous cells by forming transmembrane pores or by dispersing cell membranes into micelle particles in a manner similar to their antibacterial affect (94, 430, 431).

Non-membranolytic mechanisms of action against cancer cells have also been discovered for AMPs. For example, mutation of the *ras* gene can cause hyper-activation of pathways related to cell proliferation, angiogenesis, metastasis and immortality: essentially transforming a healthy cell to a malignant cell (432). Melittin selectively activates the phospholipase A2 enzyme present on *ras* oncogenic cells, which in turn leads to necrosis of the cell. It has been shown that

melittin treatment selects against the mutated *ras* genotype in cancer cells and eventually the population will revert back to a healthy phenotype (433, 434). In a separate study, the AMP tachyplesin 1, isolated from the horseshoe crab, was shown to bind to carcinoma cells rich in the glycosaminoglycan hyaluronan and to cause cell necrosis via the complement pathway of the immune system (435).

Despite this, recent studies have shown that some AMPs can increase tumour cell growth, proliferation, spread, and can even transform normal cells into cancer cells (436-438). The angiogenic and mitogenic properties of the cathelicidin peptide LL-37 seem to play an important role in tumorigenesis with the expression of this peptide in tumour cells significantly increased over that of healthy cells (437, 439). Furthermore, treating cancerous breast, ovarian and melanoma cells with LL-37 *in vitro* greatly increased their proliferative and invasive tendencies, suggesting that the peptide acts as a growth factor for the cells (418, 437, 439). A study conducted by Li *et al.* (438) has also shown that murine myeloid cells that express a peptide similar to LL-37: cathelicidin related antimicrobial peptide (CRAMP), are far more likely to develop into tumour tissue than their counterparts which lack CRAMP expression (438). In summary, it appears that the anticancer properties of AMPs show promise but certain peptides may exacerbate the oncogenic potential of some cells. If the factors that govern the tumorigenic activity of the peptides could be identified and mitigated, they may prove to be a safe chemotherapy for localised and systemic cancer.

5.8.3 Anti-viral Properties of Antimicrobial Peptides

The activity of clinically applied antibiotics generally involves inhibiting biological mechanisms related to metabolism within the pathogen. Viral replication is dependent on host cell machinery, leaving no pathogen-specific metabolic processes that can be tackled with medication. Therefore, despite the high rate of clinicians prescribing antibiotics for viral infections (440), antibiotics cannot directly target a virus. As an alternative treatment, AMPs have been shown to inhibit viral activity by direct or indirect interactions with the viral envelope or the host cell (138). The vaccinia virus is an enveloped virus, related to the small pox virus, which became an effective vaccine for *Variola minor* and *major* infections (441). Vaccinia induces high levels of LL-37 expression in keratinocytes, which suggests that the peptide has immunoresponsive roles against the virus (442). Transmission electron microscopy of vaccinia exposed to LL-37 shows significant

damage to the outer viral membrane, indicating a direct deleterious activity against the virus (442). Likewise, LL-37 has been shown to destroy influenza virus type A by disruption of the viral membrane (443). Analogues of melittin have also been shown to inhibit viral infection of plant cells by obstructing the viral coat protein from binding to viral RNA (444).

Recent studies found that melittin can also inhibit human immunodeficiency virus (HIV) replication in infected T-cells by disrupting viral genome expression (445). At sub-cytotoxic concentrations, melittin was shown to inhibit transcription of the HIV structural protein, group-specific antigen, in T-cells and fibroblasts without affecting the transcription of host cell genes (445). The mechanism behind this has not been fully elucidated, but it may be that the peptide is affecting the activity of one or more of the host enzymes exploited by HIV. For example, Salvatore *et al.* (446) have shown that α -defensin prevents the activation of protein kinase C (PKC) in cells infected with the influenza virus. Besides aiding in viral entry, PKC stimulates a variety of transcription factors that bind to and activate promoters of HIV replication (447). Like α -defensins, melittin may inhibit PKC, thereby preventing activation of HIV promoters and limiting its replication inside the cell. Carriel-Gomes *et al.* (448) found that pre-treating mammalian cells with a variety of AMPs inhibited the entry of herpes simplex virus type 1. Although the mechanism behind this was not investigated, it is likely that the peptides act by competitively binding to viral entry proteins on the cells, inhibiting internalisation of the viron. This has been demonstrated with other peptides and viruses. For example, HIV entry into T-cells was inhibited by β -defensin competitively binding to the CXCR4 receptor required for internalisation of the virus (449). In contrast, θ -defensin inhibits HIV entry into T-cells by binding to glycoproteins on the viral envelope which are required for envelope fusion to the cell membrane and subsequent release of the viron into the cytoplasm (450).

