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Using phospholipid vesicles to assay bacterial lytic agents, examining factors and identifying virulence loci which alter toxin production in Staphylococcus aureus

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Using phospholipid vesicles to assay bacterial lytic agents, examining factors and identifying virulence loci which alter toxin production in *Staphylococcus aureus*

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Declarations

This dissertation is the result of my own work and includes work done in collaboration which is specifically indicated here and in the text.

Chapter 3:

A) Figure 3.9 Circular dichroism (CD) spectra of synthetic PSMs were performed in collaboration with Yi Yang, University of Bath, UK. [‡]

[‡] **Laabei M*,** Jamieson WD*, Yang Y, van den Elsen J, Jenkins AT. Investigating the lytic activity and structural properties of *Staphylococcus aureus* phenol soluble modulin (PSM) peptide toxins. Biochim Biophys Acta. 2014. Sep 3 [Epub ahead of print]

Chapter 6:

A) Figure 6.2: Oxacillin-induced expression of PBP2a in CA-MRSA strain LAC (USA300) and MW2 (USA400), performed by Dr. Justine K. Rudkin, NUI Galway, Ireland

B) Figure 6.4e HPLC/MS analysis of PSM proteins, performed by Dr. Hwang-Soo Joo and Dr. Micheal Otto, NIAID, NIH, Bethesda, Maryland, USA. [‡]

[‡] Rudkin JK*, **Laabei M*** Edwards AM, Joo HS, Otto M, Lennon KL, O'Gara JP, Waterfield NR, Massey RC. Oxacillin Alters the Toxin Expression Profile of Community-Associated Methicillin-Resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2014 Feb;58(2):1100-7.

Chapter 7:

A) Genome sequencing of ST239 strains was performed by Zeynep Gulay, Department of Clinical Microbiology, School of Medicine, Dokuz Eylul University, 35210 Konak, Turkey;

B) The identification of genetic variation within this set of clinical isolates was performed by Prof. Ed Feil and Dr. Santiago Castillo-Ramírez, University of Bath, UK;

C) Figure 7.4b: Construction of the Maximum likelihood tree was performed by Dr. Daniel Wilson, University of Oxford, UK

D) Figure 7.5: Predicted toxicity correlates with disease severity *in vivo*, animal experiments were performed by Dr. Elisabet Josefsson, University of Gothenburg, Sweden

E) Figure 7.9: SNP2174068 has a major impact on the response of AgrC to AIP nd hence toxicity was performed by Dr. Tim Sloan, University of Nottingham, UK. [‡]

‡ **Laabei M***, Recker M*, Rudkin JK, Aldeljawi M, Gulay Z, Sloan TJ, Williams P, Endres JL, Bayles KW, Fey PD, Yajjala VK, Widhelm T, Hawkins E, Lewis K, Parfett S, Scowen L, Peacock SJ, Holden MT, Wilson D, Read TD, van den Elsen J, Priest NK, Feil EJ, Hurst LD, Josefsson E, Massey RC. Predicting the Virulence of MRSA from its Genome Sequence. *Genome Res*. 2014. 24: 839-849

Acknowledgements

Abstract

Burns represent one of the most devastating forms of injury with infection complications representing the highest risk of mortality. The primary objective of the *Bacteriosafe* project was the development of a smart wound dressing that would respond to the presence of bacteria in burn wounds. The basis of this sensing system employed the use of phospholipid vesicles, containing a self-quenchable fluorescent dye. These vesicles mimic the eukaryotic cell membrane and as such are susceptible to bacterial cytolytic factors which lyse the vesicles, generating an observable and measurable fluorescent response. My primary role in this project was to identify the vesicle lysing agents secreted from the two most frequent burn wound colonisers, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We identified the small amphipathic alpha helical peptide toxins from *S. aureus* and glycolipid molecules derived from *P. aeruginosa* as the agents responsible for vesicle lysis. The identification of these molecules led to the development of two novel phenotypic assays designed to measure these important virulence factors, as discussed in chapter 3 and 4. In chapter 5 we examined the role of toxic shock syndrome toxin-1 (TSST-1) in repressing global exoprotein expression. Our results demonstrate that TSST-1 does not repress toxin secretion and strains expressing TSST-1 retain their ability to lyse vesicles. In chapter 6 we explored the use of subinhibitory oxacillin in inducing the alternative penicillin binding protein 2a (PBP2a) in community-acquired methicillin resistant *S. aureus* (CA-MRSA) strains to down-regulate toxicity. Previous work in the Massey lab demonstrated that the expression of the *mecA* gene, which encodes PBP2a, resulted in reduced toxicity in hospital-acquired (HA) - MRSA. CA-MRSA strains are considered highly toxic and have a considerably lower level of PBP2a expression. Treatment of CA-MRSA strains with subinhibitory oxacillin did result in a down-regulation of some toxins but also the up-regulation of others, highlighting the pleiotropic effect oxacillin had on virulence regulation. In chapter 7 we developed an approach that uses the genome sequences of a set of related clinical *S. aureus* strains to identify novel virulence loci by associating genetic polymorphisms with specific virulence phenotypes using a genome wide association study (GWAS). This analysis resulted in the identification of four novel loci which when mutated lead to a reduction in toxicity. We demonstrate that the

GWAS approach is an effective method in identifying candidate SNPs which may be important in altering virulence but do highlight limitations of this approach, primarily the generation of false positives.

Publications

1) **Laabei M***, Jamieson WD*, Yang Y, van den Elsen J, Jenkins AT. Investigating the lytic activity and structural properties of *Staphylococcus aureus* phenol soluble modulin (PSM) peptide toxins. *Biochim Biophys Acta.* 2014. Sep 3 [Epub ahead of print]

2) **Laabei M**, Jamieson WD, Lewis SE, Diggle SP, Jenkins AT. A new assay for rhamnolipid detection-important virulence factors of *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol*. 2014. Aug;98(16): 7199-209

3) Thet NT, Jamieson WD, **Laabei M**, Mercer-Chalmers JD, Jenkins AT. Photopolymerization of polydiacetylene in hybrid liposomes: effect of polymerization on stability and response to pathogenic bacterial toxins. *J Phys Chem B*. 2014. 118(20):5418-27

4) **Laabei M***, Recker M*, Rudkin JK, Aldeljawi M, Gulay Z, Sloan TJ, Williams P, Endres JL, Bayles KW, Fey PD, Yajjala VK, Widhelm T, Hawkins E, Lewis K, Parfett S, Scowen L, Peacock SJ, Holden MT, Wilson D, Read TD, van den Elsen J, Priest NK, Feil EJ, Hurst LD, Josefsson E, Massey RC. Predicting the Virulence of MRSA from its Genome Sequence. *Genome Res*. 2014. 24: 839-849

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6) Rudkin JK*, **Laabei M*** Edwards AM, Joo HS, Otto M, Lennon KL, O'Gara JP, Waterfield NR, Massey RC. Oxacillin Alters the Toxin Expression Profile of Community-Associated Methicillin-Resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2014 Feb;58(2):1100-7.

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9) Edwards AM, Bowden MG, Brown EL, **Laabei M**, Massey RC. *Staphylococcus aureus* extracellular adherence protein triggers TNFα release, promoting attachment to endothelial cells via protein A. *PLoS One*. 2012;7(8):e43046.

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11) Zhou J, Tun TN, Hong SH, Mercer-Chalmers JD, **Laabei M**, Young AE, Jenkins AT. Development of a prototype wound dressing technology which can detect and report colonization by pathogenic bacteria. *Biosens Bioelectron*. 2011 Dec 15;30(1):67-72.

(*equal contributors)

List of Abbreviations used

Abbreviation	Description
ACME	Arginine catabolic mobile element
ADAM-10	A Disintegrin and Metalloprotease Domain-containing protein 10
Agr	Accessory gene regulator
AHL	Acylhomoserine lactone
AIP	Auto-inducing peptide
APC	Antigen Presenting Cell
CAMP	Synergistic haemolysis test
CA-MRSA	Community-associated MRSA
CC	Clonal complex
CD	Circular dichroism
CHIPS	Chemotaxis inhibitory proteins
CHO	Cholesterol
CF	5(6)-carboxyfluorescein
CoNS	Coagulase-negative staphylococci
CTAB	Cetyltrimethylammonium bromide
CWA	Cell wall anchored
CYY	Casein hydrolysate and yeast-extract containing medium
DPPC	1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPE	1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DLL	Dock, lock latch mechanism of protein binding
ECM	Extracellular matrix
EF-2	Elongation factor-2
EMRSA	Epidemic MRSA
EPS	Extracellular polymeric substances
Fb	Fibrinogen
Fn	Fibronectin
FPR	Formyl-peptide receptor
GWAS	Genome wide association study
H_2O_2	Hydrogen peroxide
HA-MRSA	Hospital-associated MRSA
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatograph
IEC	Immune evasion cluster
IL	Interleukin
InDel	Insertion/Deletion
IFN-γ	Interferon γ
IR	Infrared spectroscopy
LAC	Los Angeles County clone (USA300, ST-8, SCC*mec* type IV)
MB	Methylene Blue
MGE	Mobile genetic element

MHC	Major Histocompatibility Class
MLST	Multilocus sequence typing
MSCRAMMs	Microbial Surface component re recognising adhesive matrix molecules
MSSA	Methicillin sensitive *Staphylococcus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MV	Membrane Vesicle
MW2	Mid-West Clone (USA400, ST-1, SCC*mec* type IV)
NAD	Nicotinamide adenine dinucleotide
NGS	Next generation sequencing
NO	Nitric oxide
PFGE	Pulse-field gel electrophoresis
PFT	Pore-forming toxin
PIA	Polysaccharide intercellular adhesin
PMN	Polymorphonuclear leukocyte
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PSM	Phenol-soluble modulin
PQS	Pseudomonas quinolone signal
QS	Quorum Sensing
RL	Rhamnolipid
SaPIs	*S. aureus* pathogenicity islands
SNP	Single nucleotide polymorphism
SSTI	Skin and soft tissue infection
ST	Sequence type
TCDA	10, 12-tricosadiynoic acid
TCS	Two-component system
TFE	2,2,2-trifluoroethanol
TLC	Thin-layer chromatograph
Tn	Transposon
TNFα	Tumour necrosis factor α
TNFR	Tumour necrosis factor receptor
TSS	Toxic shock syndrome
VLT	Vesicle lysis test
vWF	Von Willebrand factor
UV	Ultra-violet

Table of Contents

1

Introduction

1.1 Development of active wound dressing for detection of pathogenic bacteria in burns patients

B urns represent a complex and traumatic event and are classified as one of the most debilitating forms of injury due to the adverse local and systemic effects on normal homeostasis. In the United States alone, 1.2 million people suffer burn injuries each year with a mortality rate of 5-10% in those patients suffering from moderate to severe burns (1, 2). Although there has been a huge improvement in the treatment of burn victims in areas such as fluid resuscitation and nutritional support, 75% of all deaths are related to infection complications (3, 4). The patients' age group can have a major influence on the cause of trauma. The annual admission of children under the age of five suffering from burn injury in England and Wales is 4,300 and 53% of all scald related injuries are suffered by this age group (5). Furthermore, this age group is particularly susceptible to burn-related infection, due partly to a thinner dermal layer increasing the propensity for deeper burns and invasive infection and an immature immune system (6). These statistics represent a growing need to tackle burn-related injury especially in young children, as it is the third leading cause of accidental death (5).

1.1.1 Biology of a burn wound

The skin plays a major role as a protective barrier to infection but is also extremely important in fluid homeostasis, thermoregulation and various immunological and metabolic functions. Thermal injury dismantles this protective function and causes a rapid degree of cellular damage. The process which leads to burn eschar begins with exposure to high temperature, stimulating increasing molecular collisions, alteration

and disruption of intramolecular bonds causing protein denaturation, liberation of oxygen radicals, disruption of cell membrane integrity and eventual cell death (7, 8). The depth of the burn is based on the intensity and duration of heat exposure and can be classified into three main degrees as shown in figure 1.1, each with an increasing predisposition to infection (9). Superficial or first degree burn only affects the outermost epidermal layer and is characterised by a painful erythematous appearance without blistering. Partial thickness or second degree burns can be divided into two subgroups; superficial and deep partial thickness. Superficial partial thickness affects the papillary dermis illustrated by a weeping moist texture and blistering with minimal scarring, whereas the deep partial thickness burns affect the reticular dermis usually causing a loss of 50% of the dermis (10). Common features of these burns are white dry texture with minimal pain as nerve fibres have been destroyed, with healing taking up to 6 weeks if no complications occur. It is these types of burns that are most associated with scald related injury (9). Full thickness or third degree burns penetrate the skin into the subcutaneous fat and sometimes deeper. They have a waxy leathery appearance, with no bleeding due to the thrombosed vessels and are at the highest risk of infection. As the body tries to maintain homeostasis after burn injury, the clinical picture of necrotic coagulation unveils.

Three distinct zones are associated with burn wounds as described by Jackson *et al* (11). The zone of coagulation is at the centre focus of the injury nearest the heat source, consisting of devitalised tissue which forms the burn eschar. The zone of stasis represents the tissue adjacent to the necrotic area and is still viable but at risk of irreversible death because of ongoing ischemia due to decreased perfusion, while the zone of hyperaemia is the most peripheral zone which consists of normal skin with minimal structural damage, and increased blood flow due to the injury.

First Degree Burn   **Second Degree Burn**   **Third Degree Burn**
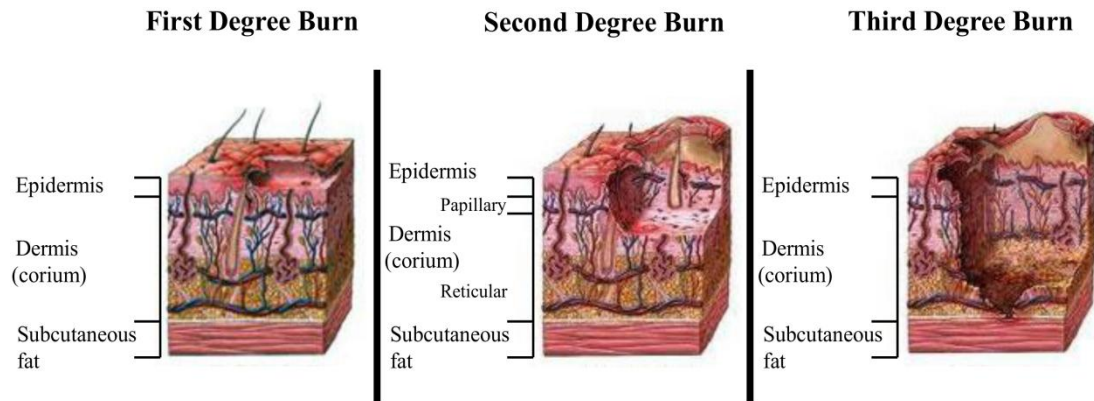
Figure 1.1: Classification of burn wounds: First degree burns are localised to the epidermis layer of the skin, causing a painful pink to red appearance with no blister formation. Second degree burns affect either the superficial dermis layer causing severe pain and blistering or the deeper reticular layer which generally results in scarring. Third degree burns result in charring and total destruction of the epidermis/dermis layers and can penetrate to the subcutaneous layer and deeper. Image modified from http://hospitals.unm.edu/burn/classification.shtml.

1.1.2 Systemic response to burn injury

Thermal injury causes systemic pathophysiological changes, resulting in clinical manifestations such as shock, respiratory and renal failure, hypermetabolism and catabolism, intestinal alterations and immunosupression, in which the latter two directly predispose the patient to infection (12-15). Like the skin, the gastrointestinal epithelium provides a mucosal barrier against infection. Following thermal injury, gastrointestinal motility and absorption is impaired (16, 17). Gastroduodenoscopy on patients suffering from burns revealed a high degree of stomach and duodenum lining erosion (18). Bacterial translocation and macromolecular leakage is also evident after burn injury, resulting in the dissemination of endogenous gastrointestinal flora (19-22). Intestinal ischemia triggers oxidative stress, generated from monocyte derived molecular mediators, culminating in the production of hydrogen peroxide (H_2O_2) and nitric oxide (NO) which are toxic to enterocytes, resulting in the observed increase in gut permeability (23, 24).

The immune system responds to trauma by rapidly expressing proinflammatory cytokines and other factors important in acute inflammation.

However, this proinflammatory cascade requires an equally important anti-inflammatory response to compensate and prevent further injury. Many of the chemical mediators involved in this compensatory response are highly immunosuppressive (25, 26), which can lead to the patient being colonised by opportunistic organisms (27, 28).

Directly after a burn injury a proinflammatory response is generated, characterised by increased serum levels of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor α (TNFα) (29). Both of this cytokines stimulate the expression of prostaglandin E_2 (PGE_2) and IL-6 from endothelial cells and macrophages (30), and as with IL-1β and TNFα, IL-6 contributes to the activation of T cells (31). Interferon gamma (IFN-γ), another proinflammatory cytokine, is produced by natural killer (NK) cells and Th-1 cells in response to burn trauma and is important in macrophage activation and CD4^{+} T cell differentiation into Th-1 cells (32).

Early immune suppression is evident 3 days after burn injury in patients with burns greater than 30% total body surface area (TBSA) (9, 33). This is mediated by an opposing set of cytokines which impact the adaptive immune system, particularly the T cell population. Biochemical alterations influencing the expression of steroid hormones and catecholamines inhibit the production of proinflammatory but not anti-inflammatory mediators (34, 35). Macrophages increase production of PGE_2 and decrease expression of IL-12, causing an inhibition of Th-1 but not Th-2 cell proliferation and function (14, 36). The differentiation of T helper cell population from Th-1 to Th-2 cells reduces cell-mediated immunity and leads to an increase in anti-inflammatory cytokines such as IL-4 and IL-10 (37). Increased IL-4 and IL-10 inhibit Th-1 cell activation, decrease production of proinflammatory cytokines and interfere with bactericidal and fungicidal activity (38-40). Increased PGE_2 can also lead to the reduction of IL-1β and inhibit the activity of NK cells (41). Neutrophil dysfunction, in terms of chemotaxis and intracellular killing impairment, is also evident following major burn injury (42, 43). Macrophages also display reduced

phagocytosis and major histocompatibility complex class II (MHC II) expression and antigen presentation following severe thermal injury (44-46).

1.1.3 Epidemiology of burn wound infections

Although the burn wound surface is initially sterile immediately after thermal injury, there is a temporal colonisation of the wound with microorganisms originating from either nosocomial transmission from contaminated healthcare workers, fomites or from the patient's own endogenous flora (47, 48). In the last three decades there has been a change in burn management from controlling bacterial growth on the wound surface, allowing for breakdown of burn eschar coupled with debridement and daily immersion hydrotherapy (conservative exposure) to early excision and closure of the wound and shower therapy (49). However, only two randomized, controlled trials of early excision *vs* controlled exposure have been published with neither illustrating a significant reduction in burn wound infection in burn wounds >15% TBSA (50, 51). Importantly, this change in burn wound care has altered the epidemiology of burn wound infections (49), therefore epidemiological studies from the era of conservative exposure therapy has largely been omitted from this thesis.

Exposed burned tissue is susceptible to colonisation from microorganisms typical of gastrointestinal and upper respiratory tract flora (52), but following initial thermal injury, Gram-positive bacteria are first to colonise the wound (53, 54). Coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* are the most common microorganism found in burn wounds, particularly small TBSA burns (33, 41, 47-49, 55-57). A recent study investigating the change in microbial flora in burn wounds during treatment found that CoNS (63%) and *S. aureus* (19.7%) were most prevalent upon admission, but a gradual decrease in CoNS isolates (34.7%) and an increase in *S. aureus* (37.6%) and *P. aeruginosa* (16.2%) isolates was observed at day 21 (48). Interestingly, antibiotic resistance in *S. aureus* increased with increasing hospital stay in the burns patient, while MRSA nasal carriage increased from 3.9% on day 1 to 62.7% at day 21 (48), consistent with other studies (58-60).

Gram-positive cocci are gradually replaced with gram-negative bacilli, which are hypothesised to have a greater propensity to invade and are associated with larger burn areas (33, 56, 61). Of the Gram-negative organism, the *Pseudomonas* species, particularly *P. aeruginosa*, are the most frequent colonisers [48, 55, 56, 59, 65-67], with *P. aeruginosa* associated with high mortality in burn patients (62). In a similar fashion to *S. aureus*, *P. aeruginosa* also exhibited decreasing susceptibilities to various antibiotics with increasing hospital stay in the burns patient (57). *P. aeruginosa* is inherently resistance to antibiotics due to constitutive expression of β-lactamases and efflux pumps, coupled with the possession of a low permeable outer membrane (63-65). *P. aeruginosa* also has a remarkable capacity to acquire resistance to various classes of antibiotics, and has been shown to contain practically all known mechanisms of antibiotic resistance (66).

Different geographical locations have a different prevalence and spectrum of organisms that are capable of causing burn wound infections. For example, *Enterococcus* species play an important role in burn wound infections in the US, accounting for up to 12% (67), but were significantly less prevalent (3%) in a Brazilian study (68). A recent review of 104 US burn units found that *P. aeruginosa* is the most frequent pathogen among Gram-negatives at 44%, followed by *Acinetobacter baumannii* and *Enterococcus spp* (69). In China, *A. baumannii* and *Proteus mirabilis* are responsible for the most burn infections (70). Different prevalence rates have also been shown for *Acinetobacter* spp (71), *Candida albicans* (72) and various gram-negatives including *Klebsiella pneumonia* and *Escherichia coli* (73), however, in the vast majority of burn hospitalisations, *S. aureus* and *P. aeruginosa* are the earliest and most frequent colonisers of burn wounds.

1.1.4 Pathogenesis of burn wound

The susceptibility of burns wounds to infection is the result of a combination of factors including the disruption of the skin barrier, depression of innate and adaptive immunity and an environment rich in nutrients in the form of coagulated proteins, exudate and necrotic tissue (30, 56). Incidence of ventilator associated pneumonia,

primary blood stream and wound infection are all higher in the burn patient than other intensive care unit (ICU) patients (73).

Frequently, after colonisation of the burn wound, microorganisms can penetrate the eschar and invade the underlining unburned subcutaneous tissues. *S. aureus* is well equipped to achieve this and after invasion can form abscesses with thick walls that further obstruct antibiotic therapy and an already weakened host defence, which can lead to the haematogenous dissemination of the infection (56). *P. aeruginosa* has a preference for warm, moist environments, and is well adapted to colonise the burn patients. Both of these major burn pathogens expresses and secrete a plethora of virulence factors which are fundamental in burn wound colonisation and infection.

A great deal of information on the specific virulence factors which *P. aeruginosa* utilises in burn infection has been documented (74-78). Virulence factor expression in *P. aeruginosa* is coordinated through an elegant hierarchy cell density quorum sensing system, involving the *lasRI*, *rhlRI*, quinolone signalling system and multiple accessory factors that alter these regulatory circuits at the transcriptional, post-transcriptional and translational level (79-85). It has been shown previously that the *lasRI* and *rhlRI* systems are extremely important in initial spread from the inoculation site in a burn wound and for dissemination throughout the body (75), highlighting the role of virulence factors in burn wound pathogenesis.

P. aeruginosa expresses two surface appendages, a single flagellum and type VI pili, that are involved in multiple roles including adhesion, locomotion, biofilm formation, natural transformation, DNA binding and invasion (86-89). Mutants deficient in either of these structures have reduced virulence in a mouse burn model, displaying reduced persistence at the wound site and lower dissemination to target organs (74). Rhamnolipids are glycolipids that have bio-surfactant properties and are important in motility and colonisation of surfaces, and are likely play a role in burn wound colonisation (90, 91) (see section 4.2 for additional information). Efficient dissemination in the burn wound also requires the production of elastases and other

proteases, which are present in both *P. aeruginosa* and *S. aureus* (76). The importance of elastase in burn wounds is governed by its ability to degrade collagen and non-collagen host proteins and disrupt the integrity of the basement membrane, providing an avenue for dissemination (77).

Direct evidence for the production of the *P. aeruginosa* ADP-ribosylating toxin, exotoxin A, in a burn wound mouse model has been shown (78). Exotoxin A catalyses the transfer of the ADP-ribose molecule from nicotinamide adenine dinucleotide (NAD^+) to a residue on elongation factor 2 (EF-2), inactivating this protein and subsequently inhibiting protein synthesis in the target cell (92). Exotoxin A causes cell death and necrosis at the site of colonisation (93), and is continuously expressed for up to 35 h after infection (78), underling the possibility that other toxins are also continuously produced at this rate. Additional *P. aeruginosa* virulence factors that have been reported to be involved in burn wound infection include the phospholipase C enzyme, which targets phospholipid membranes (94), and the effector molecules of the type III secretion system, namely exoenzyme S (95), another ADP-ribosylating toxin.

Detailed experiments identifying specific virulence factor involvement in *S. aureus* burn wound infection are lacking. However, *S. aureus* expresses a multitude of virulence determinants which are likely to be important in burn wound pathogenesis. These factors can be divided into three main virulence categories: adhesins, immune evasins and toxins (96, 97). *S. aureus* is well equipped to adhere to the host, utilising a diverse array of surface adhesins to interact with a myriad of extracellular matrix proteins and cell types (98). *S. aureus* also employs multiple factors which can interact and impede the various arms of the immune system (99) and evolved to expresses a plethora of membrane damaging toxins and enzymes, designed to destroy immune cells and host tissue (100). [The *S. aureus* virulence factors are reviewed in more detail in section 1.3.5].

1.1.5 Biofilm and burns

Biofilms exist as a community of organisms attached to a surface, surrounded by a polymeric matrix and are associated with persistence and antibiotic resistance (101-103). Both *S. aureus* and *P. aeruginosa* are capable of forming single and multispecies biofilms, in a wide range of artificial and natural environments (101). Generally, biofilms are associated with foreign device-related and chronic infections but recent evidence suggests that biofilm formation plays an active role in burn wound pathogenesis (102-104). Biofilm development occurs through three key stages: attachment, maturation and detachment (Fig 1.2). This classification is further subdivided into (I) initial attachment, (II) irreversible attachment, following the production of extracellular polymeric substances (EPS) or exopolysaccharide matrix, (III) early construction of biofilm architecture, (IV) biofilm maturation and (V) cell dispersion or detachment (Fig 1.2) (101, 105). The environmental cues which trigger initial biofilm formation include an abundance of nutrients, oxygen availability and osmotically balanced media (106, 107).
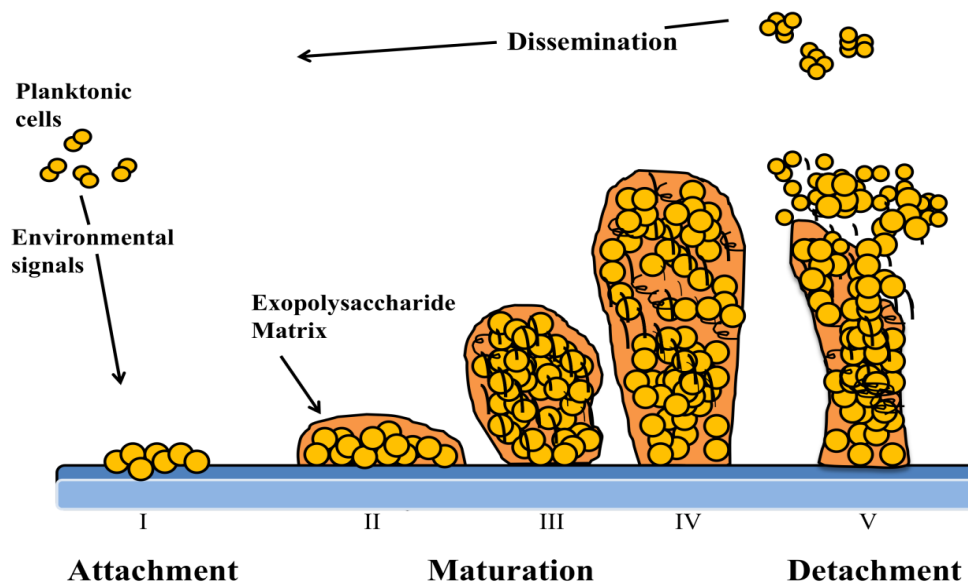
Figure 1.2: Biofilm development: Following specific environmental signals, planktonic bacteria begin to adhere to abiotic or biotic surfaces in the (I) initial attachment stage of biofilm formation. Biofilm development moves into the (II) irreversible adherence stage after the production of an exopolysaccharide matrix. Biofilms mature in stages III and IV and finally disperse or detach (V) following specific signals, leading to the dissemination of single cells with the potential to initiate new biofilm colonies.

In *P. aeruginosa* biofilms, initial attachment is largely governed by the adhesins and surface appendages such as flagella and type IV pili, which allow independent movements of twitching and gliding (108). Alteration in the O-polysaccharide of lipopolysaccharides (LPS) can alter the adherence properties, where a loss in the A-band results in reduced attachment to hydrophilic surfaces and increased adherence to hydrophobic surfaces, which may be important depending on the surface for initial biofilm attachment (109). Following the initial reversible interaction, *P. aeruginosa* commences the production of alginate and other polysaccharides (EPS) initiating the transfer into early maturation stage (110). Upon contact, flagellar synthesis is down regulated and alginate synthesis increased, suggesting a surface-mediated alteration in gene expression (111). EPS maintains biofilm architecture and protects against shear forces in fluid environments acting as a 'glue' keeping cells together (112). Alginate appears to protect the biofilm from the reactive oxygen species released from inflammatory cells and prevents phagocytosis from immune cells (113, 114). The Psl exoploysaccharide (Polysaccharide synthesis locus) is required for binding to a substratum, cell-cell interactions and maturation and maintenance of the biofilm architecture (112, 115). Another polysaccharide, Pel, was shown to be important in biofilm initiation only in non-piliated mutants, highlighting a role for both Pel and type IV pili in initial attachment (116). In addition to exopolysaccharides, extracellular DNA (eDNA) is an important factor of the matrix in early biofilms (117). The liberation of eDNA and the regulation of *pel* biosynthetic genes involved both the *las* and *rhl* quorum sensing systems, highlighting their respective roles in biofilm formation and maturation (118).

Rhamnolipids play major roles in *P. aeruginosa* biofilm maturation, maintenance and detachment (119-121). These amphipathic glycolipids have been shown to be involved in construction of channels and interstitial voids allowing a type of circulatory system to exist within the biofilm, supplying a critical avenue for efficient nutrient supply and waste removal (120). Rhamnolipids are hypothesized to be central in the development of mushroom cap formation (119) and directly involved in biofilm detachment (121).

S. aureus is also an excellent biofilm former and utilises similar gene products as *P. aeruginosa* in the construction, maintenance and dispersion of biofilms. *S. aureus* expresses a plethora of surface proteins which have the capacity to bind and interact with multiple matrix proteins (122) and are intimately involved in the initial attachment phase of staphylococcal biofilm formation (123). Although the composition of EPS or matrix of *S. aureus* differs from that of *P. aeruginosa*, its function is largely the same; important in intracellular aggregation, protection and construction of fluid-filled and mushroom shaped cell towers.

The primary molecule responsible for intracellular adhesion is the polysaccharide intercellular adhesin (PIA) (124), which functions by providing charge differences permitting electrostatic interactions between the teichoic acid-mediated negatively charged bacterial cells (125). However, PIA-independent biofilms do exist (126), and in these cases the adhesive function of PIA appears to be substituted by adhesive proteins, primarily the accumulation-associated protein (Aap) and fibronectin binding proteins (FnBPs) (127). Keeping with the importance of preserving the biofilms ability to distribute nutrients and remove waste products, *S. aureus* employs quorum sensing dependent, surfactant peptides, known as Phenol-soluble modulins (PSMs) (reviewed in section 1.3.5.2.3), to structure biofilms (128). Similar to the importance of rhamnolipids in *P. aeruginosa* biofilms, these PSM peptides are also multi-functional and are employed in an effective method for biofilm detachment and dissemination (128).

The presence of biofilms in burns has only been recently established (102-104). Biofilms have been demonstrated to form in burn wounds of thermally injured mice (102, 129). In these studies, biofilms began to form around blood vessels and adipose cells within 8 h of infection. Under these conditions, *P. aeruginosa* strain PAO1 replicates rapidly within the burn tissue increasing from 10^2 CFU to 10^9 CFU in under 24 h (75). A recent study investigating the presence of bacterial biofilm from human burn wound biopsies observed increased bacterial colonies surrounding ulcerated and surgical site lesions, which were largely accompanied by necrotic material and wound exudates within 7 days post-injury (103). These biofilms were of

mixed species and evidence of glycocalyx and EPS were observed (103). These results demonstrate that biofilm formation does occur in burn wounds which may have implications for the future development of wound dressings.

1.1.6 Current and 'smart' burn wound dressings

Wound healing progresses through overlapping stages involving a variety of cellular and biochemical processes which act together to re-establish tissue integrity (130). New advances in burn wound technology have centred on achieving an ideal wound condition: moist, warm environment conducive to growth and tissue regeneration while preventing microbial infection.

The prevention of microbial colonisation and subsequent infection is a key criterion of burn wound dressings. Silver (Ag) has very broad microbicidal activity, being highly reactive in its charged state, Ag^+, binding with high efficiency to negatively charged particles. This leads to direct killing on contact due to interference with electron transport and cellular respiration, denaturation of proteins and DNA and destabilising membrane permeability (131-133). Nanocrystalline silver (Ag^0), which is actively released from Aticoat™ wound dressings, display impressive antibacterial activity and protective covering of skin grafts, greater than that observed using silver nitrate or silver sulfadiazine (132). This greater activity has been attributed to the use of non-charged Ag, causing a less reactivity and sustained release of Ag into the wound rather than a mass active event which is short lived (131). Unfortunately, silver, even in the form demonstrated in Aticoat™ dressings, have been shown to be toxic to skin cells, fibroblasts and keratinocytes (134). Poon *et al* further warned against the use of Ag containing dressing in burn wounds where rapidly proliferating kerationcytes are exposed (134). Dressings which continuously expose antimicrobials to the burn wound may also increase the rate at which microorganism acquire resistance, leading to further complications, increasing hospitalization time and healthcare costs (135).

Occlusive dressings are an important element in modern burn management and highlight the importance of autolysis in burn care. These types of dressing provide a barrier to the external environment, allowing the body's phagocytic processes to natural debride and shape the wound (136). Hydrocolloid dressings are among the most important and widely used occlusive dressings (137). These dressings are composed of a gel forming colloidal agent combined with either adhesives or with other materials such as alginates. They are used frequently in burn care due their impermeability properties, flexibility and ease of removal, an important feature particularly in paediatric burn care (5, 137). Unfortunately, most burn wounds produces heavy exudate causing hydrocolloid dressing to swell, dislodge and fall apart. These dressings also require frequent changing, every 2-5 days, which can strip immature epithelial layers (136, 137).

In recent years, biological dressings or bioengineered skin substitutes have been introduced with great success in the management of superficial and deep partial thickness burns (138). These dressing have the advantage of containing biodegradable components, such as collagen or elastin, which play an active part of the natural tissue extracellular matrix and regeneration. One example of a biological dressing widely used in the United Kingdom is registered as Biobrane™. This dressing is composed of a nylon mesh attached to a silicone membrane bonded with purified porcine derived dermal collagen (139). Although Biobrane™ and other similar biological dressing have shown improved wound adherence, flexibility, and healing in burn wounds (140, 141) their failure rate can be attributed to wound infection, as they have no means to sense or tackle infection (142).

Currently the only methods used to investigate burn wound infection are by analysing surface swabs and tissue biopsy specimens (143, 144). This requires direct wound assessment and removal of adherent dressing. These methods are slow, cause discomfort to the patient, are costly and can increase the risk of life-long scarring and further infection (5). Assessment of the clinical condition of the patient is not sufficient as a sole method to evaluate infection, as the clinical signs of infection, particularly in paediatric patients, are non-specific (5, 145). At present time there is a

need for technology which can be used in combination with biological dressings which can sense the presence of pathogenic bacteria in the wound environment. The core principle behind the development of a 'smart' dressing, which will be discussed in this thesis, is the use of a colorimetric sensor device which responds to the presence of bacteria in the burn wound. The sensor acts as a molecular mimic of the plasma membrane of eukaryotic cells and responds to the action of bacterial secreted virulence factors, which have been shown to play a key role during burn wound pathogenesis.

1.2 Using phospholipid vesicles as biosensors

Phospholipid molecules, in partnership with proteins and nucleic acids, represent the essential factors for all living matter. Phospholipid molecules are amphiphilic, containing a polar, water-soluble, hydrophilic head group attached to a non-polar, water-insoluble, hydrophobic hydrocarbon or fatty acid chain(s). Glycerolphospholipids are phospholipids that contain two acyl chains, which can be saturated or unsaturated, linked to a headgroup by means of a glycerol backbone (Fig 1.3). These molecules are a major component in all cytoplasmic membranes as well as phospholipid vesicles (vesicles).