In vivo, HIV can spread quickly because it is internalised by dendritic cells and then transfected to T-cells as part of the antigen presenting immune pathway (451). Van Compernelle *et al.* (452) have shown that a range of AMPs isolated from amphibian skin can not only inhibit HIV by disrupting its envelope membrane, but can also disable the virus after it has been internalised by the dendritic cell. This suggests that the peptides may be able to enter the dendritic cells and attack the virus. Alternatively, the peptides may target the virus when it is presented by the dendritic cell for T-cell CD4 recognition.

Antimicrobial peptides, unlike most antibiotics, have bio-active functions that may be used to improve the treatment of a broad variety of disorders and diseases. These multifunctional roles of AMPs may complement each other so that patient comfort, cost, and treatment effectiveness are greatly improved. For example, an antimicrobial gauze or cream with α -defensins as the active component could protect topical wounds from infection while at the same time reducing the time for the wound to close. Vaginal irrigation using a wash solution with one or more AMPs could protect against bacterial, yeast, and viral infections, which may also reduce the incidence of human papillomavirus-induced cervical cancer in patients. The scope of research within this project did not cover the possibilities of using AMPs as anything other than antibacterial agents. However, evidence from the literature suggests that the clinical application of these peptides may extend beyond that of bacterial infection control. If the limitations and side-effects of these peptides can be counter-acted, AMPs may prove to be an effective solution to antibiotic resistance in pathogens while at the same time providing new options to treat viral infections, cancer, and improve existing wound healing devices.

6. Conclusions and Future Work

This work has investigated new biological sources for antibacterial peptides. From these investigations, it appears there are still antimicrobial compounds effective against MRSA which can be found in common plants and fungi. Contrary to some published results and speculation, MRSA was able to adapt rapidly to the antibacterial peptides used in this study. However this resistance was dependent on continued selection pressures suggesting that the metabolic fitness of resistant bacteria may be impaired. A melittin peptide fragment, benign to bacteria in growth media, was shown to have significant bactericidal activity if the membrane is permeabilized with low concentrations of a non-ionic detergent. This could enable peptides, with low bactericidal activity but that are also non-cytotoxic and stable *in vivo*, to become effective topical therapeutics. Finally, this study has demonstrated that the effectiveness of a clinically approved antibacterial gauze in controlling infection can be significantly improved by the addition of antibacterial peptide(s) and non-toxic compounds that enhance the action of biocidal peptides and silver nanoparticles.

In this project, it was demonstrated that the antimicrobial activities of juniper berries and cardamom seeds were protein-related, which has not been documented before. Potent antibacterial activity was also discovered in the Australian honey fungus. This fungus is considered non-toxic to humans (334) and antibacterial compounds from this source are likely to be safe for therapeutic use. From these investigations it is clear that antibacterial activity can be identified in a broad variety of plant and fungal material relatively quickly, using very basic techniques. Although characterisation of the compounds stopped at identifying their proteinaceous nature, this study was able to show that a broad spectrum of plants and fungi, which are considered safe for human consumption, have anti-MRSA properties. Preliminary chromatography separation experiments were conducted on the cardamom seed and juniper berry extracts but the results were inconclusive. Follow up experiments relying on mass spectrometry characterisation of the extract fractions showing antimicrobial activity was planned, but could not be conducted due to time constraints. It is hoped that future experiments will be performed to investigate the nature and identity of these antimicrobial compounds. It is also anticipated that the methodology developed in this project for extracting and screening antimicrobial compounds will assist in streamlining related experiments.

Multiple studies, including this one, have shown that the bactericidal efficacy of AMPs is negatively affected by exogenous proteins, high salt levels and low temperatures (186, 340-342). Based on these observations, it appears melittin and similar AMPs may only be suited for treating topical infections. Furthermore, results from these experiments suggest that the effectiveness of treatment with AMPs may be greatly enhanced by pre-washing the wound with a warm, low ionic strength buffer and by the inclusion of mild wetting agents with the peptides. However, the experiments in this study focused on treating cells in planktonic culture. The effectiveness of changing environmental parameters to improve bactericidal activity of AMPs in a wound may differ from the results obtained in this study. Future experiments using epithelial wound models to investigate debridement and heating of the surrounding tissue, to improve AMP efficacy, should give more clinically relevant data.

Perhaps one of the most important findings from this study is that resistance to melittin and bac8c in multiple MRSA and one MSSA strain can be rapidly induced. This resistance correlates with a decreased anionic charge of the bacterial surface. Growth rates and morphology appear unaffected by resistance.

However, it is likely that masking the negative surface charge was not the only resistance mechanism developed, since the peptides were still able to form transmembrane pores in resistant cells. When selection pressures were removed, resistance to the peptides diminished in the bacterial population relatively quickly. Despite the reduction in MLC seen in populations subcultured without the AMPs, a small sub-population retained a tolerance to the peptides that was slightly greater than that of wild-type cells. This sub-population appears likely to re-emerge as the dominant phenotype when the bacteria are re-exposed to an AMP, thereby ensuring the survival of the population in a dynamic environment. The short time required to bring about a substantial reduction in the MLC of melittin, which is observed in resistant cells, is in stark contrast to the reported stability of antibiotic resistance over time (353). On this basis, the results suggest that the capability for development of resistance to an AMP may not preclude its therapeutic use, as long as appropriate treatment regimes are followed.

The effect AMPs have on the cell surface can be seen clearly with SEM and transmission electron microscopy images (453, 454). However, this reveals nothing of their intracellular activity. For researchers to design more effective antibacterial peptide-based therapeutics, a more detailed understanding of the intracellular targets of these peptides is needed. Using transmission electron microscopy or synchrotron x-ray free-electron laser imaging to investigate any possible morphological damage done to internal organelles by AMP exposure, could give valuable insight into the intracellular mechanism of the peptides. The lack of membrane-bound organelles in bacteria make it unlikely that the peptides damage the cytoplasmic organelles of the bacteria in the same way they form pores in the plasma membrane. However, if the peptides have specific receptors on the bacterial organelles, peptide-receptor interactions could cause visible changes in the structure of the organelles that may be observed with transmission electron microscopy or free-electron laser imaging.

Melfrag12-26 was benign to MRSA until it was inserted into the cells by electroporation. The activity of the peptide inside the cell shows that the twelve amino acid sequence, or part of it, is capable of inhibiting intracellular metabolic processes. It appears that the factor limiting its toxicity externally is its inability to create transmembrane pores or diffuse across the membrane. Full-length melittin and bac8c also appear more effective at killing cells via intracellular means than by transmembrane pore formation. This is an interesting observation