The vesicles used in this study are artificially formulated, spherical, self-enclosed structures, composed of lipid bilayers. An important feature of this structure is the ability to encapsulate the surrounding solvent within the aqueous cavity (Fig 1.3). Vesicles vary in size from 20 nm to several micrometers, and can be generated containing one (unilamellar vesicle) or more (multilamellar vesicle) lipid bilayers, each spanning a thickness of approximately 4 nm, surrounding an equal number of aqueous spaces. The bilayers are usually composed of natural or synthetic phospholipids and cholesterol, but incorporation of other sterols, fatty acids, glycolipids proteins and other membrane factors is also possible (146).

Unilamellar vesicles make fantastic model systems for investigating the dynamics and structural features of many cellular processes including viral interactions with membranes (147) and endocytic features (148) and are used extensively in investigating toxin-membrane interactions (149-153). In addition to being important models in understanding fundamental molecular research, vesicles have been developed as vehicles for drug delivery (154) and gene transfer (155). Vesicles are very attractive structures to use in medical therapy because they are biocompatible, biodegradable, and those composed of natural phospholipids, are biologically inert and weakly immunogenic (154).
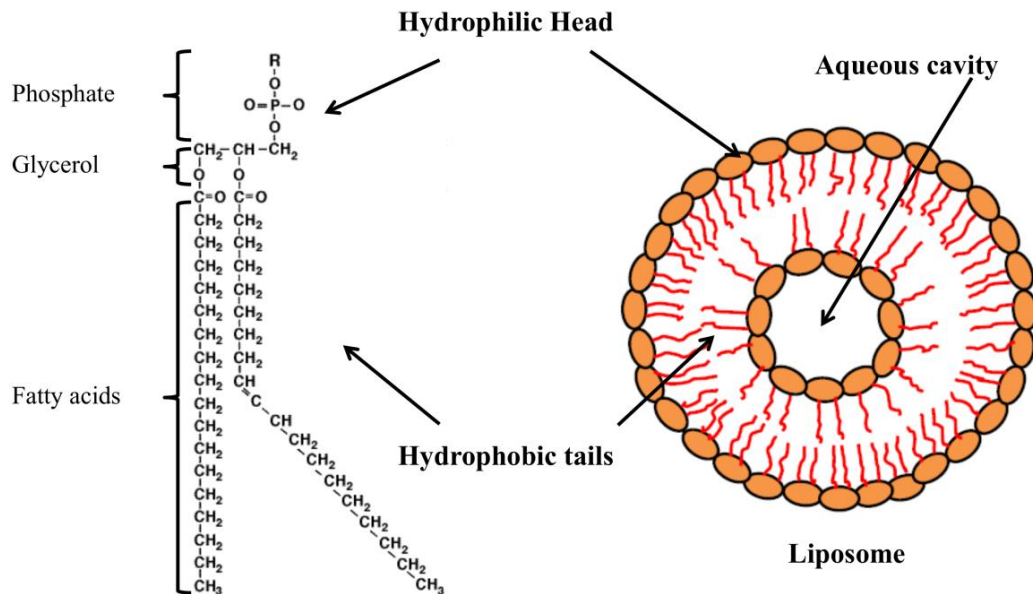
Figure 1.3: Representation of phospholipid structure and liposome. Phospholipid molecules consist of a hydrophilic phosphate head group and a hydrophobic fatty acid tail. Liposomes care spherical, self-closed structures consisting of phospholipid bilayers, which can encapsulate the surrounding environment into their interior.

1.2.1 Self-assembly

As previously stated phospholipids are amphiphiles and therefore spontaneously aggregate into a variety of different structures based on their dual solvent preference. The hydrophobic effect dictates this self-assembly, by driving the organisation of amphiphiles (156). Both ends of the phospholipid molecule imposed a thermodynamic preference which is satisfied by self-association, with the fatty acid chains in the middle, avoiding the aqueous solvent and the polar head groups at the surface as depicted in figure 1.3. This self-organisation is usually accompanied by an increase in the entropy of the system. This arises as the polar/non-polar interactions force the water molecules into an ordered state around the fatty acid chains (156). Movement of the hydrophobic parts out of the aqueous solution releases the ordered water molecules and sequesters the hydrophobic elements within the interior of the structure. This is hypothesised to lead to an overall gain in free energy, permitting spontaneous aggregation.

1.2.2 Lipid polymorphism and aggregate structure

The spontaneous aggregation of amphiphilic lipids in aqueous solution can result in the formation of many structures. Although the hydrophobic effect is important in aggregation, other parameters of the amphiphile, most notably the shape of the individual molecules and the concentration, play an important role in the formation of these structures (157). The shape of the lipid aggregates is determined by the surfactant packing parameter, S, (157):

$$S = v/al$$

where v is the volume of the hydrophobic portion of the amphiphile, l is the length of the hydrocarbon chains and a is the area of the polar head group. The relative sizes of the head group and hydrocarbon chains will dictate the way in which packaging into different geometrical aggregates occurs. By convention, if the curvature of an aggregate is around the hydrophobic portion it is considered positive, whereas if it is around the polar portion it is considered negative (158). The positive curvature forms normal aggregates and phases, whereas the latter forms reversed ones. If the surfactant packaging parameter (S) is <1/3, only spherical micelles are formed in solution. If S =1 a balance between the sizes of the polar head groups and hydrophobic tails exist, forming planar aggregates with a bilayer structure. When S >1 the surfactant form reversed micelle structures (Fig 1.4).

One of the major components of the vesicles used throughout this thesis is 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). This lipid, like most bilayer-forming lipids, has a shape parameter of approximately 1, which means it has a cylindrical shape and tends to form bilayers. As lipid concentration increases, these molecules generally form lamellar phases where planar lipid bilayers alternate with water layer (Fig 1.4). Aggregate formation in different phases depends on the free energy per lipid molecule. This differs in lamellar and hexagonal phases, in which the molecule occupies different volumes. This shape dependent free energy involves the fatty acid energies, bending of the lipid monolayers, hydration and electrostatic

potentials (158). In the hexagonal II phase (H_{II}) water molecules are sequestered within the cylindrical tubes. If the concentration of water-to-lipid increases, this results in an increase fatty acid packaging free energy. This can lead to an ordering of aggregates relative to each other or a change in the aggregate shape, which happens when monolayers curl and undergo a L_α to H_{II} phase to lower the total free energy (158).
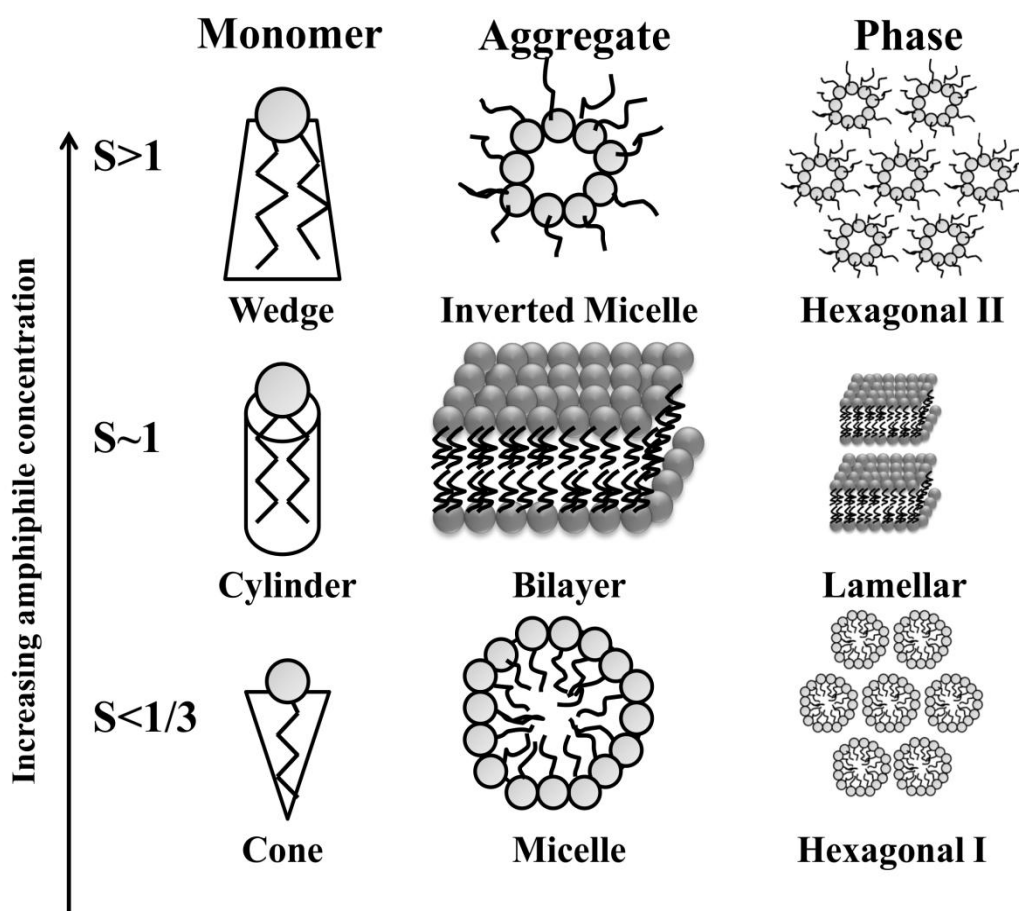
Figure 1.4: The geometrical packaging concept. The general relationship between the lipid molecule and preferred aggregate structure is illustrated, according to the surfactant packaging parameter. When the surfactant packaging parameter, S, is 1, the lipid is roughly cylindrical and can form stable bilayers (lamellar phase) when in aqueous solutions. When S <1/3 lipids tend to organise into micelles in a hexagonal phase I, whereas S>1 tends to cause lipid to form inverted micelles (Hexagonal phase II).

1.2.3 Lamellar Phases

Lipid bilayers are dynamic structures which can exist in many different physical states (159). Phase behaviour is defined as the relative fluidity of the lipid bilayer with respect to changes in lipid composition and temperature (160). Typically, there are two major phases: the lamellar liquid-crystalline or liquid phase (L_α) and the lamellar gel or solid phase (L_β). In the liquid phase, molecules freely diffuse within the two dimensional plane, rapidly exchanging position with neighbouring molecules in a process known as lipid 'flip-flop' or transbilayer lipid motion (161). Lipids in the gel phase are fixed in place and exhibit limited mobility. The attractive interactions between individual adjacent molecules are important in determining the phase behaviour. The length of the fatty acid chains permits more interactions between adjacent molecules leading to tighter packaging of lipid molecules and therefore less mobility (162). The degree of saturation of hydrocarbon tails also affects the phase behaviour, with the addition of double bonds disrupting lipid packaging and increasing mobility and flexibility (162). The melting or transition temperature (T_m) is defined as the temperature where the gel-to-liquid phase transition occurs and is a function of the chemical composition of the bilayer, particularly the acyl chains.

1.2.4 Vesicle formation

Vesicles are self-closed bilayer structures which can encapsulated the solution from which they are formed in. Bilayer edges tend to seal when they are mechanically dispersed in aqueous suspension, due to their amphipathicity, the hydrophobic effect and the assembly of hydrocarbon chains away from water molecules. When planar bilayers are dispersed in a hydrophilic environment, bending curvature minimises the high surface tension at the rim of the bilayer sheet. A thermodynamically favourable structure results when an entropically driven encapsulation of hydrocarbon chains into spherical vesicles occurs (156, 158, 163). Lipids which are used to formulate vesicles are generally cylindrical in shape containing more than 11 carbons in their acyl chain (158). Vesicles can be classified on the basis of lamella, either uni- or

multilamellar and size (163). Multilamellar vesicles (MLVs) contain concentric bilayers and are easily prepared by simple shaking of a dried lipid film in aqueous solution (163). Unilamellar vesicles (ULVs) can be sub-divided into two categories, small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) (158, 163). SUVs have diameters of 20-50 nm and are prepared through extensive sonication of MLVs, or through extrusion through polycarbonate filters of defined size. LUVs are vesicles with diameters between 100 nm and 5 μm and are formed in a similar fashion to SUVs, but with an added freeze-thaw step which induces fusion of SUV (164).

Using liposomes as biosensors for bacterial detection requires a vesicle type which exhibits stability in diverse environments, but also sensitive to secreted virulence factors. The vesicles used throughout this thesis contain 16 carbon DPPC and 16 carbon 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) phospholipids. Both of these lipids are saturated, resulting in a higher transition temperature and a more stable vesicle. The incorporation of a polydiacetylene, 10, 12-tricosadiynoic acid (TCDA) is also performed. This lipid is amphiphilic and can self assemble and incorporate into the lipid bilayer following ultra-violet (UV) induced polymerisation, providing stability (165). The addition of cholesterol has major effects of membrane fluidity causing the vesicle membrane to transition from a liquid-disordered to liquid-ordered state (166). Cholesterol influences the rigidity of the membrane controlling permeability and will affect the incorporation of bacterial toxins and the sensitivity of these vesicles. Overall the liposome recipe, as depicted in section 2.2 and highlighted above, was chosen to satisfy the two biophysical parameters of stability and sensitivity.

Carboxylfluorescein (CF) is a fluorescent molecule that is self-quenchable at high concentrations due to the formation of non-fluorescent dimer molecules at concentrations above 0.2 M (167). This trait allows this molecule to be used as a sensor dye, only fluorescing when diluted, in cases such as liposome burst. Additionally this molecule is an attractive candidate to use in such biosensors as it is cheap and relatively non-toxic at concentrations used in this thesis (168).

1.3 *Staphylococcus aureus*

*S*taphylococcus aureus was identified as a causative agent of disease in 1882, when Sir Alexander Ogston highlighted its role in abscess formation and sepsis (169). In the past century our knowledge of *S. aureus* in areas of epidemiology, physiology and pathogenicity, has increase exponentially, however this fantastically equipped organism remains a global threat to human health.

S. aureus is a Gram-positive, facultative anaerobic, coccal bacterium, and is a common commensal of mammals. This bacterium is non-flagellated, but does have the ability to spread on soft agar at temperatures between 30-43°C, a method of mobility dependent on the expression of teichoic acids and surfactant molecules such as the PSM peptides (170, 171). Exactly what advantage this colony spreading mechanism of mobility confers to bacteria is unknown, but it may aid effective colonisation over surfaces such as host tissue or inanimate objects such as catheters. Macroscopically, this bacterium generally grows with a golden colour due to the expression of the carotenoid pigment staphyloxanthin, which plays a role in resistance against hydrogen peroxide and hydroxyl radicals, compounds important in neutrophil killing (172). This carotenoid molecule acts as an antioxidant, sequestering oxygen free radicals through its conjugated double bonds (172, 173). Microscopically, *S. aureus* presents as clusters, as cell division occurs in three alternating perpendicular planes resulting in sister cells remaining attached after division, causing the irregular clumping phenotype (174). In the environment, *S. aureus* can withstand harsh conditions and is extremely versatile, capable of growing in a wide range of temperatures, from 7 to 48.5°C (175), pH between 4.0 and 10.0 and sodium chloride up to 3 M (176).

1.3.1 Genome and evolution of *S. aureus*

The genomes of *S. aureus* consist of a single circular chromosome which range in size from 2.7 to 3.1 Mb (177, 178). With the success of next generation sequencing,

a plethora of *S. aureus* strains have now been sequenced, allowing comparative genetics to identify conserved and variable features of the genome.

1.3.1.1 Core genome

The *S. aureus* genome can be divided into two parts; the core and accessory genomes. Approximately 75% of the genome is core, defined as genes conserved in all strains (179). Unsurprisingly, the majority of these genes are required for essential functions, central in metabolism and survival (179). However, genes involved in virulence are also contain within these genetic blocks, coding for surface and capsule proteins and as well as adhesins, toxins and enzymes (179).

At first instance it would seem that genes within these region do not play major roles in the diversity observed between *S. aureus* lineages, however, subtle minute changes in genes can have drastic effects on gene function and overall phenotypes of strains (177). Isolates from the same clonal complex or lineage have very conserved core genomes while isolates from different lineages can have major differences with variability in hundreds of genes (179). Genetic diversity can arise due to single base-pair changes (single nucleotide polymorphisms SNPs), insertion or deletion (InDels) of a single or run of bases pairs within genes or intergenic regions, or by large scale rearrangements observed through blocks of divergent sequence due to recombination (177, 180). Most of the observed diversity within the core genome is a result of SNPs, which can result in either a synonymous or non-synonymous mutation. A non-synonymous mutation results in a change in amino acid and may result in altered gene and/or protein expression. Synonymous mutations can be regarded as 'silent' mutations as codon change does not result in amino acid change, however a codon bias may result. This codon usage alteration can become substantial if it affects the efficiency of translation of essential proteins (181).

Recombination in the core genome can drastically influence bacterial physiology. Genetic information up to several kilobase pairs can be exchanged through homologous recombination, evident within the variable region between *agrB*

and *agrC* in the accessory gene regulator operon (182). This recombination event is responsible for the four different auto-inducing peptide (AIP) groups which define Agr activity (183), enabling cross activation or inhibition of other groups (184).

1.3.1.2 Accessory Genome

The variable or accessory component of the *S. aureus* genome consists of non-essential genes involved in virulence, substrate utilisation, antibiotic and heavy metal resistance and other metabolic functions (177, 185). Horizontal gene transfer (HGT) plays an important role in shaping the accessory genome, as many of the mobile genetic elements (MGE) which comprise the accessory genome (bacteriophages, staphylococcal pathogenicity islands, plasmids, transposons, and staphylococcal cassette chromosomes) can all be transferred horizontally between bacteria. Therefore, the MGEs are much more varied than the core genome, and can be key factors in driving the evolution of *S. aureus* clones.

Three mechanisms dictate HGT: transformation, conjugation and transduction (186). Until recently, *S. aureus* was regarded as unable to acquire 'free' DNA from the environment by natural transformation. However, the recent identification of a novel sigma (σ) factor, σ^H, has been demonstrated to assist RNA polymerase (RNAP) in the expression of competence genes, the *comG* and *comE* orthologues in *S. aureus* (187). Interestingly, *sigH* expression is cryptic and is not detected under normal laboratory conditions, whereas artificial overexpression can induce competence genes (187). Morikawa *et al* highlighted that σ^H is expressed in a fraction of the population involving chromosomal gene duplication rearrangements which occur spontaneously and at low frequency permitting the expression of a new chimeric *sigH* gene (188). This expression is also under post-transcriptional control, possibly by a small regulatory non-coding RNA, and requires growth in a specific medium (187). Under these conditions the successful transformation of recipient cells was observed through the acquisition by transformation of a Staphylococcal cassette chromosome *mec* (SCC*mec* II) element (188).

Conjugation involves the transfer of genetic material from a recipient to a donor cell, by pili or pores. *S. aureus* does not contain pili, therefore it is hypothesized that conjugation is pore-mediated between cells in close contact, but is not a common mechanism of HGT found in *S. aureus* (177, 189). *tra* (transfer) genes are important in the transfer encoding the necessary machinery for conjugation to occur, and this method of HGT is restricted to conjugate plasmids containing these genes (190) which were found in only 13 of the 243 sequenced *S. aureus* plasmids (191).

The main method of HGT in *S. aureus* is believed to be via transduction, which is the transfer of DNA mediated by bacteriophage. Bacteriophages are viruses which infect bacteria and either incorporate their genetic material into the bacterial chromosome (prophage) or package and deliver 'foreign DNA', derived from the bacterial chromosome or plasmids, in a process known as generalised transduction (177). After infection, phage can be induced to excise, replicate, synthesis new phage particles and lyse the bacterial cell releasing new phage leading to infection of susceptible cells. Lysogenic phage (phage which remain integrated within the host genome), are common in *S. aureus*, with most strains carrying between one and four types (177). Phage genomes are approximately 45 kb in length, which limits the amount of genetic material that can be packaged inside phage heads, prior to infection. Many bacteriophage carry with them virulence genes which code for virulence factors such PVL (192)), enterotoxin A (193), and exfoliative toxin A (194).

S. aureus pathogenicity islands (SaPIs) are related to bacteriophages; they are 14-27 kb in length, contain phage-like repressors, integrases and terminases as well as virulence genes (195). Some of the SaPIs encode superantigens, (SaPI 1 encodes *tst*, *sek* and *seq* (196)) and immune inhibitory proteins, (SaPI5 codes for chemotaxis inhibitory protein (*chp*) (197) and staphylococcal complement inhibitory protein (*scn*) (198)). However, the genes required for transfer (synthesis of capsid heads and tails) are absent. SaPIs are normally repressed and integrated within the bacterial chromosome, but during HGT, helper phages (such as bacteriophage 80α (199))

encode proteins which inactivate SaPI repressor proteins permitting efficient mobilization (195). These helper phage package SaPIs and deliver them to new *S. aureus* strains whereby SaPIs may integrate at specific site, in an analogous fashion to phage (196).

Plasmids are autonomously replicating, circular segments of DNA which encode a variety of genes beneficial to the host, including resistance to antibiotics (pT181 (200), tetracycline resistance in *S. aureus* strain COL), heavy metal resistance (pI258 cadmium resistance in *S. aureus* (201)) and exfoliative toxin B (pETB (202)). Historically, plasmids have been classified based on size and incompatibility (203). A more recent classification system has been developed based on the sequence of replication (*rep*) genes (204). Recent work has reported on the distribution of plasmids, conferring antibiotic resistance and virulence, in *S. aureus* populations, highlighting specific plasmid groups with specific lineages (191). This observation suggests that there are genetic pressures and restrictive barriers to evolution of hyper-resistant, hyper-virulent *S. aureus* strains (191).

Transposons are MGEs which encode a transposase gene, conferring the ability to excise, replicate and integrate into the chromosome or plasmid. Transposons are generally small and encode resistance to antibiotics, for example Tn*554* encodes resistance to erythromycin (205). Transposons can integrate multiple times in the chromosome and also integrate within other MGE such as SCC*mec* or plasmids (206). It is this 'hitch-hiking' on other MGE which is the hypothesised method of how the undergo HGT.

Staphylococcal cassette chromosomes (SCCs) are relatively large DNA fragments of 21-53kb in length that can encode either antibiotic resistance and/or virulence factors. SCCs can be divided into those which contain the *mecA* gene, which confers resistance to the beta-lactam antibiotics (SCC*mec*) and those that do not (non-SCC*mec*). *mecA* encodes that alternative penicillin binding protein 2a (PBP2a) which has a much lower binding affinity to semi-synthetic penicillins (207). Beta-lactam antibiotics work by preventing the crosslinking of peptidoglycan

polymers by inhibiting the transpeptidase action of native PBPs, therefore the introduction of PBP2a circumvents this inhibition. The SCC*mec* elements, in addition to the *mecA* gene, comprise *mecI* and *mecR*, which encode the repressor and signal membrane transducer respectively. Another important feature are site-specific recombinases, known as cassette chromosome recombinases (*ccr*) which catalyse the integration and excision of SCC*mec* elements within a specific attachment site (*attBscc*) at the 3' end of the *S. aureus* chromosome (208). All SCC*mec* elements share certain characteristic: presence of the *mec* and *ccr* gene complexes, integration at a specific site, known as the integration site sequence (ISS) and direct repeats flanking the ISS (209). Currently, there are eight described SCC*mec* types in *S. aureus*, as shown in figure 1.5. Classification of these elements is based on a combination of *ccr* and *mec* gene complexes (209). Presently there are 3 *ccr* genes, *ccrA*, *ccrB* and *ccrC*. The *ccrA* and *ccrB* genes have been grouped into 4 allotypes based on the designation of genes within the same allotype with nucleotide identities of ≥ 85%. There is only one *ccrC* allotype, as all identified variants show nucleotide similarities of ≥ 87%. The *mec* gene complex is composed of *mec*A, the regulatory genes and the commonly associated insertion sequence (IS*431*) downstream of *mec*A. Four *mec* gene complexes exist in *S. aureus*, based on the genetic diversity within the *mecI-mecR1* region. Class A is the prototypical class, with an intact and functioning *mec* gene complex. Classes B, C1 and C2 have truncated *mecR1* genes due to either insertion of IS*1272* within *mecR1* (class B) or IS*431* in *mecR1* (class C1 and C2 differ in the orientation of the insertion sequence). Variants of the *mecA* class also exist, as shown in the type IV variant in figure 1.5., which has a Tn*4001* insertion within the *mecR1* gene. The SCC*mec* also contains a region known as the J or joining region, which consist of non-essential components of the element, but often carry additional antimicrobial resistance factors in the form of integrated plasmids or transposons. For example the pUB110 plasmid integrated within SCC*mec* II elements harbours the *ant4'* gene, encoding resistance to several aminoglycosides (210).
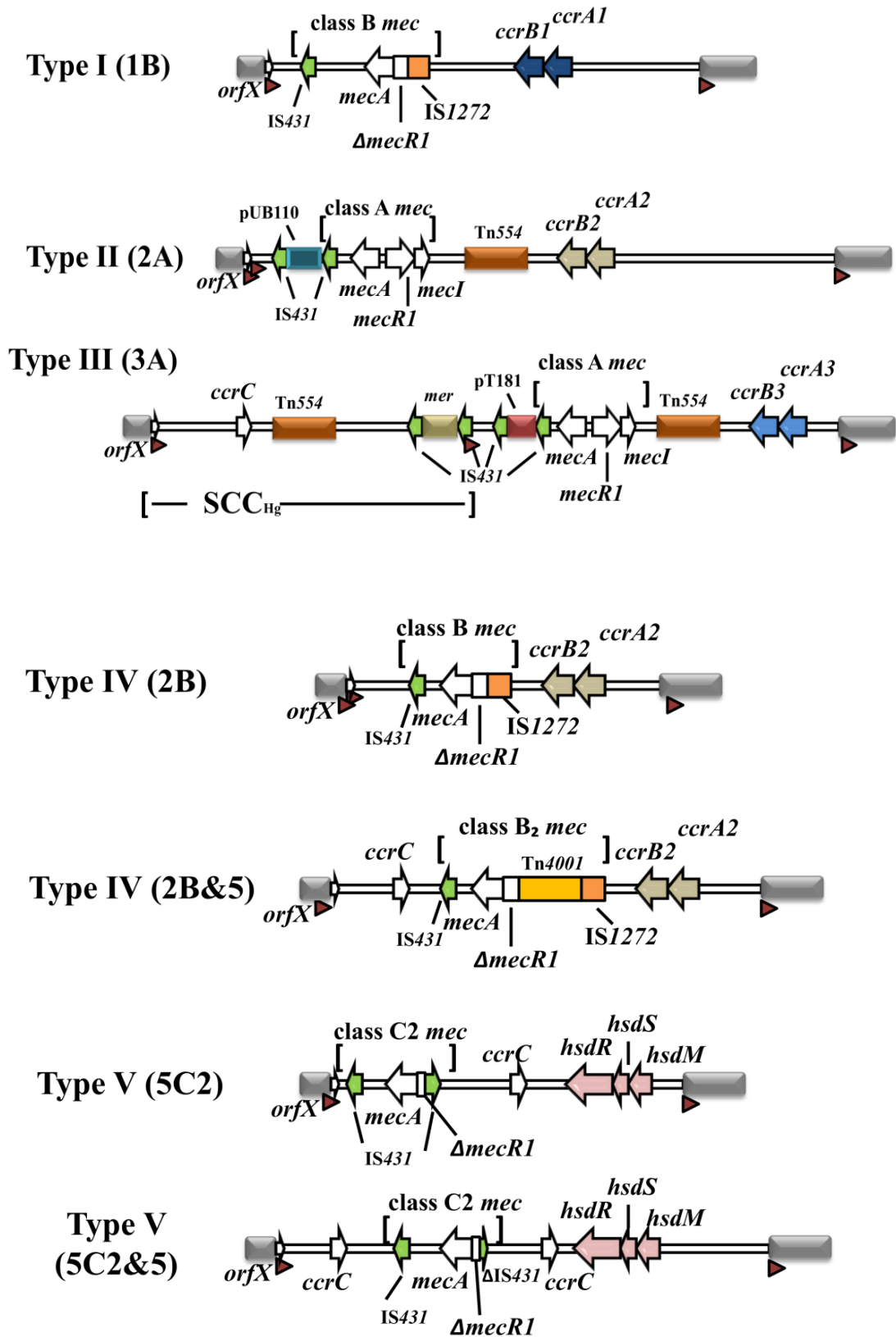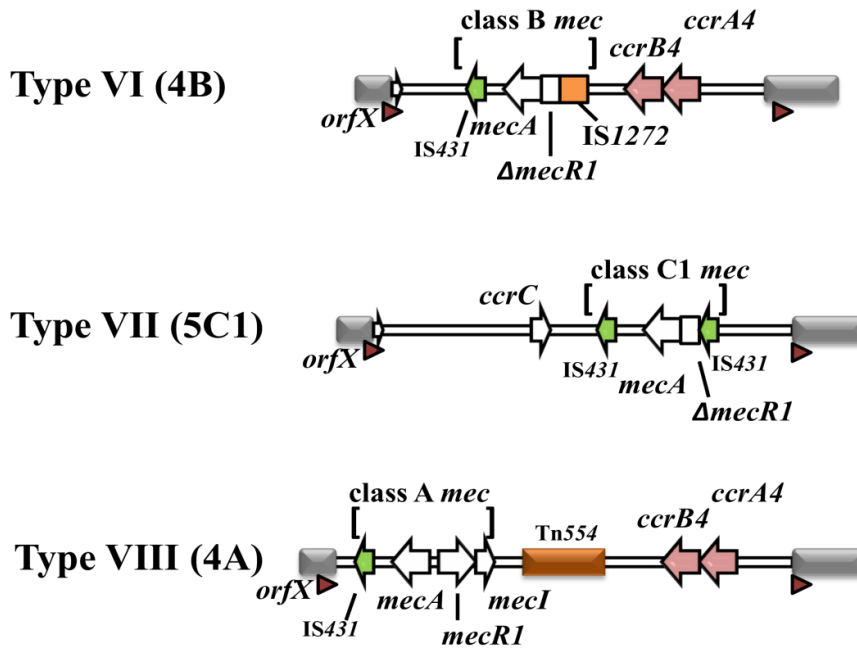
Figure 1.5: Classification of SCC*mec*. The primary structures of the eight SCC*mec* elements are shown. The different *mec* gene complexes and *ccr* groups are highlighted in brackets. Insertion sequences and common accessory genes in the J-regions are also illustrated. The red arrows indicate the integration site sequence. Adapted from (209).

Another genetic component within certain SCC*mec* elements (SCC*mec* II, III and VIII) is the phenol soluble modulin *psm-mec*, the only identified toxin within a mobile antibiotic resistance determinant (211, 212). *psm-mec* appears to have opposing functions; as a transcript it acts as a regulatory RNA inhibiting the translation of the *agrA* gene (213). Introduction of *psm-mec* into CA-MRSA or a MSSA strain decreases production of core genome PSMs (214), as PSMs are positively regulated by direct AgrA binding to *psm* promoter regions (215). Consistent with these observations, deletion of *psm-mec* from certain HA-MRSA strains increases expression of AgrA and core genome PSMs (213), illustrating the inhibitory effect of *psm-mec* in the expression of important virulence factors. The translational product of *psm-mec*, the cytolytic PSM-mec peptide, is active against human neutrophils and it has been shown that *psm-mec* has a positive effect on virulence in a mouse skin and systemic infection models (216). However, this effect appears to be strain dependent, as this positive correlation between virulence and

psm-mec is not observed in all HA-MRSA strains (213, 216). Therefore, this element appears to have both virulence inhibiting and activating properties, based on its transcriptional or translational status. The inhibitory effect of *psm-mec* is partly responsible for the increased virulence observed in CA-MRSA, due to the absence of *psm-mec* on SCC*mec* elements generally associated with CA-MRSA strains (213).

Certain SCC elements encode other factors which benefit the bacterial host in a non-antibiotic resistance fashion. For example, SCC*cap1* contains genes involved in the synthesis of capsular proteins, important in immune evasion (217). SCC*mec* elements spread relatively slowly and the exact mechanism which promotes replication and transfer is not known. It has been suggested that the SCC*mec* IV which spread more easily and frequently, maybe able to package into phage head and undergo transduction due to its smaller size (177). With respect to the larger SCC*mec* elements, the newly described proteins involved in transformation (188) may be an important method of transfer but this is not known as of yet.

1.3.1.3 Identifying related strains – Genotyping

Monitoring *S. aureus* strains is important from an epidemiological, evolutionary and preventative perspective; in light of these requirements, various methods have been designed to investigate the relatedness of *S. aureus* isolates.

Multilocus sequence typing (MLST) is a sequencing-based genotypic method of discriminating between strains based on single nucleotide variants (alleles) of multiple housekeeping genes (218). For *S. aureus*, 450 bp internal fragments of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) are sequenced, and differences in each fragment are assigned different alleles, with the cumulative alleles of the seven genes generating a sequence type (ST) (219). If five or more of the loci are identical, the strains are grouped within the same clonal complex (CC). Mutations within these housekeeping genes accumulate slowly, therefore MLST can be used to measure long periods of evolution with highly reproducible results.

Pulse-field gel electrophoresis (PFGE) is a highly discriminative typing method for *S. aureus* and was considered gold standard for investigating MRSA outbreaks in hospitals and evaluation of recent evolution among lineages. The method relies on the digestion of chromosomal DNA with the restriction enzyme, *Sma*I, and the separation of DNA fragments by agarose gel electrophoresis with an alternating voltage gradient. The resulting pattern of DNA bands are analysed using specialised software and related strains are grouped according to an 80% similarity coefficient (220).

spa typing is a sequence based analysis, which determines the variation within the polymorphic region X of the *spa* gene (encodes protein A) (221). Differences in the sequences of the variable-number tandem repeats allow discrimination between strains. This method is inexpensive, less laborious and time consuming than MLST as only one locus is sequenced. However, *spa* typing can lose its discriminative power which occurs when the same or related *spa* gene occurs in different clonal lineages, possibly through recombination (222).

The methods stated above do not fully discriminate between isolates of the same lineage and therefore do not provide the high resolution required to define specific relationships between strains. Next generation sequencing (NGS) technology has been employed to gain a more precise understanding of the relationship between strains, down to the finest of details. NGS has been used to generate ultra-fine data based on the mapping of SNPs and InDels from whole populations to that of a reference sequence (223). Each whole genome can be tagged individually allowing multiple strains to be sequenced rapidly and efficiently, with SNP and InDel outputs presented highlighting where changes to the reference genome occurred. This method was used in monitor the hospital and intercontinental transmission of a highly transmissible, multi-drug resistant clone of MRSA, ST239 (223). Results from this project illustrate the high discriminative power and superiority NGS has over other typing methods, resolving 5 strains which differed by only 14 SNPs. On the basis of SNP variation and sensitivity of the whole genome analysis, isolates can now be traced during outbreaks, and used to investigate their relatedness (224, 225).

1.3.1.4 Evolution and clonal diversification of MRSA

Molecular epidemiology reveals that the population structure of *S. aureus* and evolution of MRSA is predominantly clonal (226, 227). By analysing the sequence changes at MLST loci, it was estimated that point mutations occurred 15-fold more frequently than recombination and that this is the driving force behind the initial stages of clonal diversification (226). However, recombination plays an important role in shaping the genome of *S. aureus* and is generally restricted to the MGEs of the non-core or accessory genome, but within core genomes the highest rates of recombination are localised to cell envelope proteins (180). Long-term evolution of *S. aureus* can be influenced by large chromosomal replacements (226, 228), as seen in the mosaic chromosome of ST239, a descendent of ST8 lineage, which underwent a significant recombination event acquiring 635 kb fragment from ST30 background (178). Recently, it has been observed that the recombination rate varied significantly within a single clone of *S. aureus* (ST239), and that this variation was associated with phylogeography (180). What is ultimately causing this variation is currently unknown but could be due to a variety of factors including genetic changes in the bacterial genome affecting restriction and modification system thereby imposing barriers to recombination within specific sub-clones (180, 189).

The history of methicillin resistance *S. aureus* began with the successful introduction of the SCC*mec* element into a methicillin susceptible *S. aureus* (MSSA) host. The origins and mechanism of transfer of SCC*mec* are still unknown but an ordered progression of SCC*mec* transmission among the staphylococci has been proposed (228, 229). Considering that a PBP belonging to *S. sciuri* has the highest amino acid similarity (88%) to PBP2a, it is feasible to suggest that this organism was the evolutionary precursor of the *mecA* found in MRSA strains (229, 230). It has been hypothesised that the SCC*mec* element was first formed in coagulase negative staphylococci (CoNS), after the recombinases (*ccr*) and *mec* regulatory genes were assembled on a MGE in CoNS, which then acted as donors for transmission to *S. aureus* (228, 230). There are several lines of evidence that suggest this transmission, notably the intact nature of IS*1272* found in CoNS and on SCC*mec* elements

consistent with transmission from a CoNS, the *in vivo* development of MRSA as a result of transmission of *mecA* from *S. epidermidis* to *S. aureus* (231), the high rate of methicillin resistance found in *S. epidermidis* and the observation that SCC*mec* IV was present in *S. epidermidis* before *S. aureus* (228).

There have been multiple introductions of SCC*mec* into *S. aureus*, and although the origins of MRSA are not completely understood, it is now agreed that MRSA did not arise from a single ancestral clone, highlighted by the evidence that strains with the same ST have different SCC*mec* types (227). It has now been determined that acquisition of SCC*mec* has occurred at least 20 times, with models predicting that acquisition was 4 times more likely than replacement (232). The *S. aureus* population consists of approximately 11 different lineages or clonal complexes (CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51) with the most dominant HA-MRSA isolates belonging to CC5, CC8, CC22, CC30 and CC45 (227). *Enright et al* investigated the evolutionary origins of a vast international collection of MSSA and MRSA isolates and identified 11 major STs within the five major lineages above (227).

The ancestral MRSA genotype has shown to be ST250 which arose after the acquisition of SCC*mec* I by ST-250 MSSA, which itself descended from ST8 MSSA (227). CC8 is a successful clonal complex, containing three other major epidemic MRSA (EMRSA) STs (ST8, ST247 and ST239). Although ST8 is a successful MSSA clone, it has also acquired SCC*mec* types II and IV and is a widely disseminated EMRSA clone (227). ST247 is a single locus variant (SLV) of ST250 and has spread internationally, known commonly as the Iberian clone (233) whereas the Brazilian clone (ST239), which also has genetic roots from CC8, is highly transmissible and multidrug resistant (234). The other major internationally disseminated EMRSA STs (ST5, ST22, ST30 and ST45) are very different genetically to the CC8s and originated from epidemic MSSA lineages that acquired the SCC*mec* element (227).

The first antibiotic resistant *S. aureus* was the penicillin-resistant phage type 80/81 pandemic strain which caused infections in both the hospital and community (235). EMRSA-16 is one of the most successful MRSA clones to have emerged over the past 20 years, and belongs to the same clonal complex of phage type 80/81, CC30 (236). Interestingly, after genetic characterisation and comparison of genome sequences of isolates representing phage type 80/81, and CC30 hospital clones, it was shown that these strain diverged from a recent common ancestor (237). The shift from highly virulent phage type 80/81 to less virulent hospital restricted EMRSA-16 clone maybe due to the acquisition of the SCCmec type II element which has been shown to interfere with *agr* signalling and reduction in cytolytic toxin expression while conferring resistance to beta-lactam antibiotics (238).

EMRSA-15 represents a particularly successful clone associated with rapid and efficient transmission within the hospital environment, with evidence of spread to other countries (239, 240). EMRSA-15 belongs to the ST-22 family and is one of the most frequent found MRSA clones in Europe (240). In order to understand the genetic changes that contributed to the success of this clone, *Holden et al* sequenced the genomes of 193 ST-22 isolates from 15 countries (241). Of the many mutation and genetic rearrangements that were identified in order to explain ST-22 success, two nonsynonymous SNPs associated with fluroquinolone resistance were highlighted as key factors, with the authors suggesting that resistance to this particular antibiotic may have given ST-22 a competitive advantage and promoted colonisation in an environment where this antibiotic was frequently used. Other studies have shown that the acquisition of specific genetic elements can contribute to enhanced spreading, colonisation, virulence and success of MRSA clones. The acquisition of the ΦSPβ-like prophage containing the *sasX* gene has been implicated in the successful spreading and dominance of ST239 in China in the past 10 years (242). This 15kDa surface protein promotes nasal colonisation through increased attachment to nasal epithelial cells, bacterial aggregation and biofilm formation and impedes phagocytosis, increasing survival within the blood (242). The expression of this multifactorial protein promotes abscess formation in mice (242) and is a key

factor in increased pathogenic activity and epidemiological success of this specific epidemic clone.

Community acquired- (CA-) and hospital acquired- (HA-) MRSA are very different both in terms of genetic background and phenotype (discussed in detail in section 1.3.3), and are not from related lineages, with more clonal diversity observed within CA-MRSA than HA-MRSA (243, 244). Presently there are 5 major CA-MRSA clones in circulation globally: ST1 clone in disseminating in Asia, Europe and the US, ST8 predominantly caused by USA300 clone in the US, but becoming more frequent in Europe, ST30 clone of Australia, Europe and South America and the ST80 clone harbouring in Asian Europe and the Middle East (222). The acquisition of SCC*mec* by CA-MRSA strains most likely occurred by MSSA acquiring SCC*mec* IV, as PVL positive MSSA strains were present in Japan and acquired SCC*mec* IV (245).

The evolutionary success and dominance of certain *S. aureus* clones can be attributed to the successful acquisition of genetic elements horizontally, highlighted recently by the acquisition of *sasX* by ST239 strains in Asia. Recent evidence suggests that within certain lineages, MGEs are transferred with higher frequency than in others, potentially enhancing the successful spread of new MRSA clones (177). One method that *S. aureus* has evolved to prevent transfer of genes between different lineages is highlighted by the restriction and modification systems which targets and destroys 'foreign' DNA based on sequence and modification patterns (189). Therefore, these RM systems can dictate bacterial evolution by preventing transfer of virulence or resistance genes between different CCs. However, in certain cases, lack of a particular target sites enables genetic transfer between lineages which may be an important feature in driving the evolution of MRSA clones (246).

1.3.2 *S. aureus* and the human host

The main ecological niche for the colonisation of the human host by *S. aureus* is localised to the non-ciliated, keratinized epithelium of the anterior nares (*vestibulum*

nasi) (247-249). Other anatomical sites may also be colonised including the axillia and perineum (247), and in some cases the pharynx; however it is unclear whether this is a permanent or transient site, as *S. aureus* adherence to nasopharyngeal ciliated epithelium is poor (250). Nasal carriage is a recognised risk factor for staphylococcal disease (249, 251). Patients undergoing medical treatment are more likely to become infected if they are carriers than those who are not (252, 253). It has been widely accepted that the majority of infecting strains are identical to that of the carriage strain (254, 255) with one particular study highlighting nasal isolates as a source of bacteraemia in greater than 80% of cases (251). Elimination of nasal carriage using topical antimicrobials generally eliminates colonisation from other anatomical site, highlighting the nares as the dominant reservoir for infection (256).

Historically, *S. aureus* nasal colonisation of humans has been divided into three groups: persistent carrier, which accounts for approximately 20% of individuals, intermittent carrier (~30%) and non-carriers (~50%) (247, 248, 257). However, the rate of re-colonisation after elimination varies widely, as does the prevalence of *S. aureus* with respect to ethnicity, age, sex and patients with chronic underlying illness (255, 258-260). The identification of specific features of the different carriage types has redefined nasal carriage classification (261). Using a human colonisation model, *S. aureus* strains were observed to have a significantly higher survival rate in persistent carriers as opposed to intermittent and non-carriers, which show similar rates (261). Coupled with differences in bacterial load in colony forming units (CFU) per sample and anti-staphylococcal antibody profiles between the persistent and intermittent/non-carriers groups, nasal carriage is now grouped as either persistent or others (261). The ability of *S. aureus* to persist in the nasal cavities in certain individuals is not fully understood, but is likely to be a result of a combination of host and bacterial factors and the presence of commensal organisms, as illustrated in figure 1.6.

The pattern of carriage of *S. aureus* suggests a host genetic predisposition to colonisation, supported by the observation that nasal carriage varies with ethnicity (262). Recent studies have shed light onto specific genetic polymorphisms and

environmental nasal conditions which modulate colonisation. Nasal secretions contain anti-staphylococcal potential in the form of antimicrobial peptides (AMPs) such as lysozyme, lactoferrin, phospholipase A2 and defensins (263). Unsurprisingly, reduced expression of these AMPs have been implicated with increased nasal carriage (264). Curiously, certain individuals produced secretions which promoted nasal colonisation (265). Protease treatment and immunoprecipitation studies revealed haemoglobin as the substance conferring increased colonisation (265). It was observed that haemoglobin not only increased bacteria attachment to collagen and fibronectin, but decreased the inoculum size required for establishing successful colonisation in a rat model (265). Interestingly, this effect was independent of iron as apohaemoglobin also promoted this phenomenon. It has been observed previously that the alpha and beta chains of haemoglobin inhibit exotoxin production in *S. aureus* (266). Interestingly, it was shown that haemoglobin specifically inhibited the accessory gene regulatory (Agr) quorum sensing system (Agr reviewed in section 1.3.4.1) and that constitutive expression of RNAIII, the effector molecule of the system, inhibits nasal colonisation (265). The authors suggest that since Agr regulates proteases, the downregulation of *agr* by haemoglobin may infer an advantage by inhibiting the activation of proteases which may cleave surface proteins important in attachment and colonisation to squamous epithelial cells (265). Other host factors contributing to a persistent nasal carriage state involved polymorphisms in genes which can modulate immune activity, such as glucocorticoids, C-reactive proteins, interleukin 4 and complement inhibitor proteins (267, 268).

It is widely known that commensal bacteria can compete and inhibit pathogenic bacteria and influence colonisation (257). Recently, the impact of normal flora on *S. aureus* nasal colonisation has been reported. Here a subset of *Staphylococcus epidermidis* strains secreting a serine protease, Esp, lead to inhibition of biofilm formation and nasal colonisation by *S. aureus* (269). This inhibitory effect is augmented when Esp is combined with human beta-defensin 2 (hBD2) an AMP secreted by keratinocyes, resulting in higher biofilm destruction (269). Introduction of wild type but not *esp* mutants in human *S. aureus* positive volunteers lead to the

elimination of *S. aureus* colonisation, clearly showing the importance of *S. epidermidis* in determining *S. aureus* persistent colonisation.
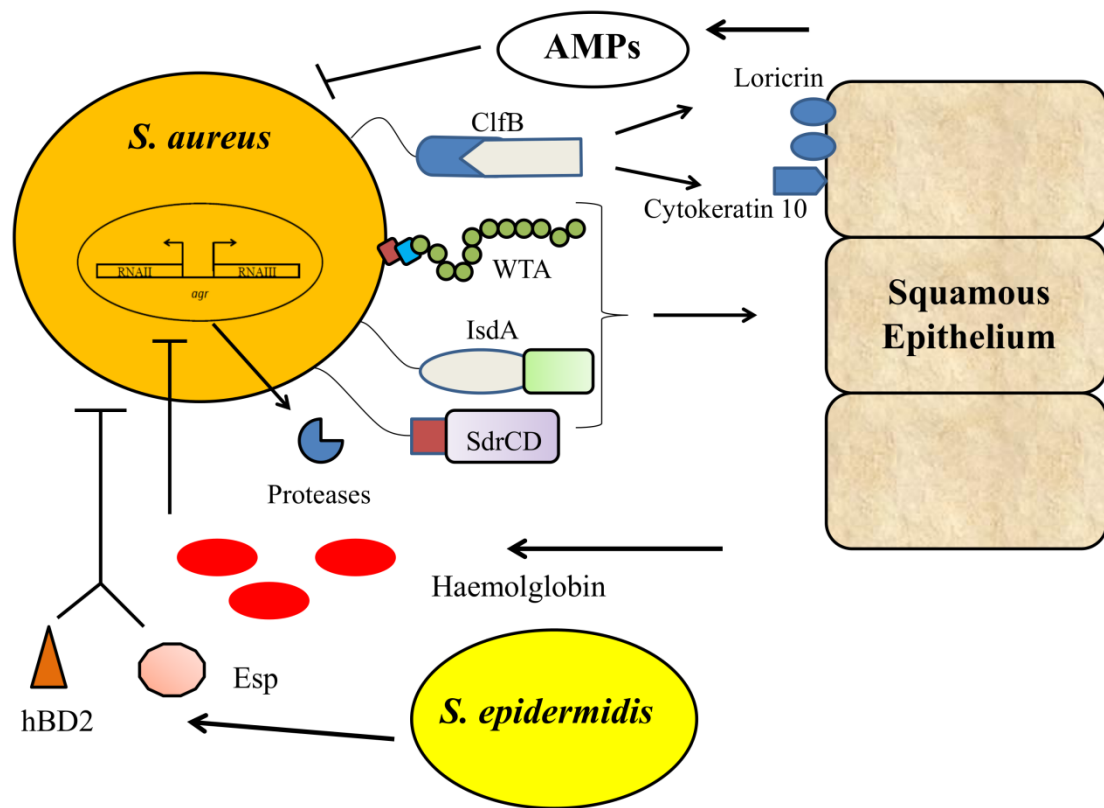
Figure 1.6: Factors affecting persistent nasal colonisation of *S. aureus*. This schematic represents some of the major factors affecting persistent nasal carriage of *S. aureus*. The expression of proteins (ClfB, IsdA and SdrC and D) and polymers (wall teichoic acids (WTA)) on the surface of *S. aureus* interact with the squamous epithelium facilitating adherence and colonisation. ClfB specifically interacts with two ligands on the surface of squamous epithelial cells, loricrin and cytokeratin. Nasal secretions contain different antimicrobial peptides (AMPs) which can inhibit the growth of *S. aureus* in the nasal cavity. Nasal secretions can also contain factors which increase colonisation, exhibited here by high haemoglobin concentrations. Haemoglobin interferers with Agr signalling, which may interrupt protease activation resulting in higher surface protein expression and increase nasal colonisation. A subset of *S. epidermidis* strains secrete the Esp protease which reduces biofilm formation and nasal carriage, which occurs through a mechanism involving human beta-defensin 2 (hBD2) AMP.

Bacterial factors are also extremely important in nasal colonisation. The most influential factor for nasal carriage is the ability of *S. aureus* to adhere to epithelial surfaces. For this interaction to take place, bacteria need to expresses the correct surface proteins to interact with the corresponding ligands present on cells. One of

the major ligands on the cell surface of *S. aureus* is clumping factor B (ClfB) (98). This protein has been shown to interact with two specific structural proteins of squamous epithelial cells, cytokeratin 10 and loricrin (270) and the absence of either ClfB or loricin in mouse models diminishes nasal colonisation (270, 271). Through the use of surrogate expression, isogenic mutants and *ex vivo* squamous adherence assays, other surface proteins have also been implicated in nasal colonisation, most notably the iron surface determinant protein IsdA, and the serine-aspartic acid repeat proteins, SdrC and SdrD (272). The *S. aureus* cell envelope also contains complex surface-exposed polymers, known as wall teichoic acids (WTA) (273). These molecules are composed of ribitol phosphate repeating units modified with N-acetylglucosamine and D-alanine (273). The role of these complex polymers in nasal colonisation was explored, with mutants deficient in key teichoic acid biosynthetic steps impaired in their adherence to primary nasal epithelial cells and human airway epithelial cells (274), however the precise receptors to which WTA in anchors with remains to be determined.

1.3.3 Epidemiology and disease of *S. aureus*

Humans are a natural reservoir of *S. aureus* and can exist in a non-pathogenic relationship with the host. Generally, initiation of infection begins with a breach in the natural barriers of the skin and/or mucosa, from which bacteria can invade the adjoining tissues or bloodstream (275, 276). *S. aureus* can cause a wide range of pathophysiological complications and is a leading cause of hospital-acquired infections (277). *S. aureus* is the primary cause of lower respiratory tract and surgical site infections (278) and among the most prevalent causes of bacteraemia (279) and pneumonia (67, 277, 278).

In the US alone, hospitalization and rates of infection increased substantially between 1999 and 2005 (277), and in 2009 an estimated 463,017 MRSA-related hospitalizations was recorded, at a rate of 11.74 hospitalisations per 1,000, illustrating the increasing burden this pathogen imposes on health care resources (280). Fatalities due to MRSA infection in hospitalised Americans approximate at

19,000 annually, a number that is equal to the combined deaths due to AIDS, tuberculosis and viral hepatitis combined (281, 282). The severity of the disease depends on the complex interplay between the multitude of *S. aureus* virulence determinants (reviewed in section 1.3.5) and the capabilities of host immune defence. Although the vast majority of *S. aureus* infections require a combination of these virulence determinants, in some cases, individual factors can be singled out as the predominant cause, such as in toxic shock syndrome (283), staphylococcal scalded skin syndrome (284) and necrotic lesions of the skin or mucosa (285).

S. aureus is notoriously difficult to treat due to antibiotic resistance. Since the first appearance of methicillin resistance in 1960, MRSA strains have spread globally and are now endemic, and in some cases, epidemic in hospitals, care facilities and communities (67, 281, 286). The high incidence of MRSA is not only localised to the US, with South East Asia, southern Europe, parts of South America and Australia all having rates of prevalence between 25-50% (228). This is in stark contrast to the Scandinavian countries and the Netherlands, which have the lowest MRSA prevalence rates, due to their effective search and destroy policies of screening, isolation of MRSA-positive carriers and eradication (287). In Europe, MRSA clones display higher levels of clustering and regional distribution than their MSSA counterparts (240). However, as MRSA strains tend to emerge from MSSA strains and considering that the acquisition of SCC*mec* is a rare event, there are fewer MRSA clones which have had less time to diversify and have had more of a selective pressure in harsher environments which may constrain their diversity. In contrast, MSSA strains have a much weaker selection which may bias the geographical clustering of MRSA (240). Of growing concern is the emergence of MRSA infection in people who had no prior contact with hospitals or health care or other risk factors, the so called community-associated MRSA (CA-MRSA).

Historically, *S. aureus*, particularly MRSA, was associated with hospital infection, so called hospital acquired/associated (HA)- MRSA (288). Risk factors for colonisation included surgery, intensive care unit admission, exposure to antibiotics or MRSA-colonised patient and prolonged hospital stay (288). Higher carriage rates

are associated with intravenous drug users, healthcare workers, patients with underlying illness such as dermatological conditions, indwelling devices and diabetes (275, 288). In recent years, MRSA epidemiology has changed from predominantly hospital acquired, to infections that are acquired in the community (289). With this switch came an increase in hospitalisations, primarily due to an increase in skin and soft tissue infections (SSTIs) caused by these CA-MRSA strains (290). The epidemiology of CA-MRSA is markedly age dependent, with CA-MRSA strains causing disease in younger patients with no previously defined health risk factors. How these CA-MRSA strains differ from HA-MRSA strains, in terms of clonal background, antibiotic resistance, virulence and transmissibility is described in the following section. MRSA is also found colonising livestock including pigs, cattle and poultry, a third epidemiological group known as livestock-associated- (LA-) MRSA, with CC398 as the most predominant clonal complex (291).

1.3.3.1 CA-MRSA

In the past HA-MRSA strains were restricted to the healthcare setting and out-competed CA-MRSA in this environment probably due to their higher antibiotic resistance potential. CA-MRSA dominated in the community setting being fitter and more virulent than their HA-MRSA counterparts. Presently, there exists a more complex epidemiology, whereby both HA- and CA-MRSA circulate in the community and highly successful CA-MRSA clones are invading hospitals and healthcare facilities (292, 293). In spite of this, certain molecular traits can be used to discriminate between the two groups, a summary of which is shown in figure 1.7.

In the early 1990s, the first documented CA-MRSA cases began to emerge in the US, with MRSA infections causing high mortality in healthy individuals in the community (294). In the following years, CA-MRSA became a global problem. Virulent CA-MRSA strains have been shown to belong to different lineages, with a geographical predominance (295). In the US, the most dominant CA-MRSA strain is USA300 (ST-8), replacing the first CA-MRSA strain observed in the US, USA400 (ST-1), in all of North America and is considered to have caused the most severe

outbreak globally (296). CA-MRSA strains are highly associated with skin and soft-tissue infections (296, 297) and under rare circumstances can cause more severe, often fatal diseases such as necrotizing pneumonia, Waterhouse-Friderichsen syndrome and necrotizing fasciitis (298).

Comparative genomics of HA- and CA- MRSA strains was essential in assessing the molecular basis for CA-MRSA pathogenic success. Two mobile genetic elements were highlighted as possible markers for the enhanced virulence associated with CA-MRSA: SCC*mec* type IV and prophage ΦSA2*pvl* (299). In general terms, HA- and CA-MRSA differ substantially in terms of antibiotic resistance. The elucidation of a new, shorter *SCCmec* belonging to CA-MRSA (types IV,V or VII), highlighted a possible reason for the observed increased dominance and transmissibility, as these SCC*mec* elements (particularly SCC*mec* IV) were shown to cause less of a fitness burden than those SCC*mec* elements associated with HA-MRSA (namely SCC*mec* I, II and III) (300). CA-MRSA isolates are usually sensitive to most non-beta-lactam antibiotics, while HA-MRSA, due to the selection imposed on them by their environment, are generally multidrug resistance (301). Importantly, differences in the antibiotic minimum inhibitory concentration (MIC) are significantly different, with HA-MRSA requiring much higher antibiotic concentrations for efficient killing (296).

The association between the Panton-Valentine Leukocidin (PVL) and CA-MRSA has been carved into the history books of *S. aureus* as one of its most controversial chapters. PVL is a bi-component beta-barrel forming leukocidin (reviewed in section 1.3.5.2.2). Enormous interest surrounding the role of PVL in CA-MRSA infections began after epidemiological studies showed a positive association between the presence of *pvl* genes and CA-MRSA (302). In an attempt to unravel the role of PVL in CA-MRSA disease, isogenic mutants of *lukS-PV* (or *lukF-PV*) were created and the effect this mutation had on either human neutrophils, or specific animal models, examined. The controversy stemmed from experimental practice: culture supernatants proved strongly dependent on growth media used (303), while different groups, using different animal models, observed contradictory

results (304-307). Recently, a receptor for PVL has been identified (C5a receptor (308)), explaining the low sensitivity of mouse neutrophils to PVL and the earlier results stating a non-involvement of PVL in CA-MRSA disease. PVL is important; however its importance is disease specific. Rabbit neutrophils are sensitive to PVL, in a range similar to humans (309), and it has been observed that PVL plays a significant role in the development of pneumonia (310). PVL involvement is less clear in skin infections, where it has been shown that α-haemolysin and phenol soluble modulins (PSMs) involvement is critical (311). Finally, the observation that increasing numbers of CA-MRSA strains do not contain *pvl* genes (301), suggests that although PVL may be important in some diseases, it is not the only factor contributing to CA-MRSA enhanced virulence.

Increased expression of core genes rather than the acquisition of novel elements is suspected to be behind the high virulence of CA-MRSA strains. Enhanced expression of two classes of toxin; α-haemolysin and phenol-soluble modulins (reviewed in section 1.3.5.2), have been implicated in the success of CA-MRSA (312, 313). α-haemolysin is one of the most widely studied and well characterised of the staphylococcal toxins, forming pores in susceptible cells such as erythrocytes and macrophages (314). Studies investigating the effects of *hla* deletion in CA-MRSA resulted in a negative impact on virulence, particularly in pneumonia (315) and skin and soft tissue models of infection (311), highlighting its central role in CA-MRSA pathogenesis. PSMs are small amphipathic peptides, which do not require a proteinaceous receptor and can lyse a variety of cells including erythrocytes, macrophages and neutrophils (312, 316). PSMs are produced in huge quantities in CA-MRSA, and although the *psm* genes are present in HA-MRSA, they are expressed at significantly lower levels (312). This higher expression of PSMs is a major factor involved in the observed increase in virulence in CA-MRSA strains, complemented by the fact that deletion of the *psmα* operon significantly reduces the mortality rates in skin infections (311) and bacteremic mouse models (312). PSMs exhibit high antimicrobial activity, which may also be important for colonisation and spread in the population in which out-competing competitors is suspected to be important (317). Both toxins are regulated by the central virulence regulator, Agr,

which is highly active in CA-MRSA (318). The specific events and minute genetic changes in central regulators, such as Agr, may be behind the increase expression of core genes, the specifics of which have yet to be determined.

CA-MRSA strains have undergone a rapid global expansion, and understanding the basis behind this is required if novel intervention strategies are to be developed. Factors that can enhance fitness and the ability to successfully colonise the host, are of major importance in the spread and epidemiological success of CA-MRSA in the population. The arginine catabolic mobile element (ACME) is specific to the USA300 strain, physically linked to SCC*mec* IV (IVa), and considering its dominance in North America and growing success in Europe (319, 320) and South America (321), it was hypothesized that ACME may be important in overall fitness, enhancing colonisation and transmissibility (322). The ACME element, originally acquired from *S. epidermidis*, encodes two main clusters; an arginine deiminase (*arc*) hypothesized to reduce the acidic environment of the skin through production of ammonia, and the oligopeptide permease (*opp*) which may be important in nutrient uptake (322). No significant impact on virulence was established for the ACME element, and its role is enhanced colonisation and skin survival remains to be established.

Polyamines are aliphatic compounds which have pleiotropic effects in many aspects of cellular biology particularly important in wound healing and inflammation (323). Interestingly, *S. aureus* is hyper-sensitive to polyamines, inhibiting rather than enhancing survival, a feature almost unique to *S. aureus* (324). However, USA300 clones are the notable exception, illustrating resistance to such compounds (325). The polyamine-resistant dependent phenotype depended upon the expression of a spermine/spermidine acetyl transferase (*speG*) located within the ACME element (325), and this polyamine resistance phenotype was shown to contribute a major fitness advantage in SSTI models (326). Considering that CA-MRSA are a major cause of SSTI, and that these infections are likely to have increased polyamine production, this particular resistance feature may contribute to the dominance of USA300 in North America.

Other factors including the presence of additional superantigen genes (*se1Q*(327)) and virulence regulators (AryK, in the Australian clone, ST93 (328)) may also impact on the hyper-virulence of CA-MRSA. The higher incidence of CA-MRSA in the US compared to Europe cannot be solely attributed to different clonal lineages as USA300 is present in Europe, but factors such as low socioeconomic standards, high rate of incarceration and lack of access to adequate healthcare (292) may all help in perpetuating the transmission and success of CA-MRSA.
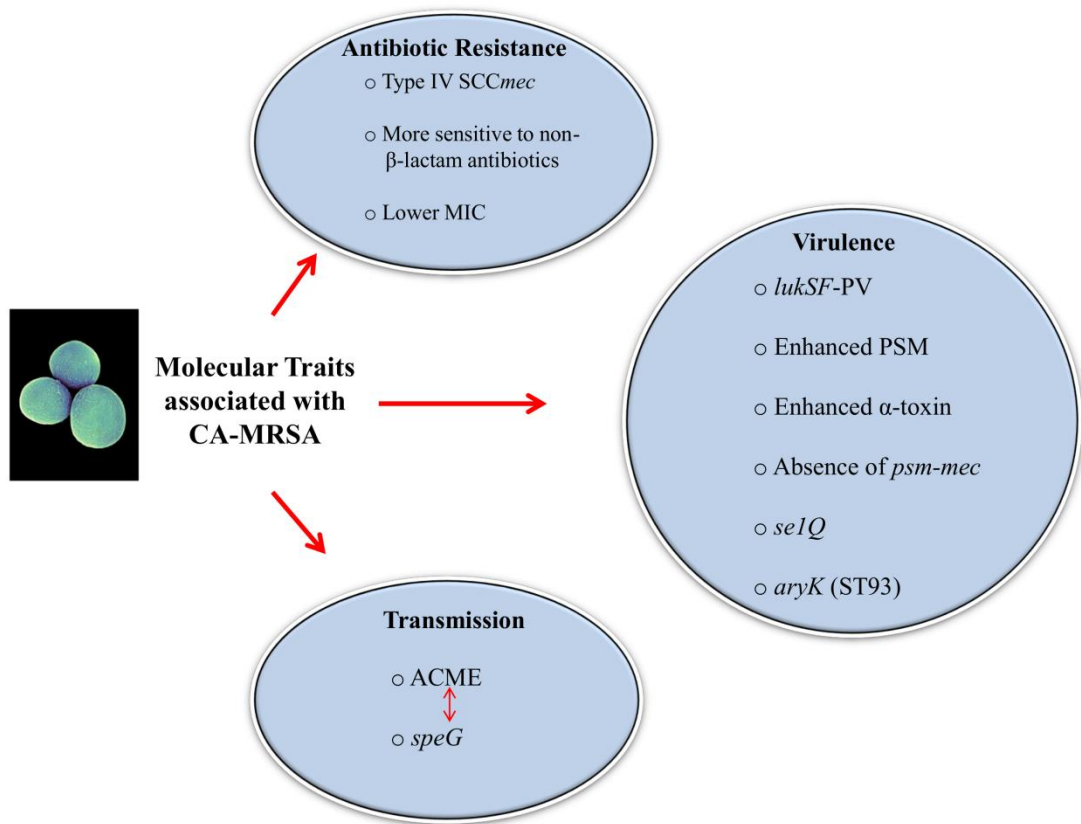
Figure 1.7: Molecular traits associated with CA-MRSA. Outlined are the general traits associated with CA-MRSA concerning antibiotic resistance and elements which may contribute to increased transmission and enhanced virulence.

1.3.4 Virulence gene regulators

Virulence gene expression in *S. aureus* is regulated in a temporal, growth-phase dependent manner, through a complex regulatory network employing two-component systems (TCS), multiple DNA binding proteins and a subset of regulatory RNAs. These systems are sensitive to environmental signals and allow the bacterium to adapt to niche habitats and are central in pathogenesis.

1.3.4.1 Quorum sensing and the accessory gene regulator (Agr)

Quorum sensing is a cell-to-cell communication system mediated by diffusible signalling molecules, or auto-inducing peptides (AIP), which allow bacteria to synchronise the expression of specific genes. This sophisticated chemical communication is used by *S. aureus* to modulate the activation of selected virulence genes (329). One of the most well-characterised genetic elements in *S. aureus* is the quorum sensing accessory gene regulator (*agr*) system, which decreases the expression of surface proteins and increases the expression of secreted toxins/enzymes as a function of bacteria density, specifically during the transition from late exponential to early stationary phase of *in vitro* growth (329, 330).

The *agr* system is encoded on a 3kb locus consisting of five genes (*agrACDB* and *hld*) composed of two divergent transcripts, RNAII and RNAIII, driven by two distinct promoters, P2 and P3 respectively (Fig 1.8) (331). The *agr*-activating ligand is a post-translationally modified peptide, encoded by the *agrD* gene, which is processed and digested by the polytopic transmembrane protein *agrB*, which secretes a mature AIP composed of seven to nine aminoacyl residues, containing a functionally critical thiolactone (332). This AIP forms the basis of the proceeding regulatory circuit, where upon reaching a critical AIP threshold, dependent on the bacterial density, the *agr* system is activated. AgrC is a transmembrane protein, consisting of several extracellular loops which interact with the AIP and a cytoplasmic C-terminal histidine kinase domain. This protein acts as the sensor component of the *agr* system, undergoing homodimerisation after AIP binding, leading to trans-phosphorylation of the histidine domain (333). This phosphate is

transferred to the response regulator, AgrA, leading to a conformational change permitting binding of this protein to the promoter regions of P2 and P3 (also binds to the promoter region of *psm* operons (215)). This activation of the P2 and P3 promoters drives the expression of the two transcripts in a positive feed-back loop manner (329).
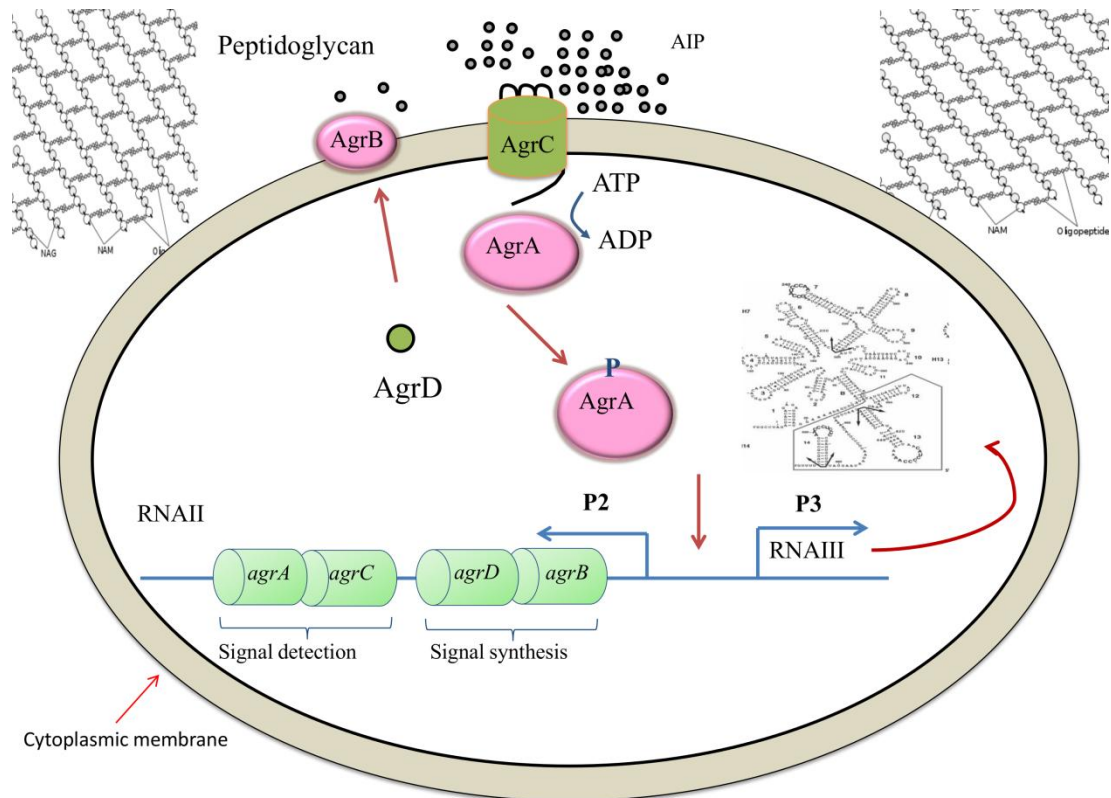
Figure 1.8: The accessory gene regulator (*agr*) system. The *agr* regulatory circuit consists of two divergently transcribed transcripts, RNAII and RNAIII. The RNAII transcript contains *agrACDB operon. agrB* encodes a transmembrane protein responsible for processing the autoinducing peptide (AIP), encoded from *agrD*. After reaching a critical threshold, AgrC (sensor kinase) senses AIP concentration, resulting in the phosphorylation of AgrA (response regulator) which interacts with promoter regions, resulting in expression of RNAIII and other genes. RNAIII is a regulatory RNA composed of 14 stem-loops and modulates the expression of virulence factors in *S. aureus*. RNA molecule was taken from (334).

The regulatory RNAIII molecule is the pleiotropic effector of the *agr* regulon and encodes a toxin gene, delta (*hld*) toxin, (335). The RNAIII molecule has a complex secondary structure containing 14 stem-loops which are important in the up-regulation of extracellular toxin transcripts and repression of cell surface proteins

during transition into the late-exponential phase of growth (335). RNAIII directly targets three specific mRNA encoding virulence factors, *hla*, *spa* and *coa* mRNA. Binding of the 5' end of RNAIII to the Shine-Dalgarno (SD) sequence of *hla* mRNA results in conformational changes, conducive to translation of *hla* mRNA (336). In contrast, RNAIII represses the synthesis of protein A via direct binding of the 3' end of stem-loop 13 to *spa* mRNA preventing ribosome interaction and initiating mRNA degradation by activation of the RNaseIII specific endoribonuclease (337). For *coa* inactivation, RNAIII utilises two distal regions which base-pair with nucleotides in the *coa* SD sequence forming an imperfect duplex complex, preventing protein activation and causing mRNA degradation through RNaseIII (338).

Genome wide analysis of several *S. aureus* strains suggest that there are at least sixteen two-component systems present in the *S. aureus* genome (339). After AgrCA, there are four major two-component systems which have been implicated in virulence gene expression: SaeRS (*S. aureus* exoprotein expression), SrrAB (Staphylococcal respiratory response), ArlRS (Autolysis-related locus) and LytRS (Lytic-related genes).

1.3.4.2 Two-component systems

The *sae* locus contains four open reading frames, two of which illustrate strong homology to response regulators (SaeR contains an aspartate phosphorylation site) and histidine kinases (SaeS contains an aspartate autophosphorylation site) (340). Upstream of the *saeRS* genes exist two additional genes, *saeP* and *saeQ,* likely to contribute to *saeRS* activity (341). Four transcripts are expressed from two promoters, P1 and P3, with P1 being strongly activated by hydrogen peroxide and α-defensins, suggesting a role of *saeRS* in activation of virulence factors important for neutrophil killing (342), with other factors such as high salt, low pH and subinhibitory antibiotics also affecting *sae* activity (329). Interestingly, the activation by α-defensins is strain specific, therefore other factors either interacting with *sae* leading to activation, or activation being a result of structural changes in the cell envelope between the different strains may be responsible for this activity (342).