because it means that enhancing the cytolytic potential of a peptide has the disadvantage of increasing its haemolytic activity but may be redundant if the peptide has intracellular toxicity and is used in conjunction with a bacterial membrane permeabilizing agent. This understanding could also lead to the creation of hybrid peptides with domains that are active inside the cell, attached to pore-forming sequences that are specific to microbial membranes. In practice, a simpler approach may be to use internally toxic peptides in conjunction with a membrane permeabilising agent such as a mild detergent or an organic solvent. In this study it was shown that the surfactants Tween-20 or Triton X-100, used in conjunction with the peptides, resulted in a substantial increase in bactericidal activity. However, it is unclear whether the detergent action could have made the bacterial membranes more susceptible to pore-forming activity or whether the increased permeability of the membrane allowed peptides greater access to intracellular targets. It is anticipated that future SEM studies analysing the morphology of the cells after AMP and detergent treatment may clarify whether the combinational treatment has an effect on the structural integrity of the cell surface, or if the damage to the cell is focused internally. However, in the case of melittin at least, it appears that Triton X-100 increases pore forming ability even at low concentrations of the detergent, which, in turn, increases the haemolytic potential of melittin. Conversely, the bactericidal activity of melfrag12-26 and bac8c was increased by the addition of a detergent with no effect on their haemolytic capability. Therefore, the therapeutic potential of AMPs may be improved by using the peptides with detergents. Additionally, the use of detergents would be most useful, therapeutically, if applied with non-haemolytic peptides. A peptide-detergent combinational treatment may be a simple and inexpensive way of enhancing AMP-therapy of topical wounds. As proposed above, the different compositions of eukaryotic membranes and prokaryotic membranes may allow the improvement of the antibacterial activity of the peptides while minimising their toxicity to eukaryotic cells. Although this study has shown that Triton X-100 can improve the bactericidal activity of an AMP without affecting its haemolytic potential, future experiments investigating a broader range of detergents with different solubilising affinities for different lipids could identify a permeabilising agent that allows any AMP to be used effectively as an antibacterial therapy below its haemolytic or cytotoxic concentrations.

The presence of “blebbing” structures on the surface of melittin-resistant cells following exposure to melittin suggests that alterations to the membrane do not

produce an impermeable barrier to the peptide. If melittin can still translocate into the cytoplasm of resistant bacteria, it is likely that the cells must have resistance mutations that protect intracellular organelles or pathways, to allow them to tolerate the peptide treatment. If this is the case, the results from this study suggest that these modifications could make some strains of *S. aureus* susceptible to certain antibiotics. It is also likely that the decreased negative charge of the membrane provides greater access to anionic antimicrobial agents, increasing their propensity to localise on the surface of the cells. If this reduced the tolerance of *S. aureus* to some antibiotics, it is unlikely that the development of widespread resistance to peptide-based therapeutics would create untreatable pathogens to the same extent as multi-drug resistant bacteria. However, resistance to the peptide bac8c appeared to make the bacteria more resistant to fusidic acid. This supports the possibility that bac8c has a different mechanism of action from melittin and that different bacterial strategies may exist for resisting this peptide. If this is the case, future experiments investigating bacterial sensitivity to a broad range of antibiotics could reveal whether the mutation that confers bac8c resistance might also make the bacteria more resistant to other antibiotics, or if this makes the cells more susceptible to other classes of antimicrobial agents.

The weak electrophoretic bands representing some of the Sma1 fragments in the pulsed-field gel made it difficult to determine with certainty whether the induction of resistance in these experiments caused major changes in the bacteria. Most importantly, CHEF electrophoresis has a fragment separation limit of around ten kilobases. Therefore, the gain or loss of smaller fragments or of as single nucleotide polymorphisms could not be detected with these methods. More comprehensive genetic investigations in this study revealed that there was great variability in fusidic acid resistance between strains and suggest that FusB/C are not the only mutations providing fusidic acid resistance. FusB mediated resistance appears to be lost in some strains that develop melittin resistance. However, FusB/C genes were not identified in all strains analysed. In addition, the presence of the FusA mutation was not investigated in this project. It is possible that the alteration of the structure of elongation factor-G caused by the mutation makes it a target for melittin. Therefore a population exposed to melittin may not have the FusA mutation which may explain why it is susceptible to fusidic acid. Consequently, more tests are needed to conclude whether melittin resistance causes some populations to lose antibiotic resistance genes or

whether the mechanisms involved in melittin resistance enhances fusidic acid activity against some strains. In any case, if some peptide-resistance mechanisms increase the bacterial susceptibility to antibiotics and others increase resistance, this could complicate potential AMP treatment for bacterial infection. The broad mechanisms of action and resistance as well as their impact on the phenotypes of exposed microbial populations requires further investigation to discern the risks of implementing AMP-based therapy.