The most abundant transcript, T2, has been suggested to be processed from transcript T1, following endoribonucleolytic cleavage, possibly acting as an important method of enhancing the half life of this transcript (342).

sae mutants are deficient in α-, β-, γ-toxins, proteases, DNase, coagulase, and surface proteins such as fibronecting binding protein A and protein A, and do not affect the transcription of *agr, sarA* or sigB. (343, 344). *Sae* locus is activated by *agr*, but repressed by *sigB* highlighting the possibility that it acts downstream in the global regulatory cascade (340, 342, 344). Inactivation of *sae* or *agr* has a reduced effect in virulence in animal models, however the respective virulence genes they regulate are different (341, 343). For example, inactivation of *agr* causes an increase in the expression of certain surface proteins whereas *sae* mutation has the opposite effect on identical proteins (345). Recently, it has been reported that a methionine residue (M31) in the predicted extracellular loop domain of SaeS is essential for transcription of toxins genes (*hla*, *lukA/B/lukG/H* and *hlgA*), mutation of which drastically reduces cytotoxicity to neutrophils, signifying its importance as a virulence regulator (346).

The *srr* locus consists of two overlapping open reading frames *srrA* and *srrB* with one transcriptional start site, producing either *srrA* or full length *srrAB* transcript (347). This system also shows homology to the *B. subtilis resDE* system, which is important for global regulation under both aerobic and anaerobic conditions (348). The *srrAB* system acts as a global regulator of virulence, down regulating RNAIII, toxic shock syndrome toxin -1(*tst*), *icaR* and protein A, particularly in low-oxygen environments (348) but enhances the level of these transcripts (except RNAIII) under aerobic conditions (349). SsrA is the response regulator of this system, and SrrA binding sites have been discovered at the *agr* P2 and P3, *spa*, *tst*, *icaA* and *srr* promoters regions highlighting a direct method of regulation by SrrA (347, 350).

Polysaccharide intracellular adhesion (PIA) molecule is an important factor involved in biofilm formation and is regulated by the *icaADBC* locus (124). IcaA

acts as an N-acetylglucosaminyl transferase important in PIA production (124). SrrAB significantly increases *icaA* transcription leading to a PIA-positive phenotype during anaerobic growth (350). This mechanism of *ica* expression is proposed to function through inactivation of *icaR*, the repressor of the *ica* locus, by both phosphorylated SrrA and SarA (350). This expression of PIA under anaerobic conditions has been shown to confer a survival advantage during *in vitro* anaerobic phagocytosis assays (350). SrrAB also plays a major role in resistance to hypoxia and radical nitric oxide, with mutants in *ssrAB* becoming hyper-sensitive to nitrosative stress (351). Under conditions of nitrosative stress *srrAB* induces the expression of genes required for anaerobic metabolism and nitric oxide ($NO^{\cdot}$) detoxification (352), conveying the importance of *srrAB* as a global regulator affecting genes important in virulence, stress adaptation and metabolism.

The *arl* locus consists of two co-transcribed genes, one coding for the histidine kinase sensor protein, ArlS, while the other for the response regulator, ArlR, which belongs to the PhoB-OmpR family (353). This locus is expressed during the transition from the exponential to post-exponential growth phase (353). Inactivation of *arlS* causes autolysis due to an increase in peptidoglycan hydrolase activity, suggesting its involvement in cell growth and division (353). The *arl* locus is also intimately involved in global virulence regulation, illustrated by mutation in either *arlS* or *arlR* increasing the secretion of specific exoproteins (α-, β-haemolysin, lipase, coagulase, serine protease) and protein A, in contrast to an *agr* mutation (354). An *agr/arl* double mutation retains the overall increased exoprotein expression, suggesting it regulates certain exoproteins upstream of *agr* (353, 354). The effect on *spa* expression is through *arlRS* alteration of DNA supercoiling, where relaxed DNA leads to increased *spa* expression (355). Recently, the involvement of *arlRS* in agglutination and development of infective endocarditis (IE) has been investigated, illustrating a positive association between *arlRS* activity and clumping in the presence of human plasma (356). ArlRS negatively effects the expression of the extracellular binding homologue, Ebh protein, allowing the formation of stable clumps important in IE (356).

The *lytRS* locus comprises two overlapping, co-transcribed genes, *lytS* and *lytR* (357). In an analogous fashion to the *arl* genes, mutation of *lytS* increases autolysis (358). Both *lytS* and *lytR* positively regulate the expression of *lrgA* and *lrgB*, proteins which are important in regulating autolysis, inhibiting the activity of murein hydrolases by structurally modifying the bacterial cell wall (359). Reduced expression of these proteins leads to increased autolysis and increased susceptibility to cell wall acting antibiotics (359).

Other, less well characterised two-component systems (TCS) which affect virulence regulation in *S. aureus* include the KdpDE and GraRS regulatory circuits. KdpDE senses external K^+ concentrations and regulates virulence genes in response to this stimulus (360). DNA microarray analyses revealed a multitude of genes were up and down-regulated in a *kdpDE* mutant, with some of these genes being important virulence factors (360). Coupled with the fact that this system is up-regulated by *agr*, the KdpDE system represents an exciting new TCS important in virulence regulation. The GraRS system is important for the induction of several genes which determine the net positive charge on the bacterial surface, where mutation of *graRS* leads to increased killing by host and bacterial antimicrobials peptides (361, 362).

1.3.4.3 The SarA protein family

The SarA family represents an array of regulatory proteins which are fundamental in coordinated virulence expression in *S. aureus*. The founding member of this family is the SarA protein, a 14.7 kDa DNA binding protein encoded from the *sarA* locus (363). The *sarA* locus consists of three overlapping transcripts derived from three different promoters which are activated during different times of *in vitro* growth, each encoding the SarA protein (364). The P1 and P2 promoters are σ^A dependent while P3 activation relies on σ^B and is activated post-exponentially (364). SarA acts as a dimeric, winged helix protein with contains multiple helix motifs allowing for efficient DNA binding to AT-rich sequences (365). SarA promotes the synthesis of fibronectin and fibrinogen binding proteins and a suite of toxins (α-, β- δ- toxins) whereby inactivation of *sarA* leads to attenuation in multiple models of infection,

(366). SarA expression peaks at late exponential phase coinciding with activation of *agr* and RNAIII in particular, and it has been observed that the intergenic region between *agr* P2 and P3 promoters contains SarA binding sites (367). Both SarA and SarR, a Sar homolog, can bind these sites, influencing *agr* activation (368). SarR is a 13.6 kDa protein which binds P1, P2 and P3 promoters, and when inactivated leads to a higher expression of *sarA* and translated SarA protein (369). AgrA is central for P2 and P3 activation, whereby SarA can interact with and enhance AgrA-mediated P2 expression, while SarR displaces this protein from the *agr* promoter region, down regulating the combined activation of SarA-AgrA (368).

Through a series of genetic investigations, comparative sequence alignments and structural data it was deduced that the SarA family consists of an another 8 homologs including Sar T, U, V, X, Y, Z, Rot and MgrA (370). The SarA protein family can be divided and classified based on structure and size, with the larger proteins such as SarS, SarY and Sar U classed as two-domain proteins, with each domain homologous to SarA, and single domain proteins, such as SarA, SarR, SarT, SarV, SarX and Rot). A further subclass is used based on the sequence similarity of MgrA and SarZ to the MarR protein family of Gram-negative bacteria (370).

Rot (repressor of toxins) is a SarA homologue, and acts as a global regulator that negatively regulates the expression of several important virulence genes, such as lipase, haemolysins, and certain proteases, and positively regulates the expression of a number of surface adhesins and genes involved in teichoic acid biosynthesis (371). Considering the opposing influences both Rot and RNAIII have on virulence, it was hypothesized that these two global regulators must interact at some level. It has been observed that RNAIII can represses *rot* mRNA translation through an anti-sense mechanism, base-pairing between multiple RNAIII hairpins and the sequence of mRNA leading to rot repression and the upregulation of toxin genes (334). Rot directly regulates the expression of *sarS*, a transcription factors important in modulating virulence expression (372). Additionally, rot directly represses the P3 promoter of *sae*, leading to repression of α-haemolysin (373).

SarS represents another SarA homolog, consisting of a monomeric, rather than dimeric structure, with two similar, but non-identical halves, important in functioning as winged helical protein (372). SarS is a 29.9kDa protein, important in the upregulation of protein A, binding directly to the *spa* promoter (372). SarS is repressed by both *agr* and another SarA homolog, MgrA and is activated by the ClpXP proteases (370).

SarT acts a regulatory mediator between genes regulated by SarA and *agr*. SarT represses *hla* transcription by binding directly to *hla* promoter region and is itself repressed by SarA and *agr* (374). SarT can positively influence the expression of SarS (374). Interestingly, repression of SarT by *agr*, is the proposed method of *spa* reduction observed in *agr* activated cells (374). Neighbouring *sarT* but divergently transcribed is *sarU*, which is repressed by SarT and is a positive activator of *agr*, observed by a significant decrease in RNAII and RNAIII transcripts in a *sarU* mutant (375). Activation by SarU may represent a secondary amplification of the *agr* regulatory cascade, strengthen by the fact that *sarT* is down regulated by *agr* (375).

SarX functions to represses *agr* activity and is up regulated by MgrA, as two consensus MgrA binding sites are embedded within the *sarX* promoter region (376). This highlights the only known SarA protein which negatively affects *agr* activity directly, highlighting another potential regulatory loop complementing the already complex interplay surrounding virulence gene regulation. SarZ positively affects *agr* activity as a *sarZ* mutant results in the reduction of RNAIII transcripts (377). *sarZ* mutant also leads to a decrease in transcription of *mgrA*, *hla* and *sspA* (encoding the V8 protease) and increased *spa* transcription (377) underpinning its importance in virulence gene regulation. The inactivation of *sarZ* also resulted in an increase in *sarA*, suggesting that both the effect of *hla* and *spa* in a *sarZ* mutant is via *agr*, and that activation of *agr* by SarZ is through a SarA-independent method (377). SarZ also represses biofilm formation through its actions on *agr* and *mgrA*, both of which effect key genes in biofilm formation (377).

MgrA is a key transcription factors which modulates virulence factors in an *agr*-like fashion (378). MgrA also affects autolysis through its repressive affect on SarV (378). SarV is a key transcription factor in modulating autolysis, whereby mutation of *sarV* results in an increased resistance of cells to detergents and beta-lactam mediated lysis compared to wild-type levels (370, 379). This decrease in susceptibility maybe due to *sarV* acting as a positive regulator of murein hydrolase (379), a decrease of which would render cells more resistance to autolysis. Mutation in *mgrA* increases autolysis, *sarS* and *spa* transcripts and decreases *agr*, highlighting its global regulatory activity (380). *mgrA* causes increased *hla* transcription through interaction with *agr*, leading to its enhanced activation, but also through direct binding at the *hla* promoter (380). MgrA downregulation of *spa* transcription occurs through increased *agr* activity as stated above, but also by direct repression of *sarS*, whereby MgrA binds directly to the *sarS* promoter (380). MgrA is also a regulator of antibiotic resistance, activated through the oxidation of a critical cysteine residue located at the interface of the protein dimer (381). Antibiotic therapy can introduce oxidative stress which leads to the downstream activation of resistance genes by MgrA.

1.3.4.4 Other regulatory proteins

Several other factors are important in virulence regulation in *S. aureus* including, but not limited to, CodY, *rsr*, ClpXP and alternative sigma factor (σ^B). In many gram-positive organisms CodY has been shown to be a global regulator, controlling the expression of genes involved in metabolism, nutrient adaptation and virulence (382). The deletion of *codY* in *S. aureus* causes the over-expression of several virulence genes and RNAIII, highlighting its potential role as a repressor of virulence (383). CodY acts as a link between metabolism and virulence owing to is activation by branch-chained amino acids (BCAAs), particularly isoleucine, leading to strong repression of target genes (383). Interestingly, under conditions of isoleucine limitation, the *agr* system is not repressed and becomes activated prematurely. This activation of *agr* has been hypothesised to work as an escape mechanism for *S. aureus*, as the expression of toxins and other spreading factors are unregulated during

times of nutrient limitation (383). The exact mechanism in which CodY regulates *agr* is not known, however it has been observed that CodY can regulate certain cell surface proteins independently of *agr* (383).

rsr (repressor of *sarR*) is located adjacent to *sarR* and acts as a repressor not only to *sarR* but also to *agr* (384). Not surprisingly, deletion of *rsr* confers a hyper-virulent phenotype in a murine abscess model due to the upregulation of virulence genes associated with *agr* activation (384). Little is known about this regulator, and whether its repressive functions act directly on *agr* or through an as of yet-unknown mediator is under investigation; however this repression is independent of *sarR*.

Clp proteolytic complexes are very well conserved in bacteria, playing a vital role in cell physiology, and recently, observed to be important in virulence regulation (385). Clp proteases consist of an ATPase factor determining substrate specificity (ClpX in *S. aureus*) and a core protease barrel-like structure (ClpP). Comparisons of DNA microarray experiments between wild-type (8324-5) and Δ*clpP* illustrate the global regulatory activity of this protein, conferring a strong impact of genes involved in virulence, stress response, DNA repair and cell homeostasis (385). Recently, a whole spectrum of ClpP substrates were identified using the 'clpP^{TRAP}' system, which uses a ClpP variant which can retain the substrate but not degrade it, highlighting central regulators such as CodY and PerR as targets (386). Specifically, mutants illustrate reduced RNAIII, *hla*, and *sspA* (serine protease) transcription and reduced virulence in a murine skin abscess model (387). Hypothetically, the link between *agr* and ClpXP may involve the degradation of virulence regulators after interaction with RNAIII. ClpX can function as an independent chaperone and is essential for *spa* transduction. Rot induces the transcription of *spa* directly by binding to the *spa* promoter and indirectly by positively activating *sarS* (388). Deletion of clpX results in a 3-fold reduction of Rot, abolishing Protein A production. This reduction of Rot was due to ClpX involvement in Rot translation, illustrating the regulatory importance of specific chaperone in virulence regulation (389).

Sigma (σ) factors are highly conserved transcription initiation factors working in concert with RNA polymerase (RNAP) to promote expression from specific genes under specific conditions (390). σ^B is the most studied σ factor in *S. aureus* and plays a role in cell physiology, stress survival and virulence (391). σ^B is transcribed from a four gene operon, *rsbUVWsigB* encoding anti-sigma factor (RsbW), an anti-anti-sigma factor (RsbV) and RsbU, an anti-RsbV phosphatase. Under certain environmental conditions, RsbU removes the phosphate group of RsbV allowing RsbV to bind RsbW, removing RsbW from sig B, freeing its interaction with RNAP to transcribe target genes (390).

Deletion of *sigB* also affects virulence. Historically, studies investigating the role of *sigB* were hampered due to use of 8325-4 strain and derivatives which contained an *rsbU* mutation. Studies have now revealed that *sigB* does play a role in repressing *agr*, *hla* and *sspA* (391). Microarray analysis of three phylogenetically distinct strains revealed *sigB* influence in excess of 200 genes, with many adhesins upregulated while many toxins and exoproteins were repressed (392). Interestingly, this data also showed *sigB* increases the activity of *sarA*, particularly during late exponential to early stationary phase (392), in contrast to Horsburgh *et al* (391). σ^B also modulates the transcription of a downstream operon, *yabJ-spoVG*, whereby inactivation leads to the repression of a set of enzymes, nuclease, lipase and certain proteases (393) again conveying *sigB* importance as a virulence regulator.

Virulence regulation in *S. aureus* is complex and involves a major cross-talk of virulence regulators comprising TCS and DNA binding proteins which can all be altered depending on growth conditions and environmental stimuli. Figure 1.9 illustrates an overview of some of the interactions of the known regulators and how communication between these elements affects overall virulence, divided on the outcome on overall toxicity (T), adhesiveness (A) and evasiveness (E) (96). Adding to the complexity of these interactions is the strain specific activities of certain regulators. Therefore, we need a greater understanding of how these regulators act in different genetically distinct clinical strains, while evaluating the inactivation of

regulators in specific animal models of infection will help fully comprehend their relevance in *in vivo* disease.
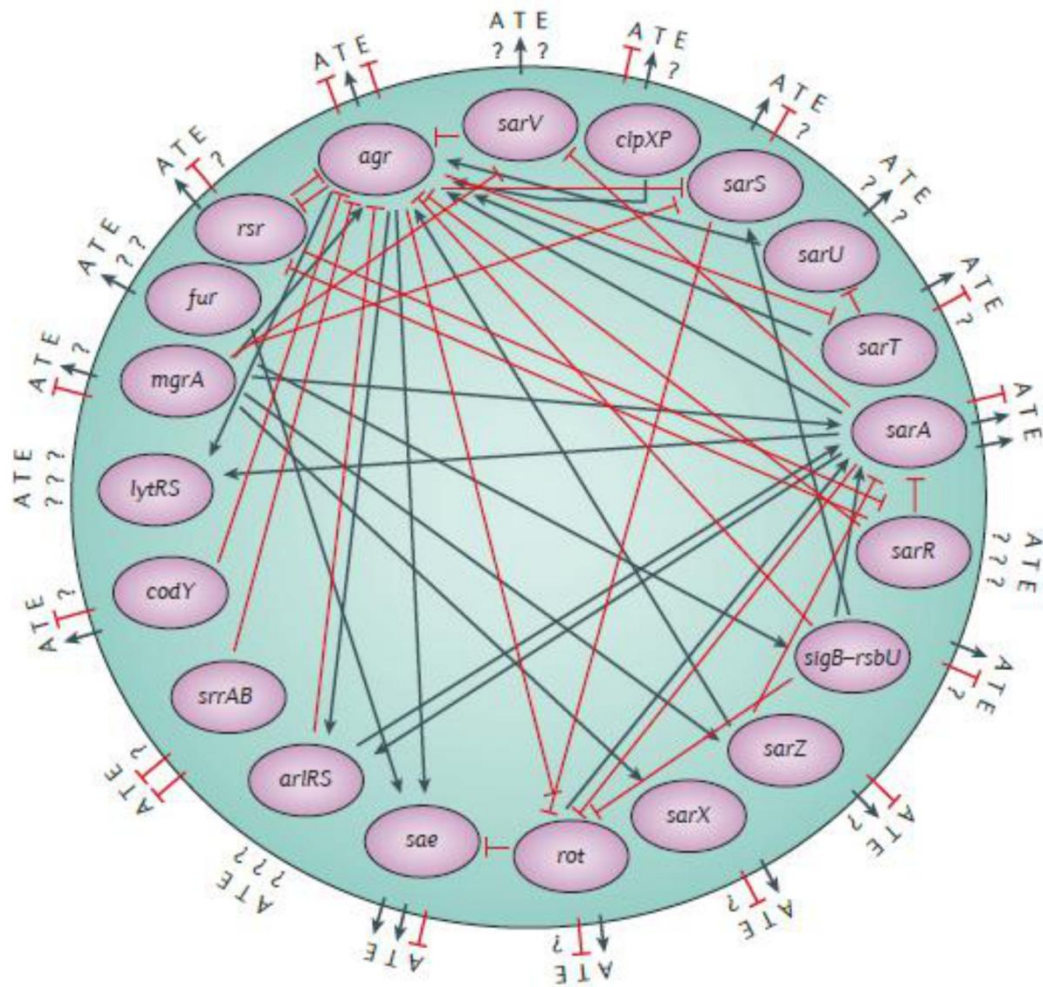
Figure 1.9: Virulence regulatory network of *S. aureus*. The known virulence regulators (sRNAs are omitted) are illustrated inside the oval shapes, where their respective effect on one another is represented by arrows (positive effect) or bars (negative effect). The outside of the circle represents the known effect each regulator has on adhesiveness (A), toxicity (T) and evasiveness (E). A question mark illustrates that either there is no information regarding its effect on a particular phenotype or the information regarding this effect is conflicting. Image taken from (96).

1.3.4.5 RNome

As stated previously, *S. aureus* adapts to a wide variety of environments using multiple TCS and transcription factors to modulate gene expression in response to several environmental cues. Another layer of regulatory complexity is added through

the use of a wide spectrum of small, non-coding RNAs (sRNAs) (394, 395). For clarity, these have been omitted from figure 1.9, however they are extremely important in rapid regulation of genes, including those coding for virulence factors. The classic representative of this group, RNAIII, which couples quorum sensing regulation to the expression of virulence genes, is the most widely studied member of this group and has been discussed in the previous sections. *psm-mec* represents another important sRNA located on certain SCC*mec* elements and exhibits virulence inhibitory properties, and has been reviewed in section 1.3.1.2. This section will discuss three other recently discovered sRNAs (*sprD*, SSR42 and *artA*) and their impact on virulence.

The SprD (**s**mall **p**athogenicity island **r**NAs) molecule is an interesting example of a sRNA, as it is encoded within a pathogenicity island, but regulates the expression of genes found on the core genome. SprD is expressed during the exponential phase of growth, and is independent of RNAIII (396). The Sbi immune evasion protein was identified as a target for SprD and molecular analysis revealed that SprD interacts with the *sbi* mRNA specifically covering (SD) sequence preventing the initiation of translation (396). The deletion of *sprD* caused a significant decrease in mouse lethality in a sepsis model of infection, highlighting the role SprD in overall virulence (396).

Another important regulatory RNA involved in virulence alteration is the 891-nt *S. aureus* regulatory RNA, SSR42, molecule (397). This molecule is part of a family of **s**mall **s**table **R**NAs (SSRs) which are produced and/or stabilised in response to specific growth phases and harsh conditions, hypothesised to help enable the organism to adapt to unfavourable conditions (398). SSR42 is expressed and predominantly stabilised during stationary phase of growth with deletion of SSR42 (in a UAMS-1 (USA200) strain) affecting the expression of 82 transcripts during this phase of growth (397). Interestingly, deletion of SSR42 resulted in the upregulation of cell surface virulence determinants such as *spa* and *sbi*. Deletion of SSR42 in strain LAC (USA300) resulted in a different set of genes being modified; highlighting specific genes are affected depending on their genetic background. In

LAC, SSR42 contributes to pathogenicity in a SSTI mouse model of infection, is important in lysing erythrocytes and confers a survival advantage against polymorphonuclear cells through its positive effect on *hla*, *hlgC* and *lukF-PV* expression (397).

A recently discovered sRNA affecting virulence has been shown to interact with other virulence regulators to modulate toxin production. The ArtA (AgrA-repressed, toxin regulating sRNA) sRNA represents another RNA molecule that is regulated, in this case repressed, by AgrA through direct binding to the *artA* promoter (399). Deletion of *artA* resulted in a decrease in α-haemolysin, which was mediated via increased *sarT* expression (399). It was shown that the regulatory activity of ArtA centred on the repression of *sarT*, via stable interaction with *sarT* mRNA providing a substrate for RNaseIII (399). This sRNA illustrates how important these regulatory molecules are in overall pathogenicity and provide clues on how the complex interplay between known regulators occurs.

1.3.5 Virulence determinants

S. aureus expresses a plethora of virulence determinants, a Swiss-army knife of surface proteins, toxins, enzymes, immune inhibitory proteins, superantigens and biofilm specific components, resulting in an extremely formidable pathogen. These factors are differentially expressed depending on growth phase and environmental conditions. Expression patterns are strain-dependent, with certain factors specific in particular diseases. Table 1.1 illustrates the known virulence factors and their primary function. In the proceeding section an overview of some of the most important virulence factors and their relevance in disease will be discussed, sectioned into the two main categories of virulence investigated in this thesis, adhesion and toxicity, focusing primarily on cell wall and cytolytic proteins.

1.3.5.1 Cell wall proteins

S. aureus comprises an impressive reservoir of 24 different cell wall-anchored (CWA) proteins which are differentially regulated depending on the environmental conditions (for example, IsdA is up-regulated during iron-limiting conditions (400)) and growth phase (98). CWA proteins exhibit conserved features. An amino-terminal secretory signal is required for Sec-dependent protein secretion, while the carboxyl-terminal end contains several domains: a wall-spanning element rich in either proline or glycine residues, or composed of serine-aspartic acid repeats, an LPXTG motif, followed by a membrane-spanning hydrophobic domain and lastly a region composed of a series of positively charged residues (401). Cleavage of the LPXTG motif between the threonine and glycine residues results covalently linkage of the protein to the peptidoglycan cell wall, carried out by the transpeptidase activity of the sortase A enzyme (402).

The most predominant proteins are those that belong to the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs), which are defined by their tandemly linked IgG-like folded domains in the A region (98). A large degree of functional redundancy exists between CWA proteins, whereby multiple proteins can bind the same host ligand (at least 5 CWA proteins bind fibrinogen). CWA proteins are fundamental for the initial attachment phase, which is a precursor for colonization and infection. These proteins are important virulence factors which can promote adhesion to the extracellular matrix, mediate invasion of specific host cells, and evasion and inhibition of the innate and adaptive immune system.

Virulence Factor	Function
Secreted Factors	
Superantigens	
Toxic Shock Syndrome Toxin -1 (TSST-1)	Immunomodulation
Staphylococcal enterotoxins (SE) SEA to SEG	Immunomodulation, gastrointestinal toxicity
Staphylococcal enterotoxin like (Sel) SelX	Immunomodulation

Cytolytic Toxins	*Cytotoxicity/Membrane requirement*
α-haemolysin (*hla*)	Monocyte, erythrocyte - ADAM-10, inflammation
β-haemolysin (*hlb*)	Monocyte, erythrocyte - sphingomyelin
γ-haemolysin (*hlgACB*)	Erythrocyte and neutrophil - unknown
δ-haemolysin (*hld*)	Neutrophils, erythrocytes, monocytes lymphocytes, biofilm *
PSMα1	Neutrophils, erythrocytes, monocytes lymphocytes, biofilm *
PSMα2	Neutrophils, erythrocytes, monocytes lymphocytes, biofilm*
PSMα3	Neutrophils, erythrocytes, monocytes lymphocytes, biofilm*
PSMα4	Weakly cytotoxic- erythrocytes, lymphocytes, biofilm*
PSMβ1	Weakly cytotoxic - erythroctyes, biofilm*
PSMβ2	Weakly cytotoxic - erythroctyes, biofilm*
PVL (*lukSF*-PV)	Neutrophil, monocyte cytotoxicity - C5aR
LukAB/GH	Neutrophils - CD11b
LukED	Neutrophils, monocyte, lymphocyte - CCR5, CXCR1/2

Enzymes	
Coagulase (*coa*)	Clotting and abscess formation
Hyaluronidase (*hysA*)	Hyaluronic acid degradation - dissemination
Catalase (*katA*)	Inactivates H_2O_2 - defence
DNase	Degrades eDNA – biofilm
Elastase	Degrades elastin - dissemination
V8 protease	Serine protease - protein degradation
Superoxide dismutase	Defence against reactive oxygen species
Exfoliative toxin (*eta*, *etb*) – serine protease	Cleavage of desmosomal cadherins
Glycerol ester hydrolases (*lip*, *geh*)	Degrades triacylglcerols
Fatty-acid modifying enzyme (FAME)	Inactivates bactericidal fatty acids
O-aceyltransferase (*oatA*)	Lysosome resistance
Phosphotidylinositol-specific phospholipase C (*plc*)	Degrades phospholipids

ACME enzymes, (*arc*, *opp*, *speG*)	Colonisation, resistance to polyamines

Immune evasins

Staphylokinase (*sak*)	Anti-opsonic
Aureolysin - metalloprotease (*aur*)	Cleavage of C3 - inhibition of phagocytosis
Staphylococcal complement inhibitor (SCIN) protein	Binds C3 convertases, inhibits C3b deposition
Extracellular fibrinogen binding protein (Efb)	Binds, Fb, C3b and C3d, inhibits opsonisation
Formyl-like 1 inhibitory protein (FLIPr)	Inhibits chemotaxis
Staphylococcal immunoglobulin-binding protein (Sbi)	Binds IgG and C3 protein
Chemotaxis inhibitory protein (CHIPS)	Inhibits chemotaxis and extravasation
Extracellular adherence protein (Eap)	Interferes with neutrophil recruitment and extravasation
Staphylococcal superantigen-like protein 5 (SSL-5)	Inhibits PMN recruitment
Staphylococcal superantigen-like protein 7 (SSL-7)	Binds C5, inhibits complement
Extracellular complement binding protein, Ecb	Inhibits C3 convertase

Cell wall associated virulence factors:

Surface proteins/polymers/polysaccharides

Fibronectin binding proteins A and B, FnBPA/B	Binds ECM proteins - colonisation and invasion
Clumping factor A, ClfA	Binds Fb - Inhibits phagocytosis
Clumping factor B, ClfB	Binds Fb, Ln and C10 - nasal colonisation
S. aureus protein A (Spa)	Binds multiple proteins - inhibits phagocytosis
Serine-aspartic acid-rich proteins (SdrC, D)	Binds ECM - nasal colonisation
SdrE	Binds complement factor H
S. aureus surface proteins (Sas)	Binds ECM - nasal colonisation
SasG	Binds ECM - nasal colonisation and biofilm
Collagen binding protein (Cna)	Binds type I and IV collagen - septic arthritis
Iron surface determinants (Isd) A	Binds ECM proteins - Nasal colonisation
IsdB	Binds haemoglobin and hemin

IsdC	Binds hemin
IsdH	Binds haptoglobin and haptoglobin-hemoglobin complex
Bone sialoprotein-binding protein (Bbp)	Binds sialoprotein and Fb
Elastin binding protein (EbpS)	Binds elastin and tropoelastin
Serine-rich surface protein (SraP)	Binds platelets
Von Willebrand factor binding protein (vWbp)	Binds and activates prothrombin, Fb and vW factor
Wall Teichoic acids (WTA)	Binds ECM - Nasal colonisation
Capsular polysaccharide (CPS)	Anti-phagocytosis
Miscellaneous	
Staphyloxanthin	Protect against reactive oxygen species
Dlt operon (*dltABCD*)	D-alanylation of teichoic acids
Mulitple peptide resistance factor F (MprF)	Lysinylation of phospholipids - prototects against neutrophil killing

Table 1.1
Abbreviations: Fb, fibrinogen; IgG, immunoglobulin; PMN, polymorphonuclear leukocyte; ECM, extracellular matrix; Ln, loricrin; C10, cytokeratin 10;
*No receptor required

Arguably the most important CWA proteins involved in pathogenicity are the fibronectin-binding proteins A and B (FnBPA/B), clumping factor A and B (ClfA/B) and *S. aureus* protein A (Spa).

1.3.5.1.1 Fibronectin binding proteins

The majority of *S. aureus* clinical strains (77%) encode both fibronectin binding proteins (FnBPs), FnBPA and FnBPB (403). FnBPs exhibit similar structural characteristics, and along with Clf, Sdr and Cna proteins, belong to the MSCRAMM family of surface proteins (404). FnBPs are composed of an amino-terminal A domain, which is subdivided into three subdomain N1, N2 and N3, and a region distal to the A domain composed of 11 and 10 non-identical repeats in FnBPA and B respectively (405, 406). The A domain of FnBPs are surface exposed and can interact

with ECM proteins such as fibrinogen (Fb) and elastin (407). The FnBP binding of Fb and elastin is hypothesised to occur through an elegant 'dock, lock, latch' (DLL) mechanism, which is based on the predicted binding of ClfA to Fb (407). This involves the docking of the γ-chain domain of Fb to region between the N2 and N3 domains of the A domain, inducing residues in the N3 C-domain to undergo a conformational change resulting in the formation of an extra β-strand, locking and trapping the Fb molecule (408). Interestingly, the N23 subdomains of FnBPB are also capable of binding an extra ECM protein, fibronectin (Fn), by an as of yet unknown mechanism (409). Fn binding by the FnBPs is also mediated by the multiple repeat regions (410). Fn acts as a molecular bridge, allowing *S. aureus* to interact with and induce clustering of host surface expressed α5β1 integrins (411). Clustering of integrins triggers intracellular phosphorylation activating a signalling cascade resulting in the alteration of the cellular architecture and uptake of *S. aureus* (412). In FnBPA, it has been shown that at least one high-affinity fibronectin-binding repeat is required for rapid and efficient invasion of non-professional phagocytes (410).

This invasive phenotype mediated by FnBPs is important in immune and antibiotic avoidance and for promoting dissemination to other anatomical sites (404). FnBPs have been shown to be important in numerous animal models of infection particularly in sepsis and endocarditis development (410, 413). Finally, FnBPs have been implicated in MRSA biofilm development under specific environmental conditions, where residues which are central for ligand binding do not play a role (414), highlighting another potential protein-protein interaction activity of these already multifunctional proteins.

1.3.5.1.2 Clumping factor proteins

ClfA and ClfB contain a series of Serine/Aspartic acid dipeptide repeats linking the subdomains of the A-domain to the carboxyl-terminal cell wall anchoring region (415). These proteins share significant sequence similarity, 41% and 47% identity at the N- and C- terminal respectively, while the A domain shares only 26% similarity

(415). ClfA and ClfB are expressed during different stages of growth with maximum *clfA* expression occurring during stationary phase, whereas *clfB* expression is highest during early exponential phase and is undetectable during stationary phase (415, 416). Given the low level similarity of the A domain it was surprising that ClfB also bound fibrinogen promoting adhesion and clumping (415). However, both proteins bind different regions of the Fb molecule, with ClfA interacting with the γ-chain of Fb, while ClfB binds with the alpha chains, both predicted to occur through the DLL mechanism (404). The ability of two related proteins binding the same molecule but to different regions is not uncommon in *S. aureus* and may be important in binding firmly to ECM proteins or thrombi when under flow.

ClfA has been shown to confer resistance to neutrophil-mediated phagocytosis, involving the binding of Fb molecules, preventing the deposition of oposins on the bacterial surface or masking any recognition of opsonins by immune cells (417). Recently it was shown that ClfA mediates this bacterial resistance in a Fb-dependent and independent manner, highlighting another role of ClfA in immune evasion. Subsequently, it was revealed that the A-domain of ClfA could trigger the cleavage of C3b to iC3b by interacting with and inducing the action of serum factor I (418). C3b is an important opsonin, central in the elimination of microbes, whereby cleavage of C3b to iC3b reduces the impact of this important clearance mechanism. ClfA can stimulate the activation of platelets leading to aggregation, an important prerequisite to the onset of infective endocarditis (419). This activation requires the binding of several Fb molecules by ClfA aided by the simultaneous interaction with immunoglobulin G (IgG) molecules leading to the clustering of platelet receptors GPIIb/IIIa and FcγRIIa inducing intracellular signalling cascades culminating in platelet aggregation (419). This multifunctional protein has been shown to be important in rat endocarditis (420), murine septic arthritis (421) and infective endocarditis (422) models of infection. ClfB plays an important role in adhesion and nasal colonisation, through its interaction with cytokeratin 10 and loricin as described previously in section 1.3.2.

1.3.5.1.3 Protein A

S. aureus protein A (SpA) is structurally distinct from the other CWA proteins, containing five homologous modules, designated EABC and D. Each of the modules contains a separately folded three-helical bundle structure which can interact with multiple ligands (404). Separating this region and the cell wall domain is the Xr region composed of octapeptide repeats which vary in number followed by a constant region known as Xc (404). SpA is best known for its ability to bind to the Fcγ region of the IgG, decorating the bacterial surface with IgGs orientated in the incorrect fashion, preventing recognition by neutrophils and activation of the classical complement pathway impairing phagocytosis (423). However, SpA also performs an immunomodulatory function, interacting with the Fab variable regions, V_H3, of IgM molecules which are exposed on the surface of B cells (424). This interaction can lead to the cross-linking and stimulation of B cells resulting in apoptosis, leading to B cell depletion and immunosuppression (424).

S. aureus is a primary cause of pneumonia in infants and the immunocompromised and evokes an intense host response characterised by high recruitment and infiltration of PMNs to the site of infection (425). This effect is mediated by the expression of SpA and its interaction with tumour necrosis factor receptor-1 (TNFR-1) on lung epithelium, inducing TNF-α like pro-inflammatory responses resulting in PMN mobilisation and recruitment (425). This hyper-recruitment has an adverse effect on lung epithelia resulting in cell damage, respiratory dysfunction and pneumonia.

SpA enables *S. aureus* to bind directly to the essential blood glycoprotein, von Willebrand factor (vWF) (426). The main function of vWF is to interact with exposed collagen and capture and direct circulating platelets for immobilization at the site of damaged blood vessels, promoting the formation of blood clots (426). Thus, binding of SpA to vWF allows *S. aureus* to adhere to damaged blood vessels and exposed sub-endothelial tissue when under flow conditions (426). This adhesive feature may play a role in enhanced survival in the blood and, in conjunction with

ClfA, attachment and induction of platelet aggregation, important features in the development of infective endocarditis.

1.3.5.2 Cytolytic proteins

A key component in the *S. aureus* artillery is the secretion of membrane-damaging proteins, essential in colonization and pathogenesis. *S. aureus* strains are capable of expressing numerous polypeptides which can be classified into haemolysins (α- and β-haemolysins), bi-component leukocidins (gamma-(γ-) haemolysin, leukocidins LukAB and LukED and Panton-Valentine Leukocidin PVL) and small amphipathic lytic peptides (δ-haemolysin and Phenol Soluble Modulins (PSMs)). The vast majority of clinical *S. aureus* strains contain the genes for the haemolysins and PSM peptides (312, 427). In contrast the genes for PVL are observed in approximately 5% of clinical strains and are highly associated with CA- *S. aureus* strains. The true distribution of LukAB and LukED genes in clinical isolates is unknown but has been suggested to be between 30-70% (100). It is important to note that the presence of the gene does not mean the protein is expressed as SNPs within toxin genes, mutations in virulence regulators and environmental conditions can impact on the expression of above cytolytic proteins, as discussed in more detail in Chapter 7.

1.3.5.2.1 Haemolysins

α-haemolysin is the model pore-forming toxin (PFT) and research into its structure, mechanism of action and role in disease from the last forty years has made it one of the most well-characterized virulence factors. α-haemolysin is composed of seven monomers which initially form a homoheptamer prepore, transitioning into a mature β-barrel transmembrane pore on the surface of susceptible cells (314). This causes an osmotic imbalance leading to the movement of molecules such as K^+ and Ca^{2+} in and out of the cell, resulting in cell death. Historically, the deposition of α-haemolysin monomers was shown to occur on phosphatidylcholine containing liposomes, underlining the requirement of these phospholipids (153). However, the differences in susceptibilities of rabbits and human erythrocytes to purified α-haemolysin

suggested another important receptor required in α-haemolysin activity. Recently, the identification of A Disintegrin and Metalloprotease Domain-containing protein 10, (ADAM10) highly expressed on rabbit erythrocytes (among other cell types) was shown as an important receptor for α-haemolysin activity (428).

α-haemolysin is responsible for the lysis of several other cell types including lymphocytes and monocytes (309, 314, 429) and binding with ADAM-10 activates the intrinsic metalloprotease activity of this protein resulting in degradation of E-cadherin and disruption of the epithelial barrier (100). Sub-lytic concentrations of this toxin can induce pro-inflammatory responses through activation of the inflammasome, a complex signalling platform, resulting in necrotic tissue injury (430). The cytolytic, immunomodulatory and barrier disruptive activity conferred by this single toxin confirms α-haemolysin as a main virulence factor in *in vivo* experiments. This toxin has been implicated as a critical factor in *S. aureus* pneumonia (315), sepsis (431), brain abscess (432) and SSTI (311).

The β-haemolysin is a neutral sphingomyelinase, which targets sphingomyelin, a specific type of membrane lipid resident in the plasma membrane (433). This lipid is highly associated with cholesterol and is enriched in lipid ordered membrane microdomains (433). This enzyme hydrolyses sphingomyelin into ceramide and phosphorylcholine leading to aggregation of microdomains, possibly affecting the fluidity and stability of the membrane resulting in cell lysis, although the exact mechanism is poorly understood (433). The classic hot-cold lysis assay to investigate β-haemolysin activity highlights the relative inactivity of this toxin to erythrocytes, compared to other cytolytic peptides. Similarly, β-haemolysin is toxic to monocytes, but at a reduced level, about 50% activity compared to α-haemolysin (100). The gene for β-haemolysin, *hlb*, is also a site for bacteriophage insertion, which results in disruption of the gene. The majority of phages which integrate at this site carry with them components of the immune evasion cluster (IEC) such as staphylokinase and chemotaxis inhibitory protein (CHIPS) (434) and the majority of clinical strains carry components of the IEC and thus are β-haemolysin negative (435), questioning the relevance of this toxins contribution to overall virulence.

In spite of the low prevalence of *hlb* clinically, it has been shown to be important in promoting lung epithelial damage and inflammation in a mouse model (436) most likely through the release of ceramide after hydrolysis of the cell membrane. Ceramide is believed to activate the inflammasome through interaction with NLRP3 receptor, promoting pro-inflammation and resulting tissue damage.

1.3.5.2.2 Bi-component toxins

Bi-component toxin activity requires the involvement of two polypeptides, designated S (slow) and F (fast) based on their electrophoretic mobility. The most studied of these toxins is the γ-haemolysin, but the mechanism of action is believe to be similar for all, consistent with the significant homology shared among these proteins (437). In a similar fashion to α-haemolysin, each monomer of the bi-component toxins binds to the cell membrane before oligomerizing into a pre-pore composed of four S components alternatively arranged with four F components (438). Prepore transition into a mature homo-octamer transmembrane β-barrel results in host cell lysis.

There are four members of this family of PFTs: γ-haemolysin, composed of two S subunits, HlgA or HlgC, and F subunit HlgB; PVL, consisting of LukS-PV and LukF-PV; LukAB and LukED. These toxins are responsible for the lysis of a broad spectrum of host cells. γ-haemolysin and LukED are haemolytic whereas PVL and LukAB are not (439). The specific binding requirements of these toxins to erythrocytes is not fully understood, however for γ-haemolysin the architecture and membrane components plays a major role in pore-formation (440). γ-haemolysin and PVL are very toxic to neutrophils and macrophages, but LukAB is only toxic at the same level when the concentration is increased 100-fold (437), suggesting that this is not the primary target for LukAB. Recently, LukED has been shown to bind to the CCR5 receptors and efficiently lyse T-lymphocytes, ending a decade long investigation into its specific cell target (441). Recent works has also elucidated the specific receptors required for binding of the other bi-component toxins to susceptible cells. The common theme among these receptors is their importance in

immune function. LukAB lyses neutrophils by binding to the CD11b subunit of the integrin Mac-1, an important receptor involved in leukocyte adhesion and chemotaxis (442). Recent work has shown that after exposure to neutrophils, USA300 preferentially activates the expression of the promoter of *lukAB* operon (443). This pre-emptive attack on ensuing neutrophils is coupled with the continued expression upon phagocytosis promoting escape, underlying the dual role played by this toxin (443). *Spaan et al* showed that PVL targets the human complement receptors C5aR and C5L2, possibly underlying the reason why murine PMNs were more resistant to PVL than human or rabbit PMNs (308). CCR5, C5aR and C5L2 are G-protein coupled receptors involved in cytokine sensing and response to inflammatory mediators resulting in neutrophil recruitment to the site of infection (444).

Toxins' acting on the same cell types highlights the degree of redundancy which exists within this family, in an analogous fashion observed with some of the CWA proteins binding the same ECM proteins. However, investigating the effects of sub-lytic concentrations of these toxins opens up a new area of investigation, potentially uncovering new functions for these virulence factors (100). For example sub-lytic concentrations of PVL can lead to neutrophil activation, or priming, resulting in the secretion of potent inflammatory mediators such as IL-8 and leukotriene B4 (445). The resulting release of such chemicals triggers massive inflammation, and when occurring within the lung, contributes to the development of pneumonia, a disease in which PVL plays a central role (310). Clinical PVL positive strains also displayed enhanced adhesion to collagen and laminin, two important matrix proteins, enabling the attachment to damaged airway epithelium, potentially playing a significant role in the development of necrotizing pneumonia (446). The ability to confer the observed increased adhesion was attributed directly to the signal peptide of the LukS-PV (446).

The importance of these toxins is further shown using isogenic deletion mutants and assessing the impact in animal models. These experiments illustrate the direct role of toxins in specific diseases, further distancing the idea that these toxins

are redundant in *S. aureus*. As described above, PVL has been shown to be a key virulence factor in rabbit pneumonia model but also in rabbit osteomyelitis (447). The importance of the leukocidins in immune evasion and destruction of neutrophils is highlighted by their role in human blood survival (437) and murine model of bacteraemia (448).

1.3.5.2.3 Small amphipathic lytic peptides

The δ-haemolysin and PSM peptides represent a class of small amphipathic membrane damaging toxins affecting a broad repertoire of host cells, requiring no proteinaceous receptor for activity. The δ-haemolysin is encoded with the *rnaIII* gene while the PSM peptides form two classes encoded on the core genome; *psmα* operon encoding the small (20-25 amino acid) PSMα1-4 peptides and *psmβ* operon expressing the larger (44 amino acids) PSMβ1-2 (312).

The exact mechanism of action of the PSM peptides is unknown but assumed to be similar to that proposed for δ-haemolysin (449). The hypothesis is based on whether the toxin is expressed at low or high concentration. At low concentration, it is suggested that δ-haemolysin dimers lie horizontal to the surface of the membrane, at the interface of the polar head groups and acyl chains. These dimers can either aggregate forming small and large channels resulting in osmotic imbalance, or can self assemble on the surface causing a bilayer curvature strain leading to membrane instability in a 'sinking-raft like' model (450). At high concentration, δ-haemolysin seems to act like a detergent, leading to the rapid solubilisation of the membrane forming micelles, resulting in cell lysis (449).

PSM peptides are multifunctional and are influential in many aspects of *S. aureus* pathogenesis. These peptides have been shown to lyse neutrophils and erythrocytes (312, 316), and in this thesis, lymphocytes (T cells) (Chapter 6). Intriguingly, although the PSM peptides are very similar, possibly arising through sequential gene duplication events, PSMα3 has a significantly higher lytic activity to neutrophils than the other peptides (70). This peptide has been implicated as a key

element responsible for the highly virulent phenotype of CA-MRSA strains (312). However, the same pattern of lysis observed with PSMs and neutrophils is not observed with erythrocytes or vesicles, most likely due to differences in the membrane composition. One of the key features of highly virulent CA-MRSA is the ability to survive and lyse neutrophils following phagocytosis (296). The role of PSMs in intracellular escape from the phagosome was predicted after it was shown that serum lipoproteins reduced the cytolytic activity of PSMs (451). In an experiment similar to that illustrating the activation of the *lukAB* promoter in the presence of neutrophils, it was shown through *psmα* promoter-GFP constructs that PSMs were expressed after phagocytosis (451). The fact that *S. aureus* employs two toxins to mediate escape and lysis of neutrophils after phagocytosis illustrates the importance of neutrophils for controlling *S. aureus* survival.

At sub-lytic concentrations PSMs attract, stimulate and induce the release of cytokines from neutrophils through their interaction with the formyl peptide receptor 2 (FPR-2) (312, 452). At first this activation may seem counter-productive to survival, however overexpression of neutrophil mediators may lead to the tissue destruction and future dissemination. The FPR receptor is also present on mouse dendritic cells, whereby PSM can bind to these receptors and modulate the activity of these cells, reducing the expression of proinflammatory cytokines such as TNFα and IL-6 but inducing anti-inflammatory cytokines such as IL-10 (453). This immunomodulation results in a decreased activation of CD4$^+$ T cells and reduced Th1 differentiation, hypothesized to contribute to *S. aureus* immune evasion (453).

PSMs are instrumental in biofilm formation, particularly in facilitating intricate channel development important for nutrient and waste transport (128). The biophysical characteristics of these molecules, namely their amphipathic property, suggest that they play a central role in the release of cells or cell clusters from the biofilm surface (128). *In vivo* animal experiments highlight the importance of PSM is specific infections. Deletion of the *psmα* operon exhibited reduced virulence in a mouse bacteraemia model, whereas deletion of *psmβ* operon displayed no effect (312). In analyzing the main virulence factors involved in SSTIs, PSM and α-

haemolysin were highlighted as the main contributors in a rabbit skin infection model (311). Considering their presence in virtually all clinical *S. aureus* strains in addition to the many roles played in pathogenicity, it has been proposed that PSMs, along with α-haemolysin, are the primary virulence factors responsible for *S. aureus* toxicity (454).

1.4 Summary, aims and objectives of PhD

This thesis consists of an interdisciplinary approach to understanding, measuring and identifying factors important for bacterial virulence. For clarity in highlighting the aims and objectives of this project, the thesis is divided into two main parts:

1) Investigating bacterial mediated lysis of phospholipid vesicles: implications for the development of 'smart' burn wound dressings and novel assays to measure specific surface acting virulence factors.

2) Examining factors and identifying virulence loci which alter toxicity in *Staphylococcus aureus*

1.4.1 Investigating bacterial mediated lysis of phospholipid vesicles

The main objective of the *Bacteriosafe* project (European Commission, Seventh Framework Programme) was the development of a 'smart' or 'active' burn-wound dressing. This project consisted of a pan-European consortium, composed of interdisciplinary scientists from the fields of cell biology and wound healing, physical and nano-chemistry and microbiology. The basis of the wound dressing is illustrated in figure 1.10 and consists of a phospholipid vesicle containing a fluorescent dye. Importantly, the fluorescent dye used in this system is self-quenchable at high concentrations, as is the case within the aqueous cavity of the phospholipid vesicle. The 'activity' of the wound dressing depends on the lysis of these vesicles by 'active' bacterial exofactors (toxins, enzymes, glycolipids) causing a switch from a non-fluorescent to fluorescent state, signalling an infection, highlighting the 'smart' aspect of the dressing. Our role at the University of Bath was to develop these phospholipid vesicles as sensors for bacterial detection. Briefly, this required the identification of several vesicle types which met the criteria for stability at different pH, temperatures, and in specific polymer matrices or hydrogels, while monitoring the sensitivity to bacterial exofactors (165, 455, 456). My primary objective was to identify these 'bacterial lytic agents'. The identification of these

exofactors lead to the development of two assays to actively measure important virulence factors expression in two of the most important burn wound colonisers, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, highlighted in Chapters 3 and 4 respectively.
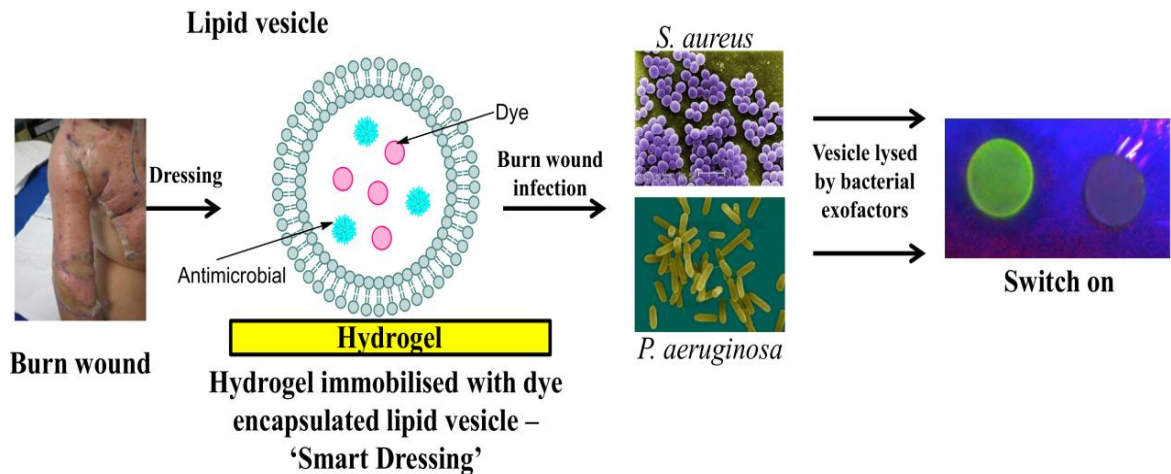
Figure 1.10: Concept behind Bacteriosafe burn wound dressing. The Bacteriosafe prototype dressing consists of self-quenchable fluorescent dye encapsulated phospholipid vesicles, which are immobilised within a protective polymeric hydrogel. Burn wounds are susceptible to infection particularly by *S. aureus* and *P. aeruginosa*. After colonisation, bacteria at the burn site express certain exofactors which are lytic to membranes. The phospholipid vesicles mimic biological membranes and are lysed by these exofactors, releasing the dye which is no longer quenched and fluoresces, signalling an infection.

1.4.2 Examining factors and identifying virulence loci which alter toxicity in *Staphylococcus aureus*

Staphylococcus aureus is a versatile human pathogen and this second project intended to gain a better understanding of how internal and external factors can modulate virulence factor expression. Keeping with the theme of the first project, one objective was to investigate the lytic activity of toxic shock syndrome toxin-1(TSST-1) positive *S. aureus* strains, as discussed in chapter 5. This was preformed as previous results suggested that TSST-1, as well as modulating its own regulation,

also down-regulated overall exoprotein expression (457), and therefore may hinder the trigger for vesicle lysis and early bacterial sensing.

Medical intervention of bacterial infection historically consists of treatment of the infection with antimicrobial agents. This comes at a cost, as antimicrobial intervention imposes a positive selection on bacteria to mutate and become resistant. Another treatment avenue is to prevent bacteria producing virulence factors by interrupting with virulence gene regulation. Chapter 6 investigates the potential of using subinhibitory oxacillin to attenuate virulence in community-acquired methicillin resistant *S. aureus* CA-MRSA (CA-MRSA) strains.

Due to the advancement in sequencing technology in the last decade, there has been an explosion of genomic data made available for various bacterial species, particularly *S. aureus*. The objective in Chapter 7 was to investigate whether it was possible to utilise the genomes sequences of 90 closely related ST239 *S. aureus* strains to identify novel virulence loci by associating genetic polymorphisms with phenotypic traits, specifically toxicity and adhesion, using a genome-wide association study (GWAS) approach. This work was done in collaboration with Dr. Mario Recker from the College of Engineering, Mathematics & Physical Sciences, at the University of Exeter.

Materials & Methods

2.1 Materials

A list of all materials can be found in Table 2.1.

Product	Description	Supplier
Acetic acid	Solvent	Sigma
Acetone	Solvent	Sigma
Agarose	DNA/RNA gels	Promega
Anhydrous tetracycline	Used for pRMC2 induction	Sigma
Ampicillin	Beta-lactam; cell wall inhibitor	Sigma
APS	Ammonium persulfate	Sigma
BHI	Brain heart infusion	Fluka
Bis-polyacrylamide	40% polyacrylamide solution	Bio-Rad
Bovine Serum Albumin	Used as a blocking reagent in binding assays	Sigma
Bradford reagent	Protein quantification	Sigma
1-butanol	Solvent	Fisher Scientific
$CaCl_2$	Calcium chloride	Sigma
Chloramphenicol	Broad-spectrum, protein synthesis inhibitor	Sigma
Chloroform	HPLC-grade Solvent	Fisher Scientific
Costar 96-well plate	96-well plate used in vesicle studies	Costar
Crystal violet	Bacterial stain	Sigma
DMEM	Dulbecco's Modified Eagle's Medium	Invitrogen
Dream Taq	DNA polymerase and PCR reagents	Fermentas
EDTA	Ethylenediaminetetraacetic acid	Sigma
Erythromycin	Macrolide antibiotic; protein synthesis inhibitor	Sigma
Ethanol	Solvent	Fisher Scientific
EZBlue	Protein gel staining solution	Sigma

FastRead counting chamber	Cell counting slide	Immune Systems
FBS	Foetal bovine serum	Gibco
Fibrinogen	Extracellular matrix protein	Sigma
Fibronectin	Extracellular matrix protein	Sigma
Gene Jet Midi Kit	Plasmid purification midi kit	Fermentas
Gene Ruler	1 kb DNA ladder	Fermentas
Gentamicin	Aminoglycoside; protein synthesis inhibitor	Gibco
Glycerol	Used in -80°C bacterial stocks	Sigma
Goat anti-*spa*	Goat polyclonal against protein A (HRP)	Abcam
GPS solution	L-Glutamine-Penicillin-Streptomycin	Sigma
Guava ViaCount	Viability stain	Millipore
HyClone DMEM	Culture media	Fisher Scientific
Isopropanol	Solvent	Fisher Scientific
LB	Luria-Bertani agar/broth	Fluka
Lysostaphin	Glycyl-glycine endopeptidase	Cell Sciences
Mouse anti-*lukS*	Monoclonal antibody against LukS-PV	IBT bioservice
NaCl	Sodium chloride	Sigma
NaOH	Sodium hydroxide	Sigma
Nap-25 columns	Sephadex G-25 DNA grade packed columns	GE Healthcare
Nunc 24-well plates	Tissue culture plates	Nunc
Nunc 96-well plates	Binding assay plates	Nunc
Olive oil	Substrate in lipase plate assay	Sigma
Opti4CN substrate kit	Colorimetric (HRP) substrate	Bio-Rad
Oxacillin	Beta-lactam, cell wall inhibitor	Sigma
Page Ruler Plus	Pre-stained protein ladder	Fisher Scientific
Paraformaldehyde	Fixative in binding assay	Sigma
PBS	Tissue grade phosphate-buffered saline	Gibco
PCR purification kit	Purification of PCR products	Fisher Scientific
Mouse anti-*mecA*	Mouse monoclonal antibody to PBP2a	Abnova
peqGreen	DNA/RNA dye	peqLab
Phusion DNA polymerase	High-fidelity DNA polymerase	New England Biolabs
Proteinase K	Broad-spectrum serine protease	Sigma
Protein G-HRP	Protein G-Horseradish peroxidase conjugate	Invitogen

PSM peptides	Peptide toxins	Severn Biotech
Rabbit anti-*hla*	Polyclonal whole antiserum against α-haemolysin	Sigma
Rabbit anti-*tst*	Polyclonal antibody against TSST-1	Abcam
Raffinose	Trisaccharide; used in sphaeroplast buffer	Sigma
Random Hexamer	Primers used in reverse transcription	Qiagen
Reverse transcriptase	Superscript II reverse transcriptase	Invitrogen
Rhamnolipid R-95	Pure rhamnolipid; glycolipid	Sigma
Rhodamine B	Fluorescent dye, used in lipase plate assay	Sigma
RNAprotect	Immediate stabilisation of RNA	Qiagen
RNeasy Midi Kit	RNA purification kit	Qiagen
RPMI 1640	Roswell Park Memorial Institute cell culture media	Gibco
SDS	Sodium dodecyl sulphate	Sigma
Skim milk powder	General blocking reagent	Sigma
T-75	Tissue culture flask	Corning
TAE	Tris-acetate EDTA buffer	Sigma
TEMED	N, N, N, N'-tetramethylethylenediamine	Sigma
Triton X-100	Detergent	Sigma
Trypan Blue	Vital cell stain	Sigma
Trypsin-EDTA (0.25%)	Cell detachment solution	Gibco
TSA	Tryptic Soy Agar	Sigma
TSB	Tryptic Soy Broth	Sigma
TSST-1	Toxic shock syndrome toxin-1	Sigma
Turbo DNAse	Fast acting DNAse	Ambion
Qubit RNA assay kit	Fast and accurate RNA quantification	Invitrogen
Virkon	Disinfectant	Fisher Scientific

Table 2.1: List of materials used

2.2 Lipid vesicle development

Aqueous buffer solutions used in the development of phospholipid vesicles are given in Table 2.2 and 2.3. These compounds were dissolved in deionised water, sonicated and left at 4°C overnight.

Compound	Mass (mg / L)
HEPES	2382
NaCl	6240
NaOH	224
EDTA	292.2

Table 2.2: HEPES buffer solution, pH 7.4
Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaCl, sodium chloride; NaOH, sodium hydroxide; EDTA, ethylenediaminetetraacetic acid

Compound	Mass (mg / 100 mL)
5(6)-Carboxyfluorescein	1877
HEPES	238.2
NaCl	58.4
NaOH	540.4
EDTA	28.4

Table 2.3 50 mM 5(6)-carboxyfluorescein buffer

Stock solutions of vesicle components were separately dissolved in 1 mL of chloroform and stored in glass vials at -20°C, according to Table 2.4.

Compound	M_r (g mol^{-1})	Mass (mg)	Concentration (mol dm^{-3})
TCDA	346.55	34.6	0.10
DPPC	734.04	73.4	0.10
DPPE	691.96	69.1	0.10
CHO	386.65	38.6	0.10

Table 2.4: Stock solutions of vesicle components: Abbreviations: TCDA, 10, 12-Tricosadiynoic acid; DPPC 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; CHO, cholesterol.

Vesicle suspensions were prepared by mixing lipid and fatty acid components in chloroform at a 3 X concentration: 1 X concentration was prepared by using 25 mol% of 10, 12-tricosadiynoic acid (TCDA), 53 mol% 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 2 mol% 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 20 % of cholesterol (CHO). This mixture of lipids and fatty acid in chloroform was dried under nitrogen. Figure 2.1a and b illustrate the various steps in the production and determination of lipid vesicles formation. The dried lipid was (**1**) rehydrated using 5 mL of 5(6)-carboxyfluorescein (CF) (molecular structure shown in figure 2.1) buffer solution, vortexed and heated in a hot water bath at 75°C for 10 min. CF is self-quenched at high concentrations (i.e. encapsulated within vesicles), but fluoresces when diluted to lower concentrations (i.e. when vesicles are lysed). Three freeze/thaw cycles were carried out by initially immersing the lipid-containing vial in liquid nitrogen and then heating the vial back to room temperature to homogenize the solution (164). A turbid solution was an indication of vesicle formation. Vesicles were then (**2**) extruded five times at 55°C using a Liposofast vesicle extruder (Avestin, USA) through 2 × 0.1 μm polycarbonate filters under nitrogen pressure. The initial opaque vesicle solution becomes translucent after extrusion. The vesicle solution was then (**3**) purified using Illustra Nap-25 columns to remove any un-encapsulated CF dye. The columns were washed with 15 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer to remove the 0.15% Kathon CG/ICP biocide. 2 mL of vesicle solution was added to each column and allowed to load. 1 mL of HEPES buffer was then added and left to drain. After this a further 2 mL of HEPES buffer was added and the resultant pure vesicle was collected and stored at 4°C overnight. These polydiacetylene containing lipid vesicles were then (**4**) cross linked and photo polymerised using a CL1000 Ultraviolet cross linker (Hamamatsu, Japan) on setting one for 6 s. Section (**4**) of figure 2.1 illustrates a TCDA molecule undergoing a conformational change after exposure to UV. The cross linking of the TCDA molecules leads to higher stability, while retaining sensitivity to bacterial supernatant (165).

A) Liposome Development

1) Rehydration

Carboxyfluorescein

High Low
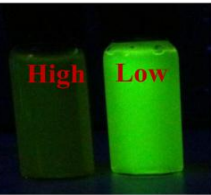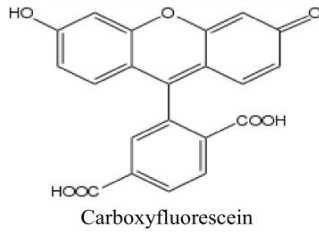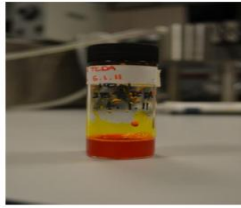
2) Extrusion
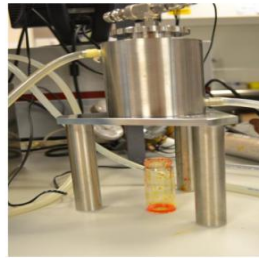
3) Purification

4) UV cross linking

UV
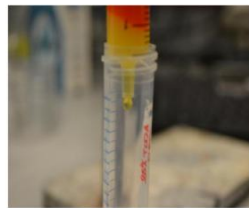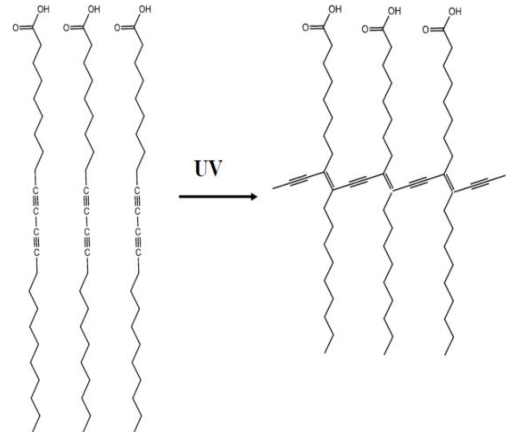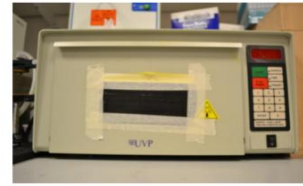
B) Size Distribution
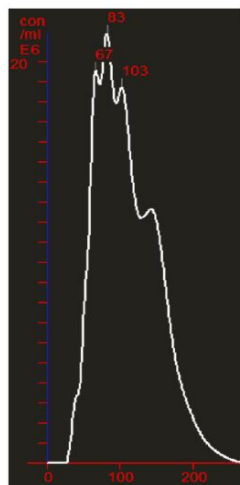
Nanosight Measurement
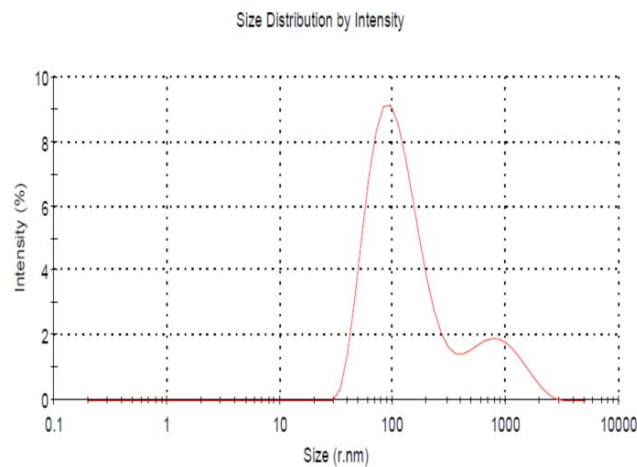
Dynamic Light Scattering Measurement
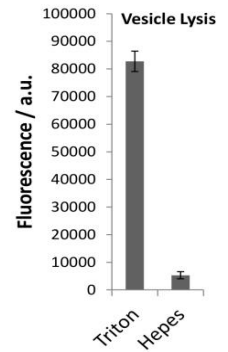
Simplified method to ensure vesicle formation

Figure 2.1: Development and validation of lipid vesicles. A) Steps in the production of vesicles beginning with **1) rehydration** of the lipid film using the 50 mM carboxyfluorescein (chemical structure shown), **2) extrusion** through 100 nm polycarbonate filters, **3) purification** using sephadex columns and **4) UV cross linking** lipid-polydiacetylene vesicles. **B) Size distribution** of the vesicles using nanosight tracking analysis and dynamic light scattering illustrates vesicle size between 90-110 nm. Use of 0.01% Triton X-100 and HEPES buffer as positive and negative controls showing maximum fluorescence release with the addition of 0.01% Triton X-100.

Size distribution and concentration of vesicle was measured via dynamic light scattering (Malvern) and nanosight tracking analysis (Nanosight Ltd) respectively, producing a size distribution of 90-110 nm and concentration of 1×10^8 particles/μL. All chemicals were purchased from Sigma-Aldrich, UK, and lipids and cholesterol from Avanti Polar Lipids, USA. These vesicles were stored at 4°C and were stable for up to 12 days under these conditions before leakage of CF was observed.

2.3 Bacteria

2.3.1 Bacterial strains and culture conditions

Bacterial strains used are listed in Appendix A. Bacterial strains were routinely stored at -80°C in 15% glycerol/broth stocks until required. Unless stated otherwise, *S. aureus* strains were streaked onto Tryptic Soy agar (TSA) and single colonies transferred to 5 mL Tryptic Soy broth (TSB). *Pseudomonas aeruginosa* and *Escherichia coli* strains were routinely plated onto Luria Bertani (LB) agar and single colonies transferred to 5 mL of LB broth. All bacterial cultures were propagated in a shaking incubator for 18 h at 37°C at 180 rpm. For detection of Panton-Valentine Leukocidin, bacterial strains were grown in casein hydrolysate and yeast-extract containing medium (CCY) (458). Bacterial strains were grown in 25 mL glass universal tubes which were decontaminated using Virkon disinfectant, washed thoroughly to prevent any residual disinfectant remaining and subsequently autoclaved. Single point bacterial growth measurements were analysed using a micro plate reader (BMG LABTECH) at OD_{600}. Where continuous bacterial growth curve analysis and/or dual absorbance/fluorescence was required, bacteria were measured at OD_{600} at 37°C and shaking at 300 rpm in Costar 96-well round-bottomed plates using a dual absorbance/fluorescence script. To identify specific roles of toxins/enzymes/glycolipids in vesicle lysis, bacterial supernatants were heated to 95°C for one hour. Where appropriate antibiotics were used with the following concentrations; *S. aureus* strains: tetracycline (10 μg/mL), oxacillin (0.5 μg/ml);

chloramphenicol (10 µg/mL), erythromycin (5 µg/mL). *P. aeruginosa* strains: tetracycline (200 µg/mL), gentamicin (100 µg/mL). *E. coli* DH5α: ampicillin (100 µg/mL).

2.4 Cell culture

2.4.1 T2 cell toxicity assay

Cell culture

In the cell toxicity assay, immortalized human T2 cells (459) were used in accordance with guidelines form the American Type Culture Collection. T2 cells are derived from a mutant TxB hybrid cell line T cells were grown in T75 tissue culture flasks containing Roswell Park Memorial Institute (RPMI) 1640 cell culture media supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1 µM L-glutamine, 200 units/mL penicillin and 0.1 mg/mL streptomycin (GPS), at 37°C in a humidified incubator with 5% CO_2 in air. Cells were routinely viewed microscopically and split every 48-60 hours. Cells were harvested by centrifugation at room temperature for 10 min at 1500 *g,* gently washed and resuspended in tissue-culture grade phosphate buffered saline (PBS) to a final concentration of $1–1.5 \times 10^6$ cells/mL using a FastRead counting chamber. This procedure typically yielded > 95 % viability of cells, as determined by trypan blue exclusion using 0.4 % trypan blue solution.

Toxicity assays

T cells were subjected to three types of treatment: 1) Bacterial supernatant (active and heat-inactivated), 2) purified phenol soluble modulin (PSM) and δ-haemolysins and 3) purified rhamnolipid. For the first two treatments, 20 µL of cells were incubated with 20 µL of staphylococcal supernatant or toxin for 12 min at 37°C.

Following incubation, 260 µL of Guava ViaCount viability strain was added and left to incubate for 5 min at room temperature. Viability of cells was then analysed using the Guava easyCyte flow cytometer (Millipore) under the following conditions: flow rate of 0.59 µL/s, with 250-300 cells/µL; adjustment of FSC threshold and viability marker to correct for cell debris and for increased discrimination between viable and non-viable cells, according to manufacturer's instructions. For treatment 3), *P. aerugionosa* supernatants and purified rhamnolipid, 15 µL of cells and 15 µL of supernatant or purified rhamnolipid were incubated for 12 min. 15 µL of 0.4% trypan blue was added and mixed with the above solution and added to a FastRead counting chamber and viability assessed through trypan blue exclusion and examination under an Olympus CX31 light microscope. This method was preferred in this treatment as neat *P. aeruginosa* supernatant and high concentrations of purified rhamnolipid caused total cell lysis and release of genomic DNA (addition of DNAse caused viscous cell mixture to return to normal fluid solution) inhibiting viability counts using the flow cytometer. Cell toxicity experiments were done in duplicate three times.

2.4.2 Erythrocyte and polymorphonuclear leucocytes harvesting and lysis assay

Polymorphonuclear leukocytes (PMNs) were isolated from heparinized venous blood obtained from healthy adult volunteers. Whole-blood samples were layered onto density gradient medium (Lympholyte cell separation medium) and centrifuged as described by the manufacturer (Cederlane). The PMN-containing band was harvested and diluted with an equal volume of culture medium (HyClone Dulbecco's modified Eagle's medium [DMEM]–10% fetal calf serum [FCS] cell medium) at a concentration of 0.5 N to restore normal osmolality. The cells were further diluted with 2 volumes of culture medium and washed by centrifugation for 10 min at 400 *g*. The cells were suspended in 5 mL of 0.2% NaCl and incubated for 120 s to lyse erythrocytes by osmotic shock, followed by the addition of 5 mL of 1.6% NaCl for 120 s to normalize the osmolality and centrifugation at 400 *g* for 10 min. Purified PMNs were then washed in PBS and enumerated by using a haemocytometer. Purity

was assessed by trypan blue and flow cytometric analyses. The final PMN count was adjusted to 1-2 x 10^6 cells/mL with culture medium. 30 μL of PMNs were incubated with 30 μL of bacterial supernatant for 30 min, and cell viability was assayed by using Guava ViaCount and easyCyte flow cytometer as above. Experiments were done in duplicate three times.