Acticoat was unable to eliminate MRSA from inoculated areas of agar or pig skin. Surprisingly the bacteria were able to survive on the surface of Acticoat. Scanning electron microscopy showed that the majority of cells were localised to what appeared to be dense biofilm communities. It appears likely that the biofilm protected the bacteria from the toxic effects of silver. However, even single cells attached to the gauze showed a healthy morphology, suggesting that they have other mechanisms to cope with the effects of Acticoat. Evidently, the infection models used in these experiments are not ideal replicates of a natural wound. Furthermore, Acticoat and similar gauzes are probably safer and just as, if not more, effective at preventing topical infections than using broad spectrum antibiotics. However, patients with deep wounds, compromised immune systems or who have serious infections, are unlikely to benefit from this treatment if the limitations of the gauze are unknown to, or ignored by medical practitioners. This study has shown that Acticoat can be made far more effective for treating infected pig skin if used in conjunction with a wetting agent, a cell wall hydrolysis enzyme and AMPs. This combination may allow more silver ions to diffuse from the gauze while at the same time increasing the antibacterial effectiveness of silver due to lysozyme and peptide-induced weakening of the cell wall and plasma membrane. In their native form most AMPs are likely to be unsuitable for systemic use. However, from these preliminary experiments it appears that antimicrobial peptides could have applications in treating topical wounds with medical gauzes. Currently it is unknown whether using the gauze in combination with AMPs will augment, compromise or have no effect on their bactericidal activity in clinical applications. It is anticipated that this work will lead to more advanced trials to test the true effectiveness of Acticoat with and without complementary compounds, such as glycerol, in clinical applications. This research may help to improve the clinical effectiveness of Acticoat, with the potential for reducing patient scarring, discomfort and mortality.

7. References

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8. Appendices

Appendix A Storage and Culture of Bacteria

Long term storage of bacterial stocks was in 25g/l bactopectone medium supplemented with 15% v/v glycerol at -80°C. Bacteria used in experiments were spread on 1.5% bacteriological agar, 25g/l bactopectone medium to produce single colonies. These inoculated plate stocks of bacteria were stored at 4°C for fourteen days before being discarded.

Appendix B Temperature Gradient PCR for FusB/C Primers

DNA from W17S, Bengal bay and WBG 8287 was amplified with FusB or FusC primers using reagent concentrations outlined in table 8.1. A gradient of annealing temperatures for the PCR reactions was used to find the optimal annealing temperature for the primers (Table 8.2). Although no amplification was seen with either primer sets for the W17S or BB strains, single amplicons were visible for both primer sets when used with WBG 8287 DNA, at annealing temperatures ranging from 55-63°C (Figure 8.1). Therefore, 59°C was chosen as the optimal annealing temperatures for FusB/C studies.

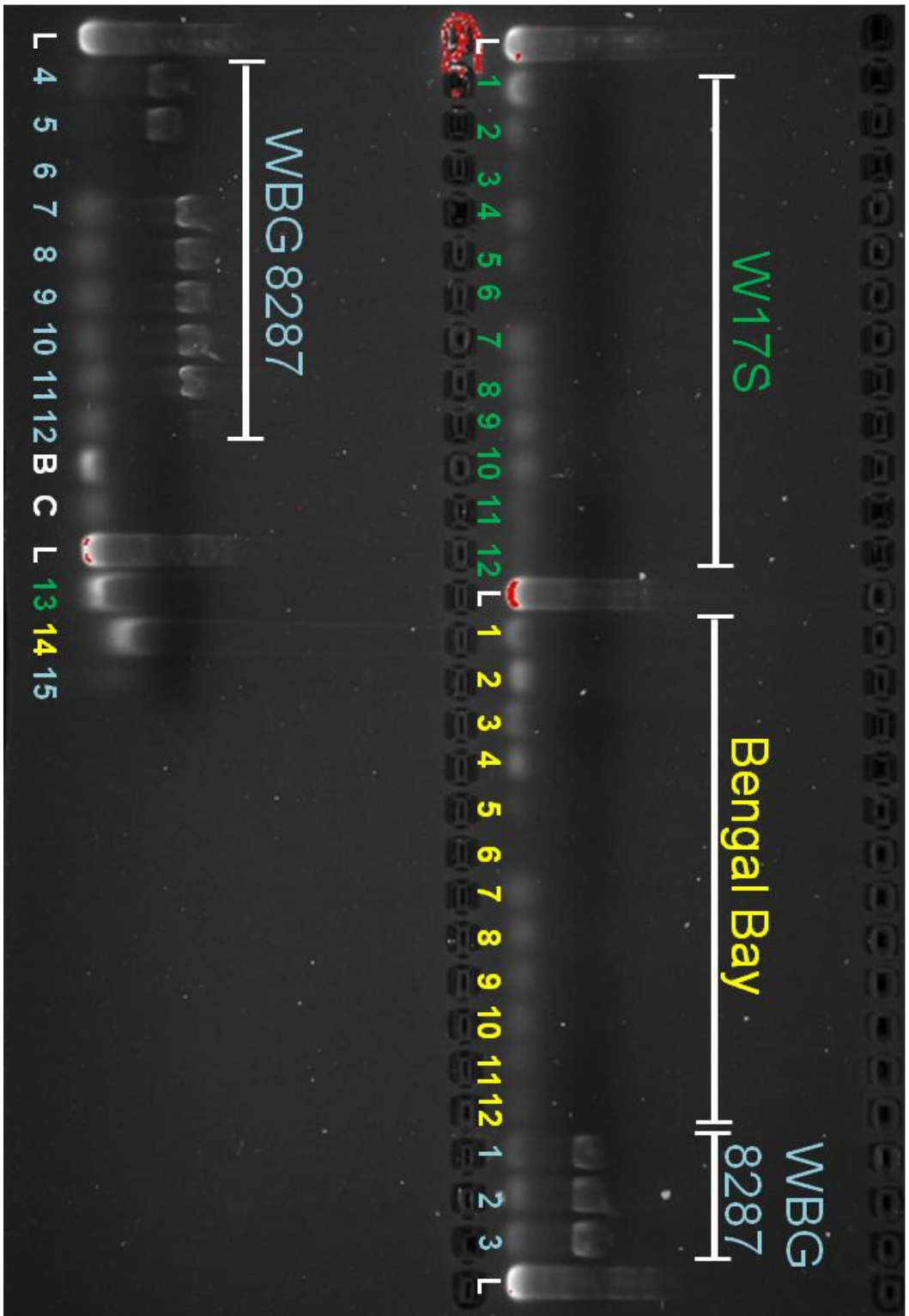


Figure 8.1 Amplified products from temperature gradient PCR

Lane	DNA Origin	Primers	Temperature
L	100 Bp Ladder	NA ¹	NA
1	DNA Extract	FusB	55°C
2	DNA Extract	FusB	57°C
3	DNA Extract	FusB	59°C
4	DNA Extract	FusB	61°C
5	DNA Extract	FusB	63°C
6	DNA Extract	FusB	65°C
7	DNA Extract	FusC	55°C
8	DNA Extract	FusC	57°C
9	DNA Extract	FusC	59°C
10	DNA Extract	FusC	61°C
11	DNA Extract	FusC	63°C
12	DNA Extract	FusC	65°C
13	W17S DNA Extract	NA	NA
14	BB DNA Extract	NA	NA
15	WBG 8287 DNA Extract	NA	NA
B	NA	FusB	59°C
C	NA	FusC	59 °C

¹None added/Not applicable

Table 8.1 PCR Reagents for Temperature Gradient PCR

Reagent	Volume/20μl Reaction	Concentration
5xMyTaq Reaction Buffer	4 μ l	1x
Forward Primer	0.4 μ l	2 μ M
Reverse Primer	0.4 μ l	2 μ M
Template	1 μ l	5ng/reaction
Polymerase (5U/μl)	0.1 μ l	0.025U/ μ l
HpH₂O	14.1 μ l	70.5%

Table 8.2 Thermal Cycle Parameters for Temperature Gradient PCR

Step	Temperature ($^{\circ}$C)	Time (Seconds)	Cycles
Initial Denaturation	95	60	1
Annealing	55, 57, 59,61,63 or 65	15	
Extension	72	15	30
Denaturation	95	15	
Final Extension	72	60	1
Hold	4	∞	1