Erythrocytes were isolated from the same blood samples and washed twice by gentle resuspension with a 10 mL volume of sterile saline (0.9% NaCl) and centrifugation at 1,000 g for 10 min. Erythrocytes were diluted to 1% (vol/vol), and 200 μL of cells were incubated with 50 μL of 30% bacterial supernatant for 30 min, using free saline as a negative control and 1% Triton X-100 as the positive control. Intact cells and cellular debris were removed by centrifugation at 1,000 g for 10 min. RBC lysis was assayed by determining the absorbance of the resulting supernatant at an OD404 nm using a micro plate reader (BMG LABTECH). These assays were performed in duplicate three times.

2.4.3 Endothelial cell invasion assay

Cell culture

The EA. Hy926 cell line was constructed by the fusion of human umbilical endothelial cells (HUVEC) and the permanent lung epithelial carcinoma cell line A549 (460). EA. Hy926 cells were routinely cultured in DMEM media supplemented with 10% FCS and GPS at 37°C and 5% CO_2. These cells were cultured in T75 flasks to approximately 95% confluency and routinely observed microscopically. Cells were liberated using trypsin-EDTA, resuspended in culture medium and approximately $5x10^5$ cells (in 0.5 mL medium) were seeded into each 24-well tissue culture plates. Following incubation for 48 h, cell culture medium was aspirated and cell monolayers were washed gently in PBS. New cell culture medium was added with the exception of the GPS supplement (450 μL per well).

Invasion assay

To each well, 50 µL of washed bacteria were added (approximately 1×10^7 CFU/mL) and incubated for 15-60 min. After the specific time point, the medium was aspirated and wells gently washed once in PBS and replaced with 500 µL DMEM/10% FBS supplemented with 200 µg/mL gentamicin and incubated at 37°C in 5% CO_2 for 60 min. This medium was removed and wells gently washed twice in PBS. 500 µL of 0.5% Triton X-100 was added and resuspended to fully lyse the endothelial cells and the CFU was enumerated by serial dilution and plating onto TSA agar plates and incubated overnight at 37°C. Invasion assays were perfomed in duplicate three times.

2.5 DNA/RNA

2.5.1 RNA isolation

Overnight cultures of *S. aureus* were diluted 1:1000 into fresh TSB and grown at 37 °C for 8 h (late exponential phase), at which time, samples were collected for RNA isolation after normalisation of optical densities. Cultures were treated with two volumes of RNAprotect, incubated at room temperature for 10 min, centrifuged and resuspended in Tris 0.05 M (pH 7.5). The pellet was further treated with 300 µL of 0.5 M EDTA/ lysostaphin (5 mg/mL) and incubated for 1 h. RNA was then isolated using the Qiagen RNeasy Midi Kit according to the manufacturer's instructions with the addition of Turbo DNase after the purification step. RNA purity was checked by running 5 µL of sample on a 1% agarose gel and absence of DNA further verified by PCR using standard primers. Typical RNA samples are shown in figure 2.2. The RNA was quantified using the Qubit RNA assay kit.
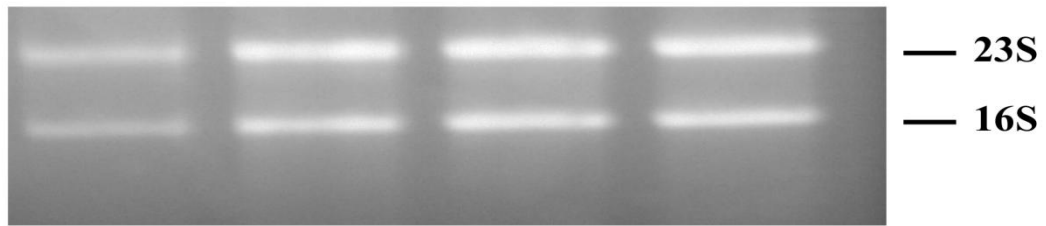
Figure 2.2: RNA isolation. Four representative RNA samples isolated from *S. aureus* cultures after DNAse treatment. 23S and 16S ribosomal RNA are indicated.

2.5.2 Reverse transcription and qRT-PCR

Reverse transcription of messenger RNA to complementary DNA (cDNA) was generated using the SuperScript II Reverse Transcriptase according to manufacturer's instructions using random hexamers. Primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3/) to produce a PCR product between 80-120 bp with an annealing temperature of 60 ℃. Primer binding sites were constructed on the RNA sequence in areas with no complex secondary structure using mfold software (http://mfold.bioinfo.rpi.edu). Primers used for gyrase B (gyrB) and RNA III are listed in Appendix A. Standard curves were generated for both primer sets on serial dilutions of cDNA to determine primer efficiency. The reverse-transcriptase PCR (RT-PCR) was performed as follows: 5 mL cDNA, 7.5 mL SYBR reagent, 0.5 ml forward and reverse primer and RNase-free water to a total volume of 15 mL. The cDNA was subjected to real-time PCR using the Applied Biosystems Step-One Real Time PCR detection system (Applied Biosystems). Cycling conditions were 95℃ for 10 min followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min and a dissociation step 95℃ for 15 s and 60℃ for 1 min. Cycle threshold values were determined for 3 biological repeats in duplicate. For each reaction, the ratio of RNA III and gyrB transcript number was calculated as follows: $2^{(Ct\ gyrB\ -\ Ct\ RNAIII)}$. RT-PCR experiments were performed in duplicate three times.

2.5.3 PCR and cloning techniques

In order to examine the effect of single nucleotide polymorphisms (SNPs) on virulence factor expression, plasmid constructs were created containing wild type *sarS* (amplified from N315, p*sarS*1) and *sarS* containing two amino acid substitutions, N221D and N243D (amplified fromMRSA252, p*sarS*2). Plasmid constructs were also created to examine the effect of SNP presence in *mecA*, containing wild type *mecA* (amplified from TW20, p*mecA*1), and *mecA* containing two SNPs at nucleotide positions T78097C (E736G) and C78222A (K611N), or the 'CA' SNPs, (amplified from strain HU24, p*mecA*2), *mecA* containing the 'CA' SNPs and SNP T78396A (K437N), or the 'A' SNP, (amplified from strain IU11, p*mecA*3) and *mecA* containing the 'CA', 'A' and SNP C78119T (E714K), (amplified from strain HU13, p*mecA*4).

Primers designed to amplify the whole *sarS* and *mecA* genes with the respective restriction enzyme cleavage sites are listed in Appendix A. Polymerase chain reaction (PCR) conditions were similar for all PCR reactions, apart from annealing temperature and duration and elongation duration. General PCR parameters consisted of: **1)** initial denaturation (95°C, 1 min), **2)** denaturation (95°C, 30 s), **3)** annealing temperature (dependent on primers (*sarS* (62°C) and *mecA* (65°C)) **4)** elongation (72°C, duration dependent on PCR product; sarS (45s) mecA (1.5 min), 30 cycles of step 2-4, 5) final elongation (72°C, 5 min) and 6) storage (4°C, unlimited). High-fidelity phusion polymerase was used to generate PCR products. PCR products were cleaned using PCR purification kit, subjected to double digest using BglII and KpnI in NE buffer 2 for *sarS* and KpnI-HF and SacI-HF in CutSmart buffer for *mecA*. Digested PCR products were cleaned in PCR purification kit and ligated into previously digested, tetracycline inducible pRMC2 plasmid (461).

2.5.4 Production of competent cells

Two 50 mL conical flasks of LB broth were inoculated 1:1000 with an overnight culture of *E.coli* DH5α and incubated for 2-4 h at 37°C with shaking at 180 rpm until an OD_{600} of 0.5-0.7. Cells were chilled on ice for 30 min before centrifugation at 3,000 rpm for 10 min at 4°C. Cells were washed in ice-cold deionised H_2O, centrifuged and gently resuspended in 15 mL of ice-cold, sterile 0.1M $CaCl_2$. Cells were left on ice for 1-2 h, centrifuged at 4°C and resuspended in 500 μL ice-cold, sterile 0.1M $CaCl_2$ containing 15% glycerol. 50 μL aliquots were snap frozen using 1.5 mL Eppendorfs previously placed in dry ice and stored at -80°C until use.

2.5.5 Plasmid transformations

Following ligation reaction, 5 μL of mixture containing ligated plasmid/PCR product was transformed into $CaCl_2$ competent DH5α by heat shock at 42°C for 2 min. Cells were recovered in 900 μL of LB and incubated for 45 min after which they were centrifuged and resuspended in 100 μL and plated onto LB agar plates containing 100 μg/mL ampicillin.

Transformation of plasmid into *S. aureus* requires initial transformation into the restriction and modification deficient *S. aureus* RN4220 strain (462). Transformation of plasmids into RN4220 and all subsequent *S. aureus* strains was performed by electroporation using a Micro Pulser (Bio-Rad) and 1mm gap pre-sterilised electroporation cuvette (Molecular BioProducts). Overnight cultures were subcultured 1:500 and were grown in BHI for 2-4 h to an OD_{600} of 0.4-0.6. Bacterial cells were washed three times in ice-cold 500 mM sucrose to remove salts. After centrifugation at 4°C, the pellet was resuspended in 500 μL of 500 mM sucrose and left on ice for 30 min. Cells were further centrifuged and resuspended in 100 μL of 500 mM sucrose. 100 μL of bacterial cells were added to a pre-chilled cuvette with 5-10 μL of plasmid and pipette mixed. Cells were electroporated using the settings for *S. aureus* (25 μF, 2.5 KV and 100 Ω; pulse time of 2.5 msec), and recover cells in

750 μL of BHI. Cells with plasmid were incubated for 1 h at 37 ℃, centrifuged for 1 min and resuspended in 100 μL and plate on TSA containing chloramphenicol (10 μg/mL). For bacterial cells transformed with pRMC2 plasmid, 0-100 ng/mL anhydrous tetracycline was added to the growth medium.

2.6 Protein and Glycolipid Analysis

2.6.1 Protein extraction techniques

Whole cell lysate

Overnight bacterial cultures were grown and optical densities normalised. 20 mL of culture was harvested by centrifugation for 10 min at 5,000 rpm after which cells were washed with 50 mM Tris, 150 mM NaCl and 5 mM MgCl (pH 7.5) and resuspended in 500 μL of the same buffer. Lysostaphin (200 μg/mL), RNase (10 μg/μL) and DNase (20 μg/μL) were added to the cell suspension and incubated at 37°C for 45 min. The cells were disrupted by sonication on ice and the insoluble cell fraction was pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. Supernatant was then transferred to a new Eppendorf and protein concentration calculated using Bradford reagent after generation of a standard curve using known concentrations of bovine serum albumin. 5 μL of sample and 250 μL of prewarmed Bradford reagent were mixed and left for 30 min at room temperature after which the absorbance was read at OD595nm.

Surface expressed proteins

Bacteria were grown to specific growth phases; either to exponential phase (OD_{600} of 0.45-0.55) or stationary phase, which was defined as 18 h growth. Cultures were normalised and 25 mL of exponential phase or 10 mL of stationary phase were harvested by centrifugation as above. Bacterial pellet was washed twice in PBS and

resuspended in 500 µl of sphaeroplasting buffer (SB) and incubated for 1 h at 37°C with shaking at 180 rpm. SB consisted of: 30% raffinose, 0.05 M Tris (pH 7.5), 0.145 M and 200 µg/mL. Bacterial cells were centrifuged for 15 min at 5,000 rpm, after which supernatant was transferred to a new eppendorf and further centrifuged for 10 min at 14,000 rpm. Protein concentrations were calculated using Bradford reagent as above.

Supernatant proteins

Bacteria were grown for 18 h and optical densities were normalised as above. Bacteria were removed by centrifugation at 14,000 rpm for 10 min and supernatant was transferred to a new eppendorf. Supernatant proteins were precipitated using trichloroacetic acid (TCA) at a final concentration of 20 % for 1 h on ice. Samples were centrifuged at 14,000 rpm for 20 min at 4°C and washed three times in 300 µL of ice cold acetone. Protein pellet was resuspended in 80 µL of solubilising buffer (8M urea with 6.25 mM NaOH).

2.6.2 Western blot

For analysis of α-haemolysin, toxic shock syndrome toxin-1 (TSST-1), LukS-PV (Panton-Valentine Leukocidin), protein A and penicillin binding protein 2a (PBP2a), 20 µL sample protein was mixed with 10 µL 2X concentrated sample buffer and heated at 95°C for 5 min before 10 µL of sample was subjected to 12% SDS-PAGE. Separated proteins were electroblotted onto nitrocellulose membrane using a semi-dry blotter at 25V for 30 min (BioRad). Membranes were blocked overnight at 4°C with 5 % semi-skimmed milk and then incubated with rabbit polyclonal antibodies specific for TSST-1 (1:1000) or α-haemolysin (1:3000) or mouse monoclonal antibodies specific for LukS-PV (1:500), or PBP2a (1:1000) or goat polyclonal antibodies specific to protein A (1:1000) for 2 h at room temperature. Immunoblots were washed 5 times in PBS and incubated with horseradish peroxidise-coupled protein G (1:1000) for 1 h at room temperature (apart from antibodies directed against protein A as these were HRP-conjugated). Proteins were detected using the

Opti-4CN detection kit and left to develop for 30 min. Band intensities were measured using Image J software (version 1.43). Experiments were done in triplicate and the mean band intensity was used for statistical analysis.

2.6.3 Peptide extraction and quantification

Overnight *S. aureus* cultures were diluted 1:1000 into 50 mL TSB in a 250-mL conical flask and grown for 20 h at 37 °C to an OD_{600} of 2.0. Bacteria were removed by centrifugation at 5,000g for 10 min and supernatant passed through a 0.22 μM pore. 30 mL of cell-free supernatants was mixed with 10 mL 1-butanol. Extraction was performed by shaking mixed solutions for 3 h at 37 °C. Samples were then centrifuged for 3 min for complete separation, and the upper organic phase was collected, aliquoted into 1.5-mL eppendorf tubes, and concentrated by using a vacuum overnight. Dried samples were dissolved in 200 μL of 8 M urea. Proteins (10 μL of each sample) were mixed with 2X-concentrated sample buffer and heated at 95°C for 5 min before SDS-PAGE (12% acrylamide) was performed. The individual PSMs were quantified by using mass spectroscopy as described previously (317) . This assay was performed in triplicate.

2.6.4 Circular dichroism and helical wheel analysis

The structures of synthetic PSM peptides were analyzed by circular dichroism (CD) using a Chirascan spectrometer (Applied Photophysics) and a path length of 0.2 cm. Solutions of PSMs were prepared in 1 mL PBS at concentrations between 30-60 μM. Due to the hydrophobic nature of the PSMβ1 and 2 peptides, they were dissolved in 10 μL DMSO initially and then to the required concentration with PBS. This buffer was also used in subsequent studies with these respective peptides. PSMβ peptides were also dissolved in DMSO 50% Trifluoroethanol to induce secondary structure. Experiments with PSMβ peptides in 50% TFE were conducted using a 0.5 mm cuvette. Measurements were converted to mean residual molar ellipticity (θ) and were performed in triplicate and the resulting scans were averaged, smoothed, and the buffer signal was subtracted. Analysis of secondary structure content was

performed using the Pro-data and CDNN software (463). Helical wheel projections were constructed using helical wheel analysis software (http://rzlab.ucr.edu/) and the primary amino acid sequence of the PSMs in Appendix A.

2.6.5 Thin layer chromatography

Rhamnolipids were extracted from filtered culture supernatants using ethyl acetate in a 1:1 v/v ratio. Samples were mixed by vortexing with subsequent phase separation by centrifuging for 1 min at 14,000 rpm. The upper, rhamnolipid containing phase was transferred to a new Eppendorf tube and the procedure repeated three times. The organic solvent was removed by evaporation using a vacuum centrifuge. For detection of rhamnolipids, this dried pellet was dissolved in 10 µL of ethanol. 5 µL of this solution was spotted on silica 60 TLC-plates (Fisher). In addition, 5 µl of a 0.1% rhamnolipid solution containing mono- and di-rhamnolipid (R-95) was used as a standard. TLC was performed using chloroform/methanol/acetic acid in a ratio of 65:15:2 as a running buffer. For visualisation, the dried plate was briefly submerged in a detection agent composed of 0.15 g orcinol, 8.2 mL sulphuric acid (60% v/v) and 42 mL deionised H_2O. The plate was left to dry at room temperature and then the sugar moieties were stained by incubating the plates at 110°C for 10 min.

2.6.6 Dry weight analysis of rhamnolipids

Dry weight analysis was based on the procedure by *Gunther et al* (464). Briefly, supernatant was separated from overnight culture (100 mL) by centrifugation at 7,000 rpm. Supernatant was then acidified to pH 2.0 by the drop-wise addition of 12 M hydrochloric acid. This solution was then centrifuged at 13,000 rpm and the RL containing precipitate was extracted three times with a chloroform-ethanol (2:1) mixture. This was then evaporated away leaving the characteristic honey-like appearance. This oily residue was dissolved in methanol and transferred to a previously weighted container, where the methanol was evaporated giving the total rhamnolipid yield.

2.7 Phenotypic assays

2.7.1 Vesicle lysis assay

The vesicle toxicity assay was designed *de novo* were experimental conditions were optimised before use. Fluorescence intensity was measured at excitation and emission wavelengths of 485–520 nm respectively on a FLUOROstar fluorimeter (BMG Labtech). Depending upon the experiment, two different assays were employed, one utilising whole bacterial cells and the other using bacterial culture supernatant/purified PSM/pure rhamnolipid. In the first method, bacterial culture was normalised to a specific CFU/mL achieved through correlation with optical density. 250 μL of bacterial culture was added to 50 μL of vesicle solution in triplicate. The fluorescence of each sample was then measured at 5 min intervals for 18 h. Positive and negative controls were pure vesicles with 0.01% Triton X-100 and HEPES respectively.

For the second assay, bacteria were grown for 18 h, supernatant (neat and heat-inactivated) was harvested by centrifugation at 14,000 rpm for 10 min and filter sterilized through a 0.22 mm filter. 50 μL of vesicle solution was incubated with 50 μL of bacterial supernatant or differing concentrations of purified PSMs (Biomatik; 99% purity) or rhamnolipid and measured for 30 min with the above parameters. Normalised fluorescence was achieved using the equation (Ft–F0) / (Fm/F0) where Ft is the average fluorescence value at a specific time point, F0 is the minimum and Fm is the maximum fluorescence value in that particular experiment. All vesicle lysis assays were performed in triplicate three times.

2.7.2 Solid-phase fibrinogen/fibronectin binding assay

The adhesion of bacteria to human fibronectin (Fn) and fibrinogen (Fb) was assessed using a protocol adapted from Edwards *et al* (410). Fn or Fb (1 μg/100 μL PBS per well) was immobilised onto Nunc Maxisorp Immuno plates and incubated at 4°C

overnight, with the remaining binding sites blocked with 300 µL 5% bovine serum albumin (in PBS) at room temperature for 2-3 h. Well contents were discarded, and 100 µL of bacteria corresponding to approximately 1×10^8 CFU/mL (and corresponding to a specific phase of growth, section 2.6.1) were added to wells and incubated at 37°C for 1 h. Non-adherent bacteria were removed through three rounds of washing with PBS. Adherent bacteria were fixed with 50 µL per well of 2.5% paraformaldehyde for 5 min. Wells were washed a further two times before the staining of fixed bacteria with 50 µL of 0.5% crystal violet for 2 min. After a further three PBS washes, adherent, fixed bacteria were enumerated by solubilisation of crystal violet with 100 µL of 7% acetic acid. Quantification was achieved through measurements of well absorbance at 595nm using a microplate reader. Both Fb and Fn reading were blanked against bacteria bound to BSA coated wells. Absorbance measurements were converted to bacterial numbers by use of standard plots of known bacteria numbers against absorbance 595nm reading (410). All adherence assays were perfomed in duplicate three times.

2.7.3 Lipase plate assay

Lipase plate assay was designed as described previously with some minor modifications (465). Olive oil (1 %) and rhodamine B (0.001 %) were used as substrate and added to the agar medium after sterilization and cooling to 60°C. 3-mm-diameter holes were punched into the agar and 50 mL of cell-free supernatant harvested from 18 h stationary phase bacterial cultures were added and left to incubate for 18 h at 37°C. Plates were irradiated with a UV light and images captured on a Nikon camera.

2.7.4 CAMP assay

The conventional method to determine or measure Agr activity is via δ-haemolysin production. This was determined by streaking the β-haemolysin positive *S. aureus* RN4220 strain on washed sheep blood agar (SBA) plates. The test strains were streaked perpendicular to this strain and any enhanced zone of haemolysis where the

δ-haemolysin (466) or PSMs (316) overlaps with the β-haemolysin zone was scored Agr positive.

2.7.5 Beta-haemolysis assay

Haemolysis plates consisted of TSA with 5% defibrinated Sheep blood (Thermo Scientific). Sheep blood was added under sterile conditions after TSA was autoclaved and cooled to 40-45°C. Bacterial strains, previously plated onto TSA plates, were streaked to achieve single colonies on blood plates and incubated overnight at 37 °C. After overnight incubation at 37°C, plates were incubated and 4°C for 2 h.

2.7.6 Orcinol assay

Culture supernatants were obtained by centrifugation and 300 μL of this was extracted twice with 1 mL of diethyl ether. The samples were pooled and evaporated to dryness using a vacuum centrifuge and then 0.5 mL of sterile H_2O was added. To each 100 μL sample, 900 μL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. This solution was heated to 80°C for 30 min, after which the samples were cooled at room temperature for 15 min. The absorbance of the samples were measured at 421_{nm} (BMG labtech) and concentration of rhamnolipids compared to those generated using a standard (R-95 Sigma). These assays were performed in duplicate three times.

2.8 Statistical and genotypic analysis

2.8.1 Statistics

For adhesion, invasion, cell and vesicle lysis assay and protein expression experiments, statistical analyses were performed with a Student's *t test.* Values that were stastically significantly different than the wildtype or control values are indicated by asterices in the figures. Error bars indicate the mean average ± the 95% confidence interval of multiple independent experiments.

2.8.2 Genome wide association studies using PLINK

We conducted a quantitative association study on a set of 90 isolates of the *S. aureus* clone ST239 to identify single nucleotide polymorphisms (SNPs) that were significantly associated with toxicity, using the PLINK software package (http://pngu.mgh.harvard.edu/purcell/plink/) (467). From the original set of 3060 intragenic SNPs we identified 102 SNPs with a statistical significance of $p < 0.05$ after quality control (using PLINK options -geno 0.9 and -maf 0.05, which restricted SNPs which were present in 90% of isolates or less than 5%). Association study was undertaken using command:

./Plink__file(name)__assoc__adjust

where all files were in .map format. SNPs were only chosen that has a statistical significance of $p<0.05$ after Bonferroni correction. P-values are used to determine the significance of a result. These values allow the interpretation of the result based on rejecting the null hypothesis, the default position where there is no relationship between two measured factors. Bonferroni correction undertakes an assessment of the statistical significance of multiple assessments. With multiple assements there is a higher probablility that one of the assessments is statistical significant by pure chance. Therefore in cases with multiple assessments a lower p-value is used to assess whether something is stastically significant and this is achieved by using

Bonferroni correction, where .05 is divided by the number of tests that are done. A similar association study was performed using the insertion and deletion polymorphism data (InDel), where inserts, deletions, and wild types were coded as +1, -1, and 0, respectively. This identified 22 unique InDels quantitatively associated with toxicity and present in at least five strains.

Staphylococcus aureus Interaction with Phospholipid Vesicles – A New Assay to Determine Accessory Gene Regulator (Agr) Activity and Insights into PSM Activity

3.1 Abstract

Accurate and sensitive measurement of Agr activity is central in understanding the virulence potential of *Staphylococcus aureus*, especially in the context of Agr dysfunction, which has been linked with persistent bacteraemia and reduced susceptibility to glycopeptide antibiotics. Agr function is typically measured using a synergistic haemolysis CAMP assay, which is believed to report on the level of expression of one of the translated products of the Agr locus, δ-haemolysin. In this study we develop a vesicle lysis test (VLT) that is specific to small amphipathic peptides, most notably delta and Phenol Soluble Modulin (PSM) toxins. To determine the accuracy of this VLT method in assaying Agr activity, we compared it to the CAMP assay using 89 clinical *Staphylococcus aureus* isolates. Of the 89 isolates, 16 were designated as having dysfunctional Agr systems by the CAMP assay, whereas only three were designated as such by VLT. Molecular analysis demonstrated that of these 16 isolates, the 13 designated as having a functional Agr system by VLT transcribed RNAIII and secreted δ-haemolysin, demonstrating they have a functional Agr system despite the results of the CAMP assay. The Agr locus of all 16 isolates was sequenced, and only the 3 designated as having a dysfunctional Agr system by the VLT method contained mutations, explaining their Agr dysfunction. Given the potentially important link between Agr dysfunction and clinical outcome, we have developed an assay that determines this more accurately than the conventional CAMP assay. Additionally, this study provides an insight into how the PSMs differ in lytic potentials against specific vesicles, confirming that differences in their alpha helicity impact on peptide-mediated vesicle lysis.

3.2 Introduction

*S*taphylococcus aureus expresses many different secreted and surface - associated virulence factors which are utilised during all stages of infection (276). The staphylococcal accessory gene regulator (Agr) quorum sensing system is central in the ability of this organism to promote infection, through coordinated, temporal expression of specific virulence genes, in which cell surface adhesins are synthesised and expressed before secreted toxins and enzymes (329, 468). Previous studies have shown that a functional Agr system is important in several infection models including a murine arthritic (469) and subcutaneous abscesses model (470) as well as rabbit endocarditis (471). The shift in gene expression is tightly correlated with population density and sensing of a diffusible signal molecule by the Agr two-component system (see section 1.3.4.1 and Fig 1.8 for more details). This complex alteration in expression of virulence genes occurs in conjunction with many global regulators namely the DNA-binding Sar family of proteins (363, 370), the alternative sigma factor (472) and other two component systems such as *saeRS* (340) and *arlRS* (354) (see section 1.3.4 for more details).

Recently, there has been much research into Agr dysfunction, particularly on the outcomes of infection with isolates containing mutations in the Agr locus, which can range in prevalence from 10-20% of *S. aureus* clinical isolates (473, 474). These mutations can occur during infection in a patient (475) and have been implicated in an increased mortality for patients suffering from bacteraemia (474, 476). The increased survival of Agr defective strains observed in persistent bacteraemia has been hypothesised to involve a defect in autolysis (477), owing to the fact that several murein hydrolase genes are regulated by Agr (358). This phenotype has been implicated in the increased survival of Agr dysfunctional strains in the presence of platelet derived antimicrobial peptides and reduced sensitivity to gylcopeptide antibiotics, namely vancomycin (477).

The conventional method of assessing Agr function is to demonstrate the haemolytic activity of δ-haemolysin using a blood agar plate assay in combination

with a β-haemolysin positive strain, usually RN4220 (466). Additionally, Agr activity can be monitored through other methods such as Northern blotting or quantitative reverse transcriptase-PCR (qRT-qPCR) with probes or primers directed at RNA III, and recently, by whole-cell matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (478). However, Northern blotting and qRT-PCR are time consuming and expensive, but considerably more sensitive than the CAMP assay, and a MALDI-TOF mass spectrometer costs several hundred thousand dollars to purchase, although running costs are low (479).

Here we describe the development of a new methodology that determines Agr activity in a fast, high throughput, sensitive and quantitative manner. This method is based on the interaction of delta and Phenol Soluble Modulin (PSM) toxins, both used as surrogate markers for RNAIII and RNAII activity respectively, with lipid vesicles containing encapsulated self-quenched fluorescent dye. One fundamental element in the transition from inactive toxin monomers into fully functional membrane-damaging agents is the lipid and protein composition of the target membranes, particularly important in artificial membranes. We have formulated a lipid vesicle system that is responsive to specific peptide toxins; the small alpha helical, amphipathic delta and PSM toxins. When compared to the conventional CAMP assay this method proved more accurate, identifying all the isolates in a collection of 89 clinical *S. aureus* isolates that had mutations in the Agr locus, unlike the CAMP assay that had 13 false positives. As such, if Agr dysfunction becomes more widely accepted as being a critical determinant in the clinical outcome of infection, the accuracy of the VLT assay suggests that it should be used ahead of the plate based CAMP assay.

In additional experiments, we observed differences in the lytic potentials between the different PSM peptides (alpha and beta classes) and within the PSMα class, contrary to previous studies (316). In an attempt to understand this observation, experiments were designed to investigate peptide amphipathic properties and secondary structure. Ultimately, the nature of peptide secondary structure and lipid bilayer composition dictate the lytic capacities of these peptides. Here we highlight

differences in the degree of alpha helicity through circular dichroism experiments and hypothesize how this structural property explains why certain PSM are less, or non-cytolytic.

3.3 Results and Discussion

3.3.1 Vesicle breakdown correlates with early stationary phase growth

Methicillin-susceptible *S. aureus* strain MSSA476 is a community-acquired invasive isolate for which a genome sequence is available (480). To determine if and at what stage of growth MSSA476 lysed lipid vesicles, a range of concentrations of bacteria were used to inoculate broth containing vesicles. Toxin production in bacteria is strictly growth-phase regulated and density dependent, with bacteria relying on the secretion of auto-inducing peptides to communicate and regulate genes (330). Figure 3.1 shows the vesicle breakdown/fluorescence response, observed for the three start inocula were growth phase dependent; being triggered in the early stationary phase in each case.
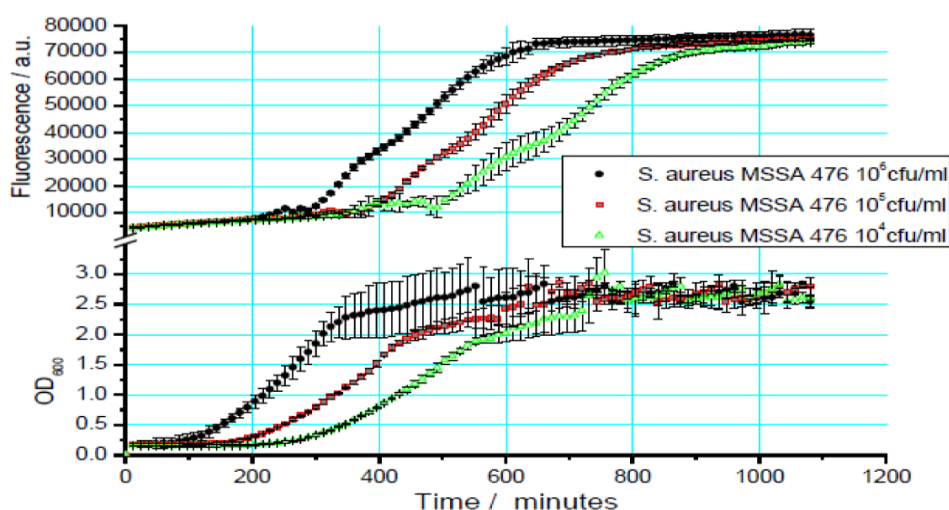
Figure 3.1: Vesicle lysis occurs during early stationary phase growth. Different starting inocula (10^4, 10^5, and 10^6 CFU/mL) of MSSA 476 were used to assess vesicle lysis during bacterial growth. Bacteria were grown for 18 h with lipid vesicles and by monitoring optical density and fluorescence it was evident that vesicle lysis occurs at early stationary phase. Experiments were done in triplicate three times and error bars represent the 95% confidence interval.

3.3.2 Identification of the toxins lysing the lipid vesicles

S. aureus expresses a number of toxins that are up-regulated in the stationary phase by the Agr system. These toxins differ in their mode of action and host receptor specificity, resulting in different toxins lysing different cell types. To determine which staphylococcal toxins were causing vesicle lysis, a number of isogenic mutants were assayed.

α- and β-haemolysin

The *S. aureus* 8325-4 laboratory strain and the isogenic *hla* (α-haemolysin) and *hlb* (β-haemolysin) mutants were used to assess the role of these two toxins (Fig 3.2a). It has been known that α-haemolysin damages protein free liposomes and that phosphatidylcholine head groups play a vital role in initial association (149, 314). α-haemolysin mediated lysis requires the clustering of phosphatidylcholine head groups within membrane microdomains enriched in cholesterol and sphingomyelin while the absence of either membrane proteins or the absence of sufficient clustering lead to the inhibition of monomer heptamerization (149) and therefore no lytic event. 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is a major component of the vesicles under study (53%) and for this reason we explored the role of α-haemolysin mediated breakdown of vesicles. Figure 3.2a clearly shows that both the alpha and β-haemolysin knockout strains caused vesicle lysis at virtually identical levels as the wild type strain, showing that neither toxin were critical in vesicle lysis or caused maximum fluorescence release in these experiments.

Importantly, in the lipid vesicles used here, 10, 12-tricosadiynoic acid (TCDA) was included to stabilize the membrane via cross-linking of the acyl chains of DPPC, and this is believed to prevent such head group clustering, rendering the monomers incapable of binding and penetrating the membrane. The β-haemolysin is an enzyme which acts as on sphingomyelin (481) and consistent with its activity, no difference in vesicle lysis was observed using the β-haemolysin mutant.

Bi-component leukocidins

The Panton-Valentine leukocidin (PVL), γ-haemolysin and the leukocidin family (notably LukAB and LukED) belong to the bi-component pore-forming family of toxins. In a similar fashion to the α-haemolysin, these leukocidins require specific receptor(s) for lysing biological membranes (150, 308, 441, 442) but may still lyse artificial membranes (151). Therefore, we wanted to investigate whether any measurable difference could be seen between supernatants derived from USA400 MW2 wild type and isogenic PVL mutant and strain Newman and the respective *lukAB*, *lukED* and *hlgACB* mutants. Due to the reported high levels of toxin production by these strains and sensitivity of vesicles, a series of dilutions of supernatants were tested. No measurable difference was observed between wild type and mutant strains with our vesicle type.

It has been shown previously that γ-haemolysin can permeate liposomes composed of PC head groups (151); however our results are consistent with two pieces of evidence which also proves that this toxin does not have the capacity to bind and permeabilize our vesicle type. Firstly, the lipid composition is crucial for this toxins' activity with short (less than 13 carbon atoms) acyl chains being vital for pore-forming ability (440). The vesicles in question are composed of longer (16 carbon atoms) chains causing cholesterol to integrate below the DPPC head groups permitting packaging not conducive to monomer binding (440). Secondly, this toxin, as with α-haemolysin, is susceptible to heat-inactivation at 65°C for 30 minutes (152) and since heat inactivated supernatants were still able to lyse vesicles with the same ability as un-treated supernatant (Fig 3.2e) over a defined period (20 min exposure), demonstrating that these toxins were not involved in vesicles lysis.
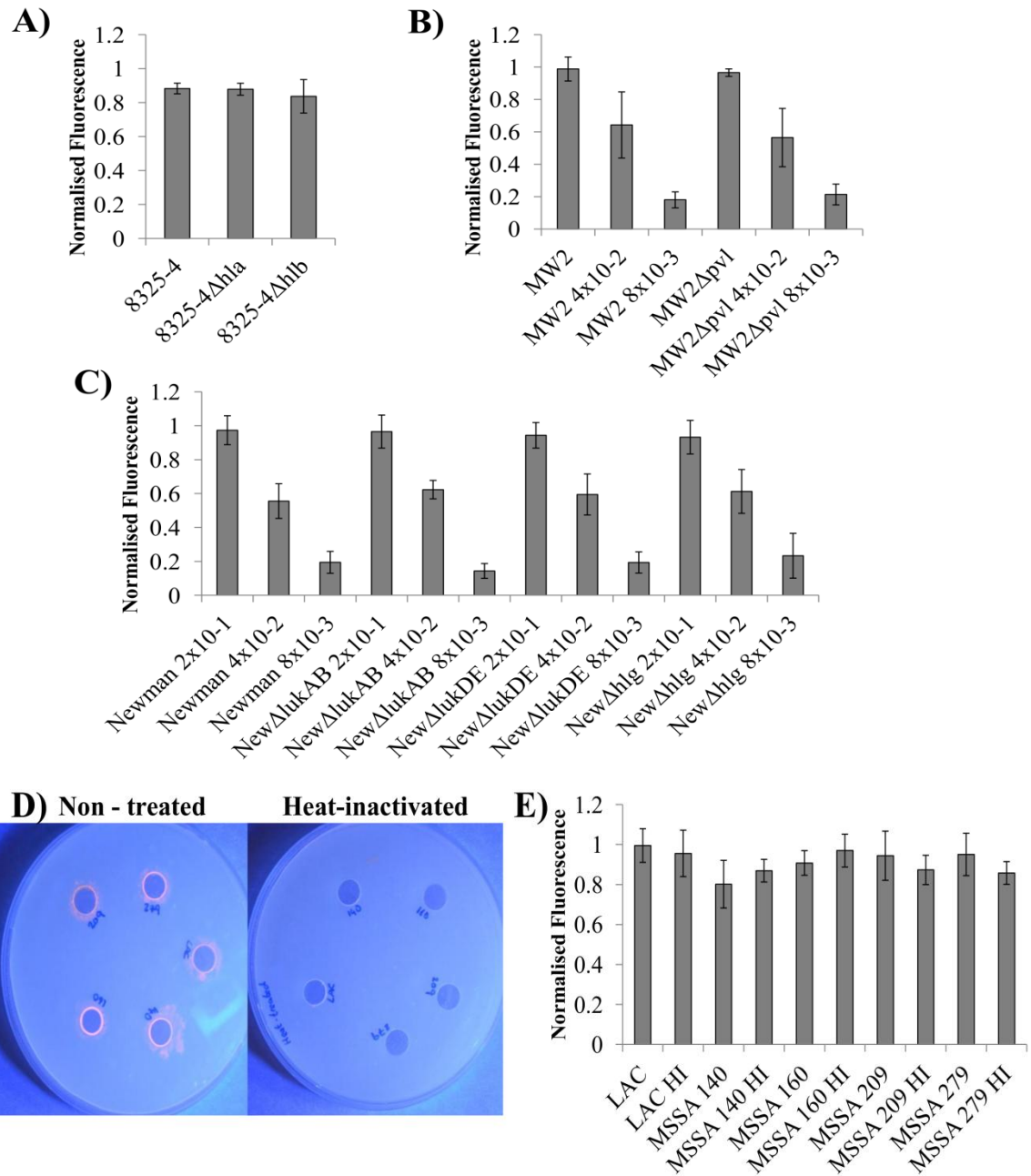
Figure 3.2: Characterising the toxins and enzyme involved in vesicle lysis. A) Deletion of α-or β-haemolysin had no measurable effect on vesicle lysis. **B)** 5-fold dilutions of MW2 and MW2Δpvl mutant and **C)** Newman, NewmanΔ*lukAB*, Newman*ΔlukED* and Newman*ΔhlgACB* illustrate the lack of involvement of the Panton-Valentine leukocidin and leukocidin AB, leukocidin ED and the γ-haemolysin in lysis of vesicles. **D)** Phospholipase plate assay showing phospholipase activity as an orange halo around un-treated supernatant filled wells in contrast to no activity with heat-inactivated supernatants. **E)** 95°C Heat-treatment of supernatants retains vesicle lysis ability, suggesting no involvement of either phospholipases or γ-haemolysin in vesicle lysis (see text for details). Lipid vesicles were incubated with bacterial supernatants for 30 min. Vesicle lysis experiments were performed in triplicate three times with error bars representing the 95% confidence interval.

Phospholipase

S. aureus also produces a number of enzymes, some with lipolytic activity such as (phospho) lipases. These enzymes are heat-sensitive and to investigate whether these enzymes had an effect on vesicle lysis, we first demonstrated the inactivation of these enzymes in the bacterial supernatant of a selection of clinical isolates following heat treatment using a lipase plate assay (Fig 3.2d). These heat-treated supernatants retained their vesicle lytic activities, demonstrating that these enzymes were not the main lytic agent of this system.

Delta and PSM peptide toxins

It has been previously shown that δ-haemolysin can retain its alpha helical structure in phosphatidylcholine bilayers and causes membrane perturbation and lysis in a concentration dependent manner (449). Therefore, we examined the effect of δ-haemolysin and related PSM toxins on the vesicles described in this chapter. Lysis of vesicles was observed with δ-haemolysin and also for the PSMα1, 2 and 3 toxins whereas PSMα4 and PSMβ1 and 2 had reduced lytic activity (Fig 3.3a). Given the effect of δ-haemolysin and PSMα3 in our assays and their published effect on polymorphonuclear leukocyte cells (312) we focused on their activity in greater detail.

Above a concentration of 2.5 µM and 1.5 µM for δ-haemolysin and PSM3α respectively, vesicle lysis occurs rapidly, whereas below this magnitude, lysis is reduced by approximately 20–30 % of maximum fluorescence (Fig 3.3b and d). The concentration of δ-haemolysin and PSM3α required to lyse 50 % of vesicles (V50) is 1.5 µM and 0.25 µM respectively. By comparing this to the LD_{50} for biologically relevant T cells which are derived from an immortalized T cell line (482) the sensitivity of the vesicles to these peptides is illustrated. A 50-fold (δ-haemolysin) and 16-fold (PSM3α) increase in toxin concentration is required to cause lysis of 50 % of T cells (Fig 3(c) (e). It has been shown previously that PSM concentrations of greater than 30 µg/mL were required to cause lysis of human neutrophils [41]. When

using PSM3α as an example, this roughly equates to a concentration of approximately 11.5 µM, whereas for the immortalized T cells, a concentration of approximately 5 µM results in lysis of 50% of T cells, highlighting the differences in susceptibility of different cell types to PSMs.
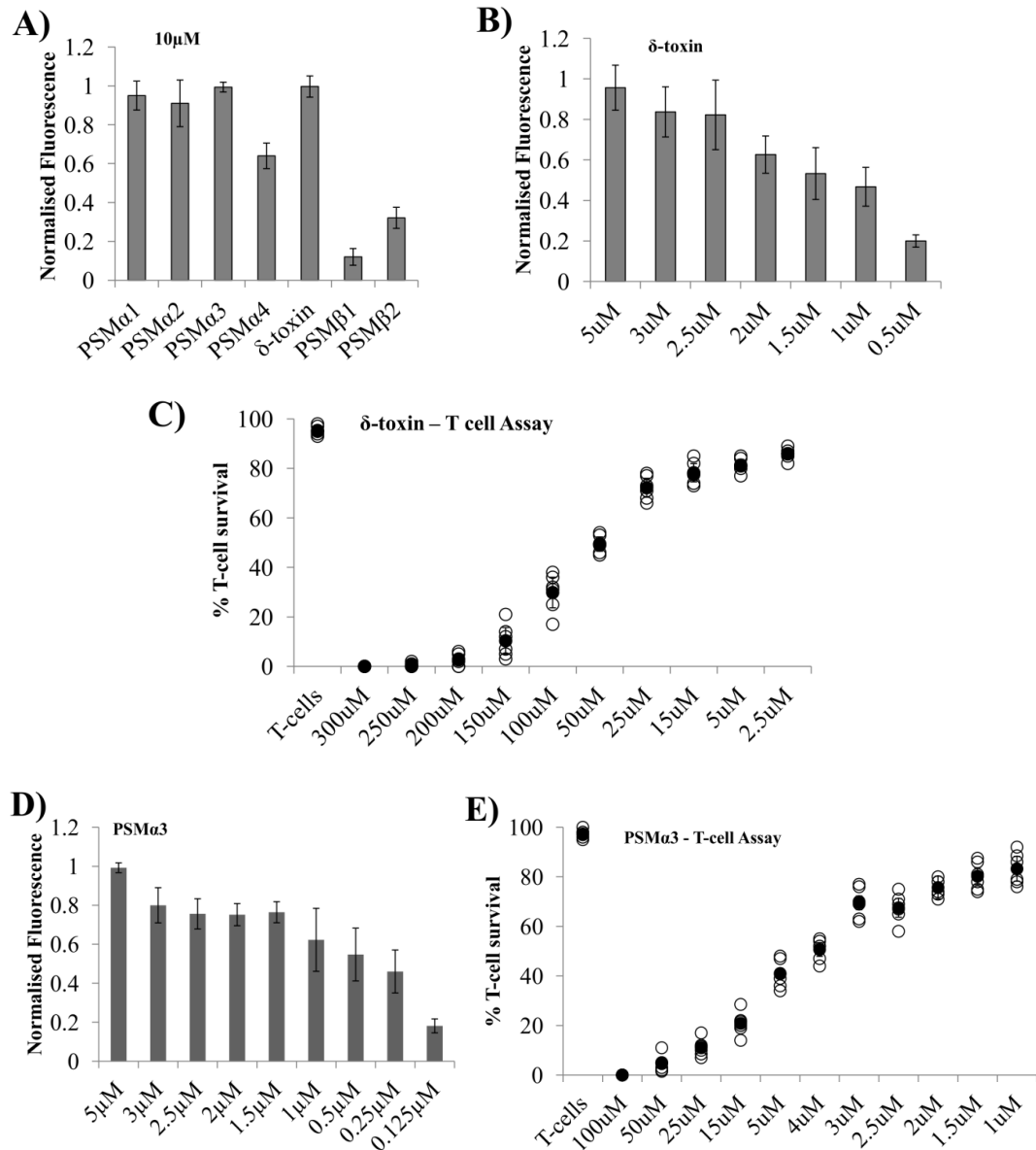
Figure 3.3: Effect of purified toxins on lipid vesicles and T – cells. A) Vesicle rupture as a result of incubation with 10 µM synthetic PSM and δ-haemolysin peptides for 30 min **B)** Lysis of vesicles and **C)** T cells subjected to selected concentrations of purified δ-haemolysin. **D)** Lysis of vesicles and **E)** T cells subjected to selected concentrations of purified PSM3α toxin. The vesicle system is highly sensitive to both toxins at low concentrations, while PSM3α is more potent at lysing both vesicles and T cells than δ-haemolysin. T cells were incubated for 15 min at 37 °C with purified toxins. Experiments were performed in triplicate three times with error bars representing the 95% confidence interval.

3.3.3 Comparison of two assays in measuring Agr activity

Having developed an assay that responds to the activity of δ-haemolysin, we next wanted to establish how this vesicle lysis test (VLT) assay compared to the conventional δ-haemolysin plate CAMP assay in determining Agr function. 89 clinical *S. aureus* strains were tested including 51 hospital acquired and 38 community acquired isolates from diverse genetic backgrounds (427). (The strains used in this study are derived from CC1 (n=7), CC5 (n=5), CC8 (n=5), CC9 (n=1), CC12 (n=4), CC15 (n=6), CC16 (n=5), CC22 (n=7), CC25 (n=10), CC30 (n=24), CC39 (n=5), CC45 (n=9) and CC51 (n=1)). Of the 89 strains assayed using the CAMP assay, 17.98% (16 of 89) exhibited no synergy between δ- and β-haemolysins on the blood agar plates (Fig 3.4a) and as such were designated as having a dysfunctional Agr system. Using the VLT only 3.3 % (3 of 89) had no lytic activity, as measured by fluorescence comparable to the negative control (HEPES buffer) and were classified as Agr dysfunctional (Fig 3.4b). Using LAC and its isogenic *hld* mutant it was also shown that the synergistic effect of haemolysis could also be observed in the absence of translated δ-haemolysin (Fig 3.4a), an observation also made by that Cheung *et al* (316) where other PSMs were shown to be involved. Of the CC used, CC30 showed the highest percentage of *agr* dysfunction when examined using the CAMP assay, with 13 of 24 strains showing no haemolysis, however no association between CC type and agr dysfunction is shown when using the VLT assay.
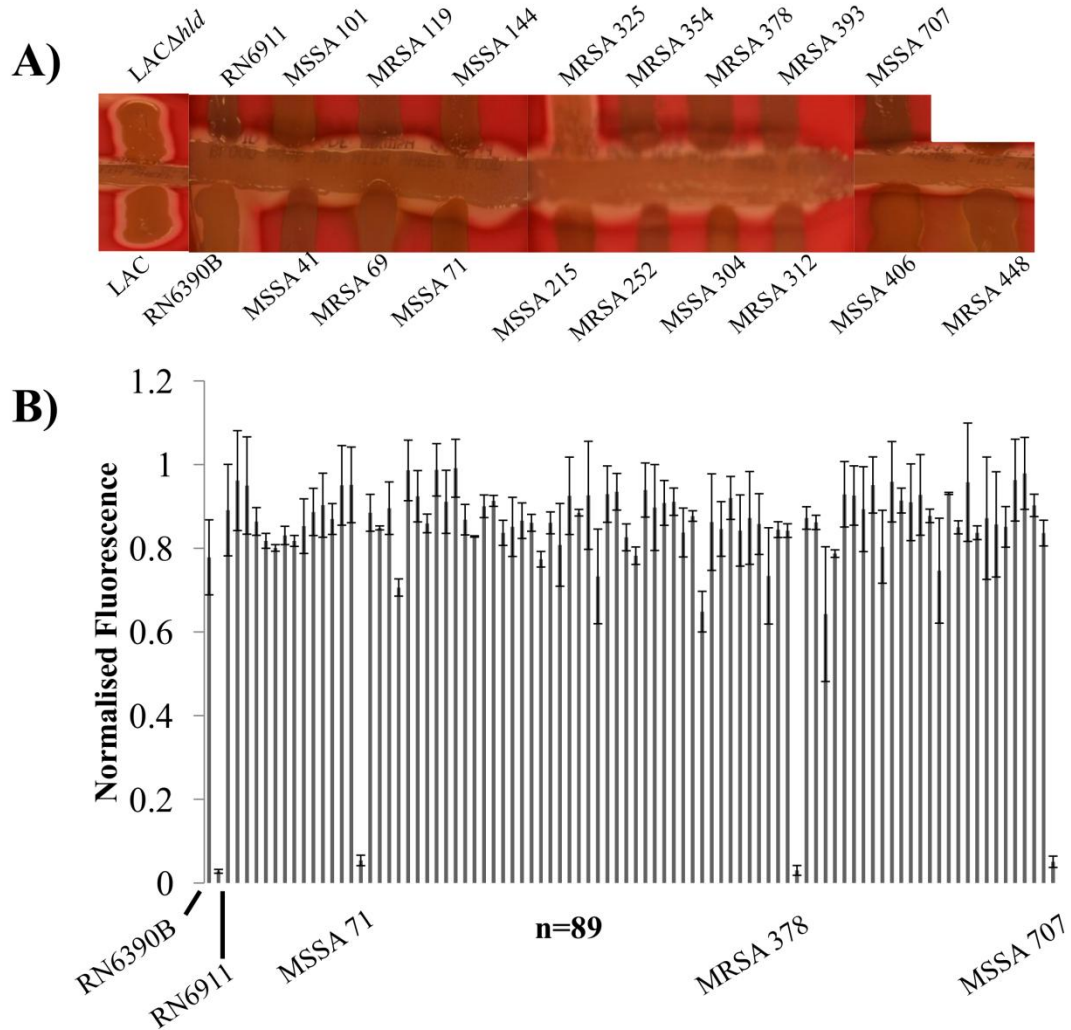
Figure 3.4: Differences in Agr activity observed using two methods. A) δ-haemolysin plate assay of *agr* positive (RN6390B) and negative (RN6911) strains, 17 *S. aureus* clinical isolates, 16 which are designated as *agr* negative, one *agr* positive isolate (MRSA325) and *agr* positive LAC and its corresponding isogenic *hld* mutant strain, signifying the effects of δ-haemolysin and PSMs on the haemolysin plate assay. **B)** Normalized fluorescence measurements of 89 clinical *S. aureus* strains using the vesicle-supernatant method, highlighting the three strains causing no vesicle lysis. Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

3.3.4 Validation of the sensitivity of the VLT test

To determine which of the two assays (CAMP or VLT) were assaying Agr activity accurately we measured the level of expression on RNAIII in all 16 isolates defined as Agr dysfunctional by the CAMP assay by qRT-PCR (Fig 3.5). Only the three isolates defined as Agr dysfunctional by both the CAMP and the VLT assays were

impaired in the expression of RNAIII (MSSA71, MRSA378 and MSSA707). We also qualitatively assayed the secretion of small amphipathic peptides (i.e. δ-haemolysin and the PSMs) by these isolates (Fig 3.6). Although variability existed in the secretion of these peptides across all 16 isolates, only the three designated as being Agr dysfunctional by VLT had no detectable amount of peptide. One of the limiting factors of using this extraction technique is that it doesn't differentiate between the PSMs and may not be sensitive enough to detect them all, especially if they are expressed at a low-level. Therefore, we extracted the sample and analysed it by liquid chromatography mass spectrometry (LC/MSMS) where only δ-haemolysin was detected in this band. (LC/MSMS was done at the Central Proteomics Facility at the University of Oxford, United Kingdom).
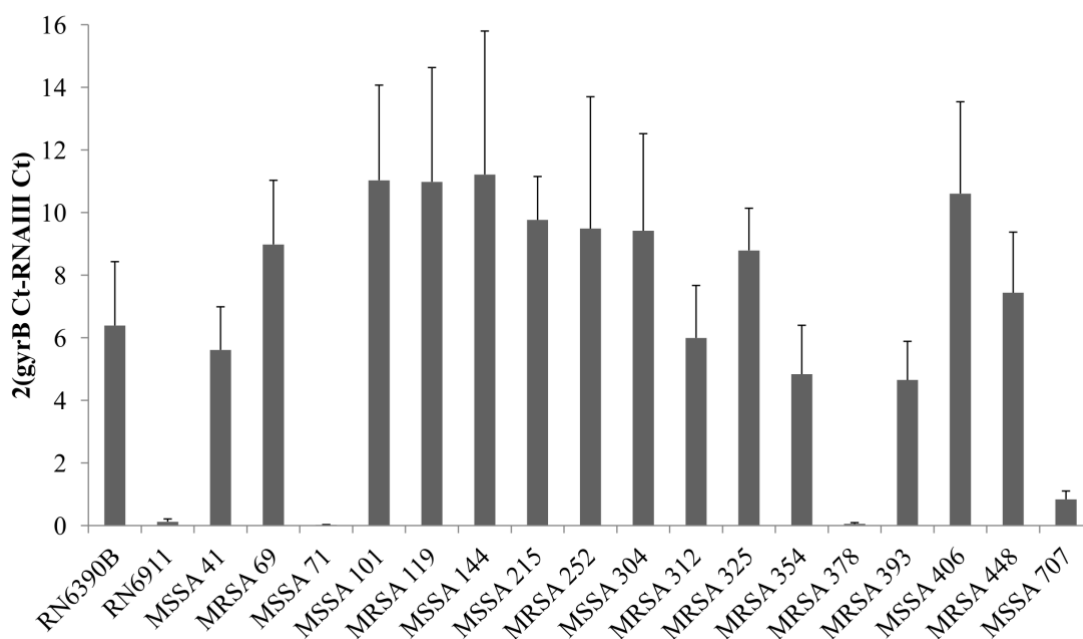
Figure 3.5: Measurement of RNAIII transcription. Real - time qPCR results of 19 strains consisting of the positive and negative control RN6390B and RN6911 respectively, the 3 negative vesicle lysis test strains (MSSA 71, MRSA 378 and MSSA 707), 13 non-haemolytic strains and the haemolytic strain MRSA 325. RNAIII transcription is observed in those strains designated *agr* positive by vesicle method and no transcription is evident in those 3 strains which show no lysis of vesicles.
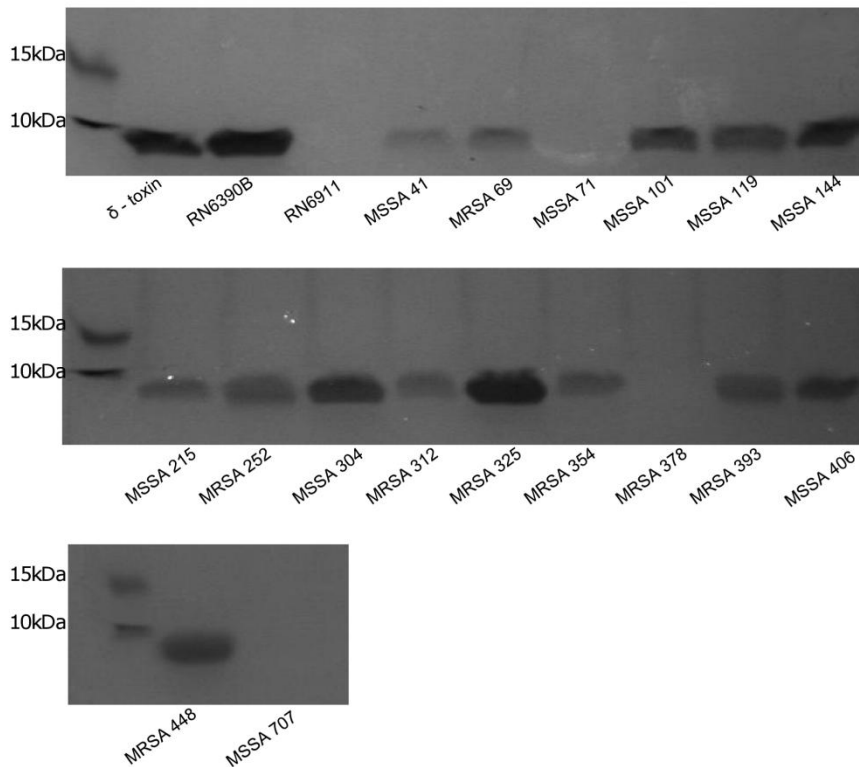
Figure 3.6: SDS-PAGE of concentrated Agr regulated peptides. Concentrated and extracted proteins using 1-butanol from several *S. aureus strains*, showing the presence of protein bands, indicating δ-haemolysin. Purified δ-haemolysin and RN6390B used as a positive control and RN6911 as a negative control. Figure shows the absence of bands in those strains which cause no lysis of vesicles.

3.3.5 Agr sequencing

The Agr loci of all the 16 isolates, designated as having dysfunctional Agr system by the CAMP assay, were sequenced. We aligned the *agr* sequence of these isolates to the known sequence of the *agr* locus in the respective clonal complex (Fig 3.7). Only the three designated as Agr negative by both VLT and CAMP were found to have mutations in this locus and the mutational effect on the protein sequence is summarised in figure 3.9. All three isolates had 1 bp deletions occurring within a run of adenine (MSSA71 and MRSA378) or thymine (MSSA707) residues. In MSSA71, this lead to a premature stop codon in AgrA truncating the protein at amino acid position 49; in MRSA378 this caused a frame shift mutation and the addition of 22

amino acids at the C-terminal end of AgrA protein; and in MSSA707 this lead to the truncation of the AgrC protein at position 176. With regards to the other non-haemolytic but VLT positive strains, (MSSA41, MRSA69, MSSA101, MRSA119, MSSA144, MSSA215, MRSA252, MSSA304, MRSA312, MRSA354, MRSA393, MSSA406, and MRSA448) and one haemolytic strain (MRSA325), had the same Agr sequence apart from MRSA69 and MRSA325 which were identical with each other and of a different Agr group (Agr-1 as opposed to Agr-3) from the rest of the strains. Therefore Agr dysfunctional strains were resolved only by the VLT assay and not the CAMP assay as summarised in table 3.1.

MW2   (*agrA*)
132-GAGCAAGCTAAAAATATGAATGACATAGGCTGT
     E   Q   A   K   N   M   N   D   I   G   C
MSSA71 (*agrA*)
132-GAGCAAGCT**A**AAAATATGAATGACATAGGCTGT
     E   Q   A   K   I STOP M   T STOP

MRSA252 (*agrA*)
699-AACGTTAAAAAAATATAA
     N   V   K   K   I STOP
MRSA378 (*agrA*)
699-
AACGTT**A**AAAAAAATATAATAAGATAATAAAGTCAGTTAACGGCGTATTCAATTGTAAATCTTGTTGG
 N   V   K   K   Y   N   K   I   I   K   S   V   N   G   V   F   N   C   K   S   C   W
  ATTTTAACAAGATAA
   I   L   T   R STOP

LGA251   (*agrC*)
478-GCATTTTTTTATATTTATTCTACAAATACCAGTTCAAAC
     A   F   F   Y   I   Y   S   T   N   T   S   S   N
MSSA707 (*agrC*)
478-GCATTTTTT**T**ATATTTATTCTACAAATACCAGTTCAAACGGTGATAGTTTAA
     A   F   F   I   F   I   L   Q   I   P   V   Q   T   V   I   V STOP

Figure 3.7: Mutations in VLT negative isolates. Isolates MSSA71, MRSA378 and MSSA707 all contained 1 bp deletions (red bold) which led to truncation or alteration of essential Agr proteins. The reference strain is given (top) along with the strain in which the mutation was shown to occur in (bottom). Nucleotide positions are given at the 5' end of the sequence.

Strain	CC	CAMP	VLT (N Fluor)¶	RNAIII Expression*	Agr locus/ mutation
MSSA41	30	Negative	0.71±.04	5.7±1.4	Identical to MRSA252
MRSA69	30	Negative	0.95±.09	8.9±2.1	Identical to MRSA325
MSSA71	**1**	**Negative**	**0.05±.01**	**0.02±.01**	**-1bp(nt147) AgrA**
MSSA101	30	Negative	0.98±.07	11±3.1	Identical to MRSA252
MRSA119	30	Negative	0.85±.02	10.9±3.7	Identical to MRSA252
MSSA144	30	Negative	0.99±.07	11.2±4.6	Identical to MRSA252
MSSA215	30	Negative	0.80±.09	9.7±1.4	Identical to MRSA252
MRSA252	30	Negative	0.73±.11	9.5±4.2	MRSA252
MSSA304	39	Negative	0.91±.03	9.4±3.1	Identical to MRSA252
MRSA312	30	Negative	0.65±.05	5.9±1.7	Identical to MRSA252
MRSA325	30	Positive	0.92±.05	8.8±1.4	Identical to MRSA69
MRSA354	30	Negative	0.73±.11	4.8±1.6	Identical to MRSA252
MRSA378	**30**	**Negative**	**0.03±.01**	**0.05±0.03**	**-1bp(nt705) AgrA**
MRSA393	30	Negative	0.64±.16	4.64±1.2	Identical to MRSA252
MSSA406	30	Negative	0.92±.07	10.6±2.9	Identical to MRSA252
MRSA448	30	Negative	0.95±.14	7.4±1.9	Identical to MRSA252
MSSA707	**16**	**Negative**	**0.05±.02**	**0.83±.3**	**-1bp(nt487) AgrC**

Table 3.1: Key characteristics of CAMP and VLT assayed strains
Abbreviations: CC; clonal complex, CAMP; synergistic haemolysis plate assay, VLT; vesicle lysis test
¶ Values shown in normalised fluorescence
* qRT-PCR after 8 hour

3.3.6 Secondary structure and alpha helical wheel analysis of PSM peptides

The differences in PSM mediated vesicle lysis observed in section 3.3.2 and PSM cytotoxicity assays shown in section 6.3.1 and by others (312), highlights that different PSMs have the different lytic activities. PSMs are generally classified as small, amphipathic, alpha helical peptides which can target cell indiscriminately, not requiring a proteinaceous receptor. For these reasons we sought to investigate which structural parameters are important in determining PSM lytic activity.

Studies of antimicrobial peptides (AMPs) suggest that there are two functional requirements needed for active membrane destabilisation: a net positive charge to facilitate interaction with the negatively charged phospholipid membrane; and the potential to form amphipathic structures which allow incorporation into the membrane (483, 484). Other parameters which can strengthen the incorporation of peptides into lipid membranes include the overall hydrophobicity of the peptide, the ratio of hydrophobic to charged residues (h:c) and the degree of structuring (485-487). In light of these parameters and using the hypothesis that PSMs act in a similar fashion to AMPs and δ-haemolysin, we investigated these properties in an attempt to understand the observed different lytic activities of these related peptides.

The δ-haemolysin and PSMα1-3 peptides consist of 46-54% hydrophobic residues, which is consistent with the requirement for an amphipathic helical structure (483). However, the PSMα4 peptide contained 70% hydrophobic residues, giving the highest hydropathicity score using the Kyte and Dolittle algorithm, where a high hydropathicity value indicates high hydrophobicity (Table 3.2) (488). Previous studies using short AMPs observed that increased hydrophobicity lead to a decrease in interaction with negatively charged membranes and under certain conditions the more hydrophobic a peptide, the lower its membrane lytic capability (483, 489). PSMα4 also has the highest hydrophobicity to charge (h:c) ratio among the PSM peptides, which may further explain its lower lytic capacity. Overall charge on the δ and PSMα1-4 toxins was positive, (Table 3.1) PSMα2>

114

PSMα1=PSMα3=PSMα4> δ, within the limits of charge typical for lytic peptides, as excessive charge is potentially damaging towards lytic activity by preventing suitable structuring (490). The PSMβ1 peptide has an overall negative charge whereas PSMβ2 displays a neutral charge while both peptides have a high h:c ratio which may in part explain the low lytic phenotype.

PSM	Hydropathicity	Charge
Delta	0.135	0
PSMα1	0.957	+2
PSMα2	0.890	+3
PSMα3	0.305	+2
PSMα4	1.700	+2
PSMβ1	0.570	-1
PSMβ2	0.607	0

Table 3.2 Hydropathicity and charge of PSM peptides

Helical wheel projections of these peptides (Fig 3.8) illustrate that like δ-haemolysin, all the PSMα peptides have a predicted helical structure with a high degree of amphiphilicity, with PSMα3 showing the largest hydrophobic moment (PSMα3 > δ > PSMα4 > PSMα2 > PSMα1, ranging from 9.16 to 5.96) and PSMβ1 and β2 relatively lower (6.23 and 4.29, respectively). PSMα3 is the most toxic towards polymorphonuclear leukocytes (PMNs) (312) and in this thesis, is most lytic to lipid vesicles and T cells (Figure 6.3b). It has been observed that ionic or salt bridges may form when negatively charged residues are spaced 3- to 4- positions from positively charged residues, which may promote helix formation (491). PSMα3 shows the largest number of charged residues (positive and negative) on the hydrophilic phase of the predicted helix, indicating the potential formation of helix stabilizing ionic

bonds. This coupled with the large hydrophobic moment for PSMα3 will likely increase both peptide agglomeration in solution and partitioning of the peptide into the lipid bilayer (492).

Circular dichroism (CD) spectroscopy can be used to investigate the alpha helicity of proteins. CD measures differences in the absorption of left-handed circularly polarized light and right-handed polarized light over a range of wavelengths, generating a distinct CD spectral signature for specific biological molecules, such as proteins. CD spectral analyses demonstrate that the PSM peptides that were highly lytic (PSMα1-3) displayed a higher degree of alpha helical secondary structure (Fig 3.9a), whereas PSMα4 and particularly PSMβ1-2 showed reduced helical characteristics (Fig 3.9b) (alpha helical content is shown by the peaks at 190 and 210 nm). Alpha helicity is important in the ability of small peptides to integrate within the lipid membrane and cause disruption as this type of structure allows for the formation of both hydrophilic and hydrophobic faces (493-495). The structuring is influenced by both the overall hydrophobicity of the peptide, which will govern initial interaction with the bilayer surface and subsequent interactions between individual residues on the hydrophobic face of the peptide and the bilayer interior. It is worth noting that the PSMα1-3 peptides possess helix stabilizing alanine residues at position 5 whereas PSMα4 contains a helix destabilizing glycine in this position which may reflect the observed difference in alpha helicity.

δ-haemolysin displays high a degree of amphipathicity and alpha helicity, with well defined hydrophobic and hydrophilic phases, but had a lower cytolytic activity than PSMα2-3 (Figure 6.3b). The lower lysis activity is intriguing and may be due to the low hydropathicity value and a neutral charge affecting efficient initial interaction and insertion within the membrane. Previous studies have demonstrated that aliphatic residues decreasingly promote alpha helix stability in the order Leu>Ile>Ala>Val> in a lipid environment (496, 497). The combined number of all aliphatic residues within each peptide (discounting glycine), varies in the order; 6 (PSMα3), 8 (PSMα2), 9 (PSMα1), 9 (δ toxin) and 12 (PSMα4). Of those residues PSM alpha 3 has proportionately the highest leucine content while PSMα4 has the

lowest. The number of aliphatic residues a peptide contains therefore shows an inverse correlation with vesicle and cell lysis.
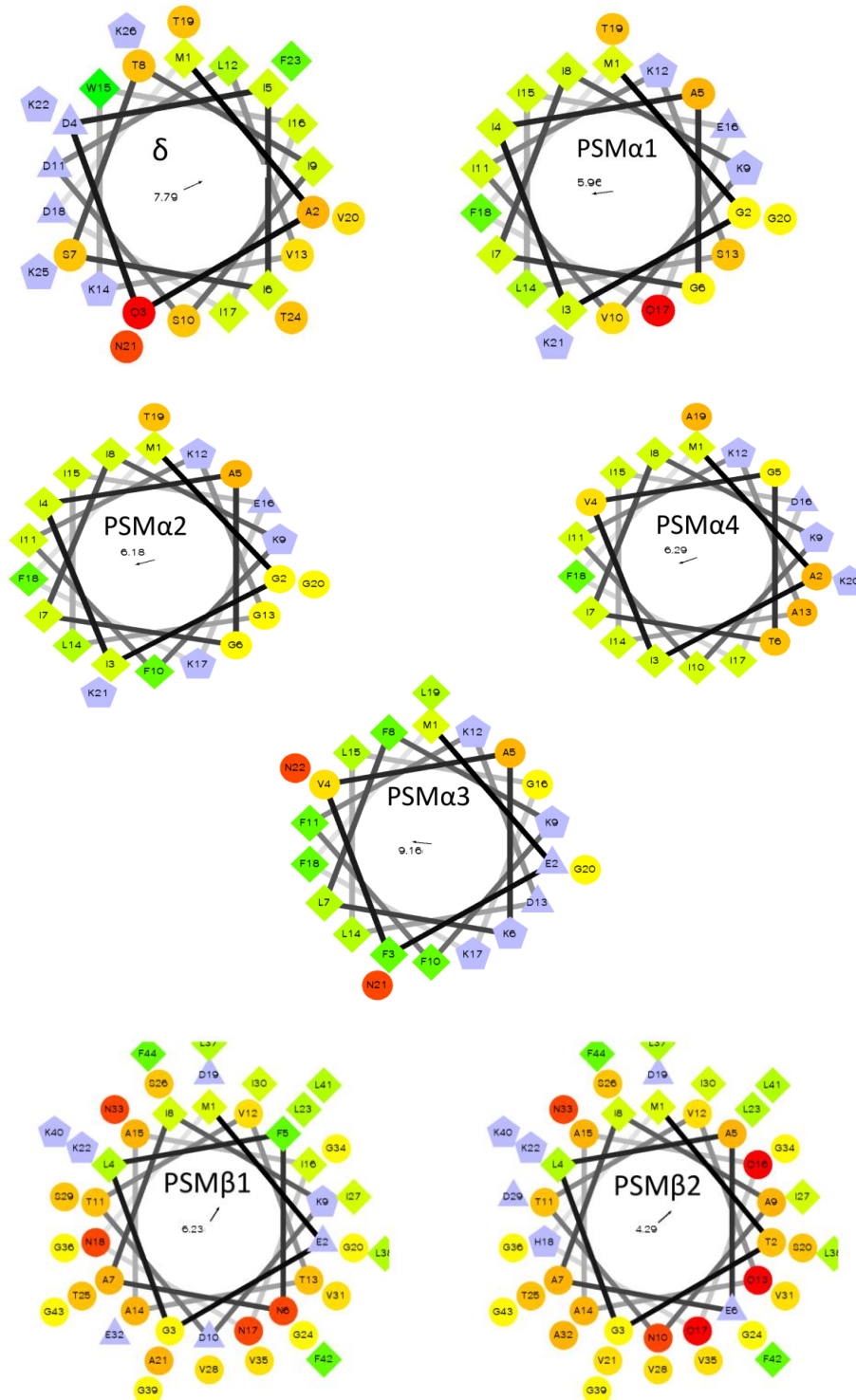
Figure 3.8: Helical wheel projections of δ-haemolysin (top), PSMα1, α2, α3, α4 (middle, from left to right) and PSMβ1 and β2 (bottom, from left to right). Hydrophobicity is color coded from dark green (most hydrophobic) to yellow (zero hydrophobicity). Hydrophilic amino acids are shaded from red (most hydrophilic) to yellow, charged residues are shown in light blue (reference; http://rzlab.ucr.edu/). The arrows indicate the directions of the hydrophobic moments.

It is important to note that δ-haemolysin and PSMα1-3 illustrated good alpha helical spectra in the absence of lipid membranes and alpha helical inducing solvents such as 2, 2, 2- trifluoroethanol (TFE). The PSMβ1-2 peptides, which show the lowest lytic potential, are relatively unstructured in the absence of TFE. The negative peaks at 202nm and minimal negative peaks at 220nm indicate random coil secondary structure, a property which is not amenable to lysis. However, under our conditions, PSMβ1-2 shows increasing alpha helicity with 50% TFE as shown in figure 3.9b table 3.3b. Previous results have shown that all PSM possess alpha helicity, however these peptides were dissolved in 50% TFE (312) which is known, and observed by us, to induce alpha helicity. Our results suggest that the ability to form alpha helical structure is extremely important in lysing CHO containing lipid vesicles, as those PSM which have poor alpha helical folding also have poor lytic activity.

A)

B)
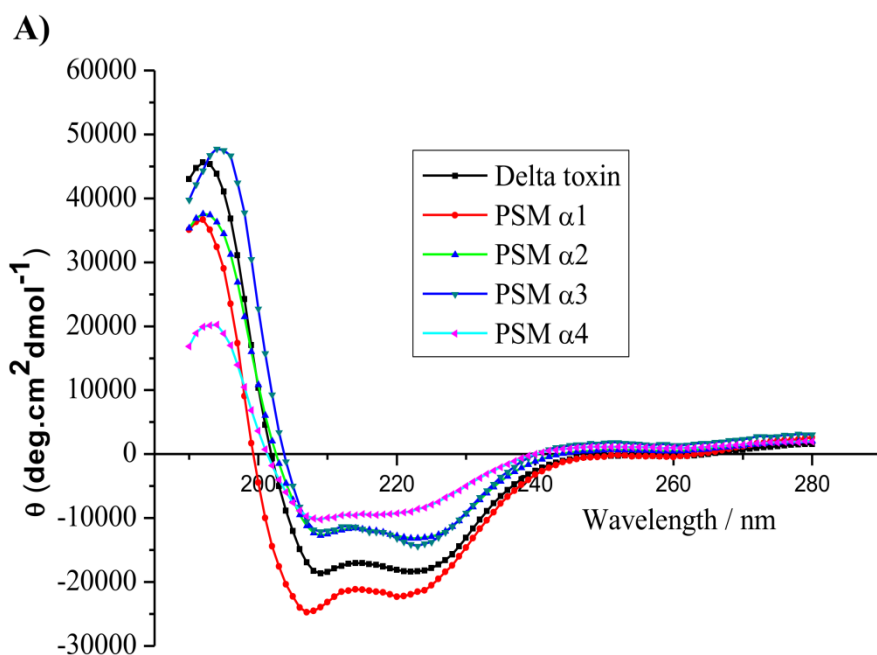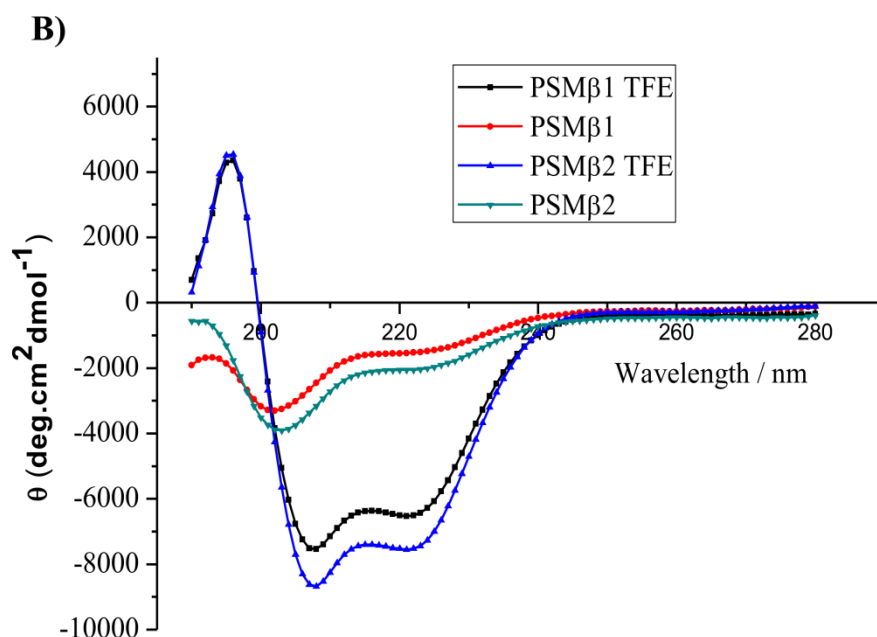
Figure 3.9: Circular dichroism (CD) spectra of synthetic PSMs. A) CD spectra of PSMα1-4 and δ-haemolysin prepared in PBS. **B)** CD spectra of PSMβ1 peptide in 50% TFE (trifluoroethanol), PSM β1 peptide in PBS buffer, PSM β2 peptide in 50% TFE and PSM β2 peptide in PBS buffer. The peaks at 190 and 210nm are indicative to alpha helicity. The negative peaks at 202nm and minimal negative peaks at 220nm indicate random coil secondary structure.

3.4 Conclusions

Lipid vesicles have been extensively used to study toxin membrane interactions (149-151, 153, 440). Synthetic lipid vesicles allow different membrane components, such as cholesterol, glycolipids and phospholipids to be incorporated at varying concentrations allowing the possibility of being able to tune the vesicles response to different lytic toxins (455). In this study we formulate a lipid vesicle that is lysed by a specific group of small, amphipathic, alpha-helical peptides, regulated by the staphylococcal Agr system but unresponsive to other known Agr regulated toxins. This vesicle contains some features consistent with certain eukaryotic membranes, most notably comprising a percentage of cholesterol reflecting that of erythrocyte membranes (20–25% (498)) and featuring two very common membrane phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine. However,

our vesicles lack any glycolipids or sphingomyelin, which can be in high proportion in certain cell types (498).

The similarity in composition of our vesicle system to some eukaryotic membranes is reflected in the similar concentrations of purified toxins required to lyse erythrocytes and our vesicles. In general, erythrocytes lysis occurs after incubation with 10 mg/mL (approximately 3.85 μM) of individual PSMs (316), whereas our vesicles require in the range of 1.5–2.5 μM depending on the peptide toxins, both systems requiring incubation at 37°C for 30 min. If we compare this to the results of PSM interaction with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles used by Duong *et al* we see a stark difference in the concentration of PSMs used and incubation time required to cause lysis (499). For POPC vesicles, PSM concentrations were in the range of 0.5–1.0 μM and incubated for 200 s to reach maximum lysis. Moreover, the order in which the PSM peptides caused lysis of POPC vesicles is quite different to the order for normal cellular lysis and order of our vesicles, with the PSM β1 and β2 peptides and PSM4α causing the highest degree of lysis in POPC vesicles whereas these peptides have the lowest lytic capabilities under physiological conditions (312) and our vesicles (Fig 3.3a). The increase in time and toxin concentration required to lyse our vesicle type in comparison to pure POPC is based a number of factors, but largely dependent on the cholesterol composition in our vesicle type. Cholesterol plays a major role in membrane fluidity and the transition temperature of lipids, which has an overall impact on the mechanical rigidity of the lipid membrane (500) and which we have shown has a profound effect on toxin-mediate lysis of vesicles (Laabei, M *et al*. 2014: Investigating the lytic activity and structural properties of *Staphylococcus aureus* Phenol Soluble Modulin (PSM) peptides, under review). The POPC vesicles are also unsaturated and are therefore packed less tightly which may influence toxin binding and disruption of the membrane; unsaturation also lowers the transition temperature and is more prone to oxidation (501) again affecting membrane integrity.

Agr dysfunction is an increasingly important issue due to its relevance with regard to persistent bacteraemia and decreased antibiotic glycopeptide susceptibility such as to vancomycin (476, 477). Loss of Agr activity is also implicated in increased biofilm formation and attachment to inert surfaces, which is important as the majority of bloodstream hospital acquired infections are catheter associated (502, 503). However, the majority of these studies looking at correlations between Agr dysfunction and clinical outcomes use the CAMP assay. We suggest that the use of the CAMP assay for determining Agr activity may not be sufficiently sensitive in determining Agr function. Using our VLT assay we correctly identified the only 3 isolates from a collection of 89 that had mutations in the Agr locus (summarised in Table 3.1). A possible confounder of the VLT method is their sensitivity to the other PSM toxins. It has been shown previously that *psm* genes can be regulated in an RNAIII independent manner, with AgrA being important in this upregulation (215). However, this also affects the outcome of the CAMP assay, as we show in figure 3.4a, where the LAC δ-haemolysin mutant exhibits a positive CAMP assay. We must also keep in mind that the vast majority of strains that produce detectable levels of PSMs are the highly toxic community acquired type IV SCC*mec* strains, which produce considerably higher concentrations of δ-haemolysin than PSM (312). No examples of Agr dysfunction have been reported amongst this group, indeed Agr function is believed to be critical to their emergence and virulence (318), suggesting that neither the VLT or the CAMP assay will be of use clinically for these types of infection. The question that this study also touches on is how much Agr activity is required in determining a strain to be functional, semi-functional or have complete inactivity? We have suggested that those strains which were VLT negative and non-haemolytic were the only true Agr inactive isolates, acquiring mutations leading to truncation of essential proteins. This VLT is a sensitive, rapid assay which is quick and easy to perform and amenable to 96-well plate high-throughput analysis and may help us in redefining the Agr activity of clinical isolates.

Physico-chemical properties of peptides affect their ability to both penetrate and lyse phospholipid membranes. On the basis of the results presented here, the degree of alpha helicity is the most important property for correct insertion and lysis

of membranes for PSM peptides. The PSMβ1-2 peptides, which show the lowest lytic potential, are relatively unstructured in the absence of TFE, instead possessing a random coil structure which is not conducive to high lytic activity. Why the PSMα4 or PSMβ1-2 peptides have lower alpha helicity is not completely understood as yet but maybe due to the presence of helix breaking residues. Therefore, we suggest refining this class of peptides, as not all of the members have alpha helicity, which is an important factor in membrane lysis.

The ability of these PSM to act synergistically with other toxins has been recently reported and shown to be involved in *S. aureus* escape from epithelial and endothelial phago-endosomes (504). This study reported the synergistic effect of δ-haemolysin or PSMβ1-2 with the sphingomyelinase β-haemolysin in disrupting the endosomal membrane. Interestingly, no synergistic effect was observed with the PSMα peptides, which are more related to δ-haemolysin than the PSMβ peptides. Currently, it is unknown if PSMs can act synergistically with each other, and what molecular interaction may dictate such effects. Experiments designed to investigate how PSMs acting in synergy affect different phospholipid vesicle membranes and how they influence the structure of one another are ongoing and will hopefully shed light onto how these interesting peptides act during pathogenesis.

A New Assay for Rhamnolipid Detection – Important Virulence Factors of *Pseudomonas aeruginosa*

4.1 Abstract

Rhamnolipids [RL] are heterogeneous glycolipid molecules that are composed of one or two L-rhamnose sugars and one or two β-hydroxy fatty acids, which can vary in their length and branch size. They are biosurfactants, predominantly produced by *Pseudomonas aeruginosa* and are important virulence factors, playing a major role in *P. aeruginosa* pathogenesis. Here, the ability to detect RL producing *P. aeruginosa* strains with high sensitivity, based on an assay involving phospholipid vesicles encapsulated with a fluorescent dye is shown. This vesicle-lysis assay is confirmed to be solely sensitive to RL, confirming the importance of the expression of this virulence factor for sensing in a putative smart dressing application. We illustrate a half maximum concentration for vesicle lysis (EC_{50}) of 40 μM (23.2 μg/mL) using pure commercial RL, and highlight the ability to semi-quantify RL directly from the culture supernatant, requiring no extra extraction or processing steps or technical expertise. We show that this method is consistent with results from thin layer chromatography detection and dry weight analysis of RL, but find that the widely used orcinol colorimetric test significantly underestimated RL quantity. This assay was used to compare RL production among strains isolated from either chronic or acute infections. We confirm a positive association between RL production and acute infection isolates ($p = 0.0008$), highlighting the role of RL in certain infections.

4.2 Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen, ubiquitous in the environment and capable of causing a multitude of infections in the immune-compromised host (505, 506). The ability of this organism to cause such a wide array of infections is based in part on the large arsenal of virulence factors it produces (507). Virulence factor regulation in *P. aeruginosa* is achieved through a density-dependent cell-to-cell communication network, involving two acylhomoserine lactone (AHL)-mediated quorum sensing (QS) systems; the *las*, *rhl* (508, 509) and Pseudomonas quinolone signal system (82, 510). The *las* and *rhl* systems are LuxRI homologues, where *lasI* and *rhlI* direct synthesis of *N*-3-oxododecanoylhomoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL) respectively; these are diffusible signalling molecules which activate their respective DNA binding response regulators LasR and RhlR, which in turn induces the expression of a wide range of genes, approximately 6% of the genome (509, 511-513). The other cell-to-cell signalling system responds to the quinolone compound 2-heptyl-3-hydroxy-quinolone (the Pseudomonas quinolone signal, PQS), acting with the transcriptional activator, PqsR (82, 514). An elegant hierarchy system predominates in this global regulatory network, with the *las* system positively regulating both the *rhl* and quinolone signalling systems (82, 83, 515).

One of the most interesting extracellular factors produced by *P. aeruginosa* are rhamnolipids [RL]. RL are surfactant-acting molecules, composed of a hydrophilic head, of one or two rhamnose molecules, defining mono- and di-rhamnolipid, and a hydrophobic tail portion of one or two fatty acids (Fig 4.1). RL have been utilised in a wide variety of industrial applications involving emulsification, detergency, wetting, foaming and solubilisation procedures, bioremediation and food additives (516). The amphiphilic nature of RL allows these biosurfactants to partition into biological and artificial membranes altering their biophysical properties, previously shown in model membranes using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine POPC vesicles and purified RL (517, 518). RL are also very important virulence factors for *P. aeruginosa*, central in immune cell and erythrocyte

destruction (519, 520), swarming and twitching motility (90, 91), biofilm formation and protection (120, 521). Furthermore, they have been implicated in the deterioration of patients with ventilator associated pneumonia (522) and disruption and permeablization of epithelial cells, a prerequisite to *P. aeruginosa* invasion (523).

Monorhamnolipid (n=6)
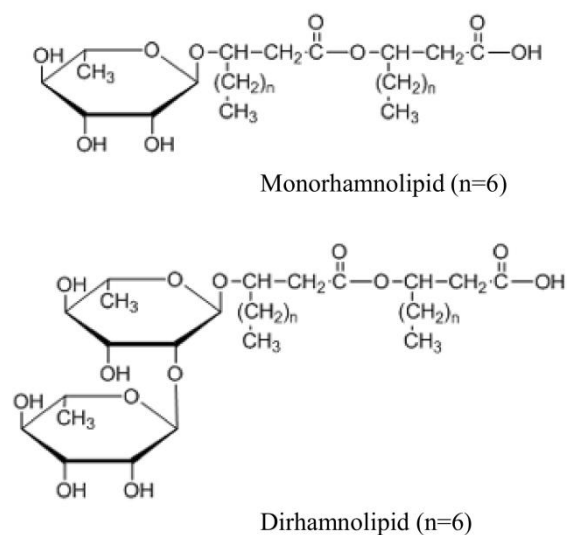
Dirhamnolipid (n=6)

Figure 4.1: Molecular structure of rhamnolipid. RL are composed of a one or two hydrophilic rhamnose sugar head groups attached to either one or two hydrophobic fatty acid chains.

Clearly an accurate and rapid method to assess RL production is important. Current methods can be qualitative; the cetyltrimethylammonium bromide (CTAB) methylene blue (MB) agar test (524), which signals RL expression through the formation of blue halos due to the complexation of the anionic RL and cationic CTAB and MB. Although this method is quick, it suffers from a time delay of incubation for 48 h for best results and also through distortion of halo formation due to fluorescent pigments produced naturally by certain *P. aeruginosa* strains. Other methods measure the tensioactive properties of the surfactant (525) however these methods employ sensitive instruments and are laborious, and not amenable to being utilised in a high-throughput manner. Quantitative methods consist of spectrophotometric analysis, using the orcinol test (526), chromatographic methods

including thin layer chromatography (TLC), high performance liquid chromatography (HPLC) (527), infrared spectroscopy (IR) (528) and dry weight analysis of rhamnolipids following solvent extraction (464).

In this chapter, a new methodology in detecting and quantifying RL using carboxylfluorescein encapsulated phospholipid vesicles is presented, which came to light through my attempts to identify the factors produced by *P. aeruginosa* to lyse vesicles as part of the larger *Bacteriosafe project*. In this report we confirm that RL can be detected and quantified directly from overnight culture supernatants using the vesicle lysis assay, decreasing preparation time, hazardous extraction techniques or expert analysis.

4.3 Results and Discussion

4.3.1 Bacterial mediated lysis of vesicles occurs during early stationary phase of growth

To investigate at what stage of growth *P. aeruginosa* PAO1 wild type lysed lipid vesicles, a range of starting inocula were used to initiate growth in a combination of nutrient rich broth and lipid vesicles (Fig 4.2a). Expression of specific genes is essential at different stages of growth, particularly in the expression of virulence factors (529), capable of lysing lipid vesicles. Therefore, we investigated the ability of *P. aeruginosa* to break down vesicles and correlated this with the respective growth rate with and without vesicles within the media (Fig 4.2b). Differences in optical density (OD_{600}) observed between the two experiments are most probably due to the vesicles impacting on light scattering, resulting in higher absorbance values. Nonetheless, vesicles did not have a negative impact on bacterial growth (Fig 4.2b). By using different starting inocula of $1x10^4$-$1x10^6$ CFU/mL we determined that a time delay exists in lysis of vesicles with respect to smaller starting inoculum. With a starting inoculum of $1x10^6$ CFU/mL bacteria reach late exponential/early stationary

phase of growth after approximately 400 min (Fig 4.2b) (blue line in both Fig 4.2a and b represent 400 min time point) the approximate time point when vesicles begin to breakdown followed by increasing fluorescence release over the next 150 min (Fig 4.2a). This signifies that bacterial mediated breakdown of vesicles occurs during early stationary phase, when the local concentrations of bacteria reach a critical threshold, leading to the expression of quorum sensing (QS) regulatory genes known to be involved in the expression of virulence factors capable of causing membrane damage.
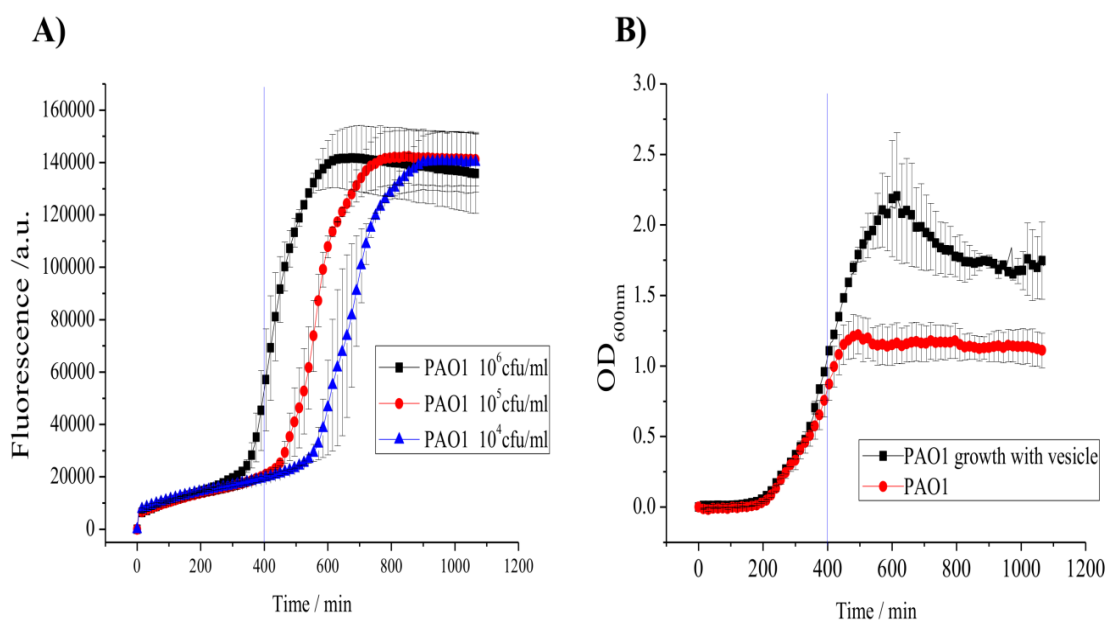
Figure 4.2: Lysis of vesicles occurs during early stationary phase of growth of *Pseudomonas aeruginosa*. A) The breakdown of vesicles by different starting inoculums (10^6-10^4 CFU/mL) was measured over 18 h of growth, shown here by the detection of carboxyfluorescein released from lysed vesicles. **B)** The growth curves of *P. aeruginosa*, with and without vesicles added to the medium, are illustrated over 18 hours from a starting inoculum of 10^6 CFU/mL. Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

4.3.2 Identification of rhamnolipids as the vesicle lysing agent

The production of toxins/enzymes in *P. aeruginosa* is governed by a hierarchical cell to cell QS system, which when activated, leads to the production of a whole suite of virulence factors, some of which are excreted into the extracellular environment. Therefore, we investigated the ability of QS mutants to lyse lipid vesicles (Fig 4.3a),

to determine the factor(s) involved. The quinolone signalling system contains the *pqsABCDE* operon, in which the *pqsABCD* section is required for the synthesis of the hydrophobic quinolone signal which interacts with a LysR-like regulator PqsR, modulating the expression of genes known to function in virulence (79, 530). Therefore, we assayed the culture supernatant of a *pqsA* mutant to identifying any deleterious effects on lipid vesicle lysis; however no difference between WT and mutant strain was evident (Fig 4.3a). It has been shown previously that a *pqsE* mutant, although not impacting on the production of the PQS signal, negatively affects PQS-controlled virulence factors (80, 531) and has been shown to enhance the *rhl* system (532). However, no difference was seen in lysis of lipid vesicles with culture supernatants derived from the *pqsE* mutant (Fig 4.3a). Unlike the signalling molecules of the *las* and to an extent, *rhl* system, the PQS is very hydrophobic, inhibiting free diffusion between bacterial communities (533). To circumvent this, these PQS signals are encapsulated within membrane vesicles (MVs), derived from the outer membrane of the bacterial cell envelope (534). Interestingly, PQS has been shown to induce membrane curvature in erythrocytes, leading to haemolysis (535). The formation of bacterial MVs requires the expression of PqsH, which is a monooxgenase, essential for the conversion of 2-heptyl-4-quinolone (HHQ) to PQS (531). Therefore, in a *pqsH* mutant there is a reduced capacity to form membrane vesicles due to reduced quinolone formation to induce membrane blebbing and structural perturbations. However, no difference was seen in vesicle lysis with culture supernatants derived from this mutant and WT, suggesting that the quinolone signalling system did not play a role in lysis of this specific vesicle type (Fig 4.3a).

The *lasRI* and *rhlRI* regulatory systems are the most well characterised QS systems in *P. aeruginosa*. To investigate which secreted factor(s) caused lysis we examined culture supernatants from deletion mutants of the signal synthase (*lasI* and *rhlI*) and the response regulators (*lasR* and *rhlR*) (Fig 4.3a). The results demonstrated that an active *rhlRI* system was required for vesicle lysis. *P. aeruginosa* expresses three quorum-regulated phospholipases C (PLC) enzymes: a haemolytic PLC (PlcH), a non-haemolytic PLC (PlcN) (536) and a PLC involved in phospholipid chemotaxis (537) (PlcB). PlcH causes cytolysis, with a preferred substrate affinity for

phosphatidylcholine (PC) and sphingomyelin (536), importantly PC is the most abundant lipid in our vesicle. For this reason, we examined the effect of a *plcH* deletion mutant on the capacity to lysis the vesicle (Fig 4.3b). By using serial dilutions of supernatants, it was evident that PlcH did not have any effect on lysis. Most proteins are denatured and inactivated at high temperatures, therefore to understand what was causing vesicle breakdown, supernatants were heat treated and compared to non -treated supernatants (Fig 4.3b), however, no difference in vesicle lysis activity was observed. This suggested that a heat-resistant glycolipid could be involved in overall vesicle lysis.

RL are biosurfactant glycolipids, in which the synthesis of these molecules is under the control of the *rhlABC* operon, where the *rhlA* gene is itself directly regulated by the *rhl* QS system (538). We performed TLC on solvent extracts of the supernatant in order to detect RL from *P. aeruginosa* strains that were either vesicle lysis positive or negative (Fig 4.3c and Table 1). In all cases where strains caused vesicle lysis, RL was detected by TLC, whereas the opposite was true in those strains which were vesicle lysis negative. To understand conclusively what caused *P. aeruginosa* mediated breakdown of lipid vesicles, culture supernatants from an *rhlA* mutant were used and no vesicle lysis was observed (Fig 4.3d). The *rhlA* gene is critical in the formation of RL precursors (90) and thus without this neither mono- nor di- RL are formed.
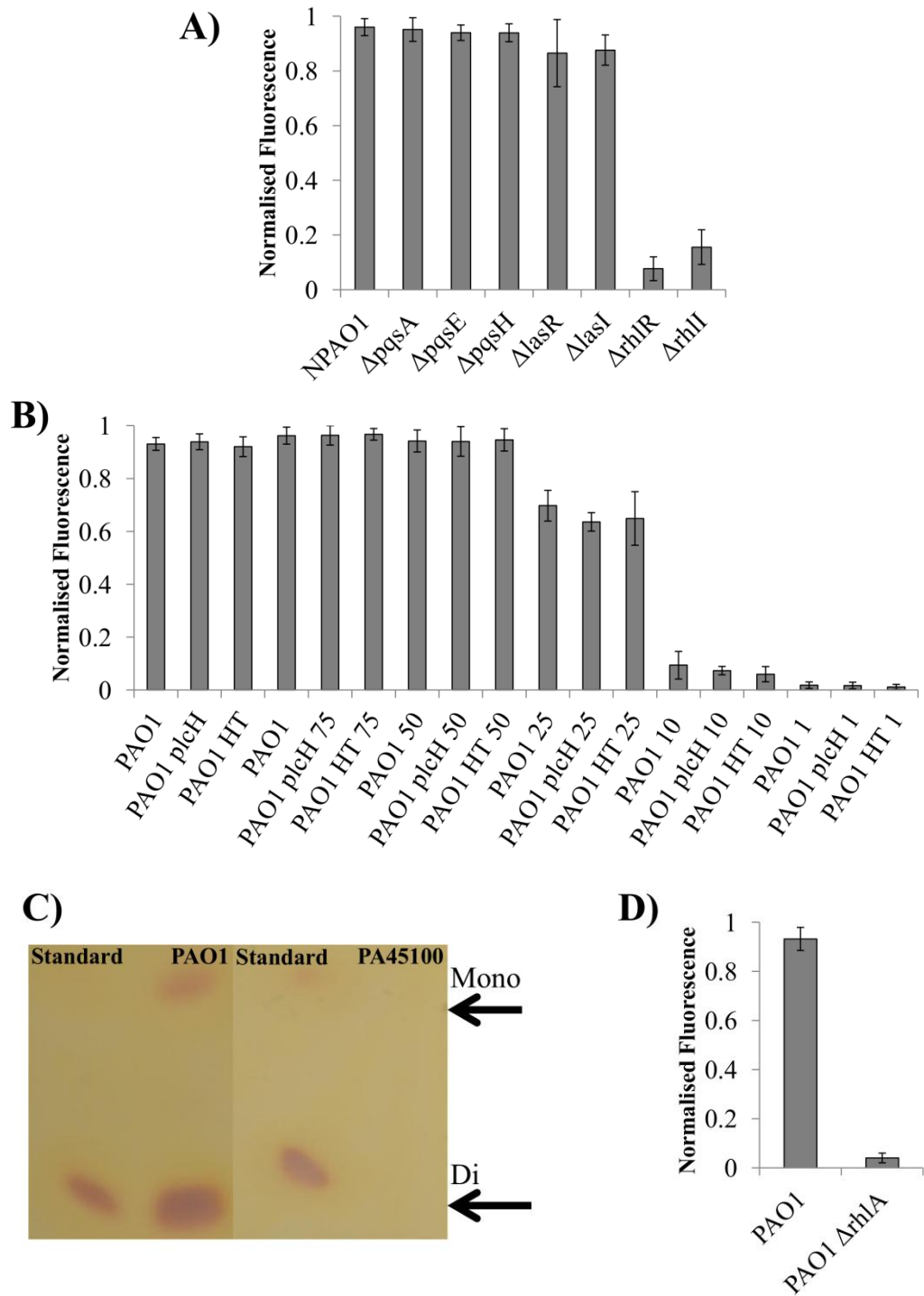
Figure 4.3: Identification of vesicle lysing agent. **A)** Culture supernatants of several quorum-sensing mutants ($\Delta pqsA$, $\Delta pqsE$, $\Delta pqsH$, $\Delta lasR$, $\Delta lasI$, $\Delta rhlR$, $\Delta rhlI$) were tested against lipid vesicles, highlighting the important role of the *rhlRI* QS system in vesicle lysis. **B)** Culture supernatants of wild-type (WT) PAO1, a lipase mutant PAO1$\Delta plcH$ and heat treated (HT) PAO1 culture supernatant at various dilutions (neat, 75, 50, 25, 10, 1) were tested against lipid vesicles, with no significant differences observed. **C)** TLC was used to detect the presence of rhamnolipid from two strains, PAO1 (vesicle lysis positive) and PA45100 (vesicle lysis negative) **D)** Comparison of WT PAO1 and the isogenic *rhlA* mutant. Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

4.3.3 Estimation of secreted rhamnolipid concentration from *P. aeruginosa*

To investigate whether this vesicle-lysis assay could be used to quantify RL secreted from culture supernatants, we created a standard curve (Fig 4.4a) incubating vesicles and purified R-95 rhamnolipid, a mixture of the highly abundant mono- and di-rhamnolipids congeners from *P. aeruginosa*. This graph illustrates the EC_{50}, the concentration of purified rhamnolipid to cause 50% lysis of vesicles, as 23.2 μg/mL (40μM). Using this value, the quantity of RL from culture supernatants can be estimated (Fig 4.4b), by graphing the respective normalised fluorescence obtained from serial dilutions of culture supernatants, using a sigmoidal curve fit, generating the best fit line, giving the dilution constant required for EC_{50} and multiplying to give an estimation of RL in the original culture (92.8 μg/mL for PAO1). This PAO1 RL value generated from the vesicle-lysis assay was relatively consistent with that of our dry weight analysis of PAO1 RL (172.5 ±56.4 μg/mL). It is important to note that quantity and structure of RL is dependent on many factors, including carbon source, reaction vessel and conditions, temperature and strain-specific details and that very high concentration of RL have been recorded using optimised growth medium and bioreactors (539, 540). The growth conditions and reaction conditions we have employed are not conducive to high levels of RL production, as this was not the aim of this study.
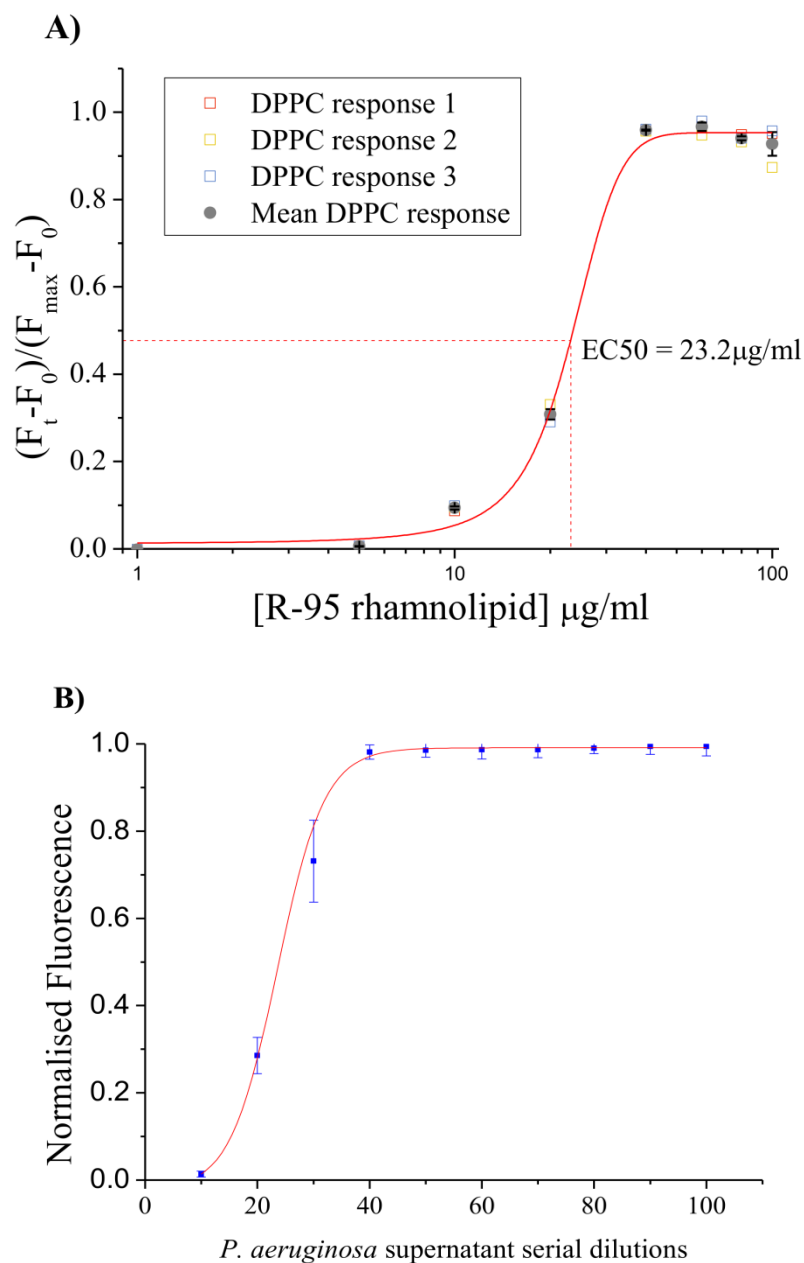
Figure 4.4: Estimation of rhamnolipids in culture supernatants. A) Vesicle-purified rhamnolipid interaction illustrating the EC_{50}, the amount of purified rhamnolipid required to cause 50% of vesicle lysis as measured by fluorescence release. **B)** Estimation of rhamnolipid concentration via serial dilutions of PAO1 culture supernatant exposed to vesicles, generating different fluorescence values. EC_{50} value obtained using 25% supernatant, thus EC_{50} times 4 giving the estimated starting RL quantity (23.2 μg /ml x 4 = 92.8 μg /ml). Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

4.3.4 Comparison of vesicle assay with orcinol and TLC assays

A comparison was made between the vesicle-lysis assay and the orcinol and TLC methods of RL detection and quantification. We selected 16 strains, 8 derived from acute infections and 8 derived from chronic infections and measured their respective RL content using the above methods (Table 4.1). The orcinol assay was chosen as this colorimetric assay is widely used. However, this method provided a significant underestimation of RL values compared to our results, perhaps due to loss of RL during the extraction procedure, which were produced at small quantities initially. This was not the first study which has highlighted erroneous results using the orcinol assay (540). The orcinol method can also suffer from contamination from the growth media and also other components of the cellular envelope which has rhamnose as a component in their structure, namely lipopolysaccharides.

The TLC method was used qualitatively and complemented the results of our vesicle-lysis assay, whereby vesicle lysis positive strains were also shown to secrete RL (Table 4.1). The CTAB method can suffer from distortion of RL complexation circles due to pigment production (524) and we were not able to generate consistent results using this method. The RL values of clinical isolates examined here (Table 4.1), are consistent with values shown by other groups (539). Although the vesicle-lysis assay is dependent on fluorescence detection from lysed vesicles, this is not influenced by fluorescent molecules being expressed by *P. aeruginosa* (data not shown): pyoverdine is typically excited by low wavelength light (UV) whilst carboxyfluorescein is excited by blue (490 nm) light (541).

Strain (acute)	Orcinol Test*	Lipid Vesicle Test*	TLC	Strain (chronic)	Orcinol Test*	Lipid Vesicle Test*	TLC
44883	21.7	125.4	+	PA00856	22.2	85.5	+
44981	8.6	25.6	+	PA00887	20.83	30.9	+
45197	16.7	86.9	+	PA00889	21.7	91.1	+
45445	32.5	150.2	+	PA00935	16.2	90.1	+
45076	2.3	NL	-	PA00261	3.2	NL	-
45100	15.2	NL	-	PA00848	3.9	NL	-
45122	1.88	NL	-	PA00918	3.4	NL	-
45394	2.83	NL	-	PA00929	3.7	NL	-

Table 4.1 Detection and quantification of rhamnolipids
* Quantification of rhamnolipids derived from these techniques in μg/mL; No lysis, NL.

4.3.5 Rhamnolipid expression is associated with acute infections

It has been shown previously that RL are important virulence factors as they have been implicated in cell death and essential in correct biofilm construction and protection. We wanted to explore the use of this assay to determine rhamnolipid expression among clinical isolates. We performed vesicle-supernatant experiments on a range of clinical strains (n=78), from a chronic (n=48; isolated from cystic fibrosis patients) and an acute (n=30; isolated from blood stream and wound infections) infection background to gain an understanding of the clinical importance of RL in these two classes of infection (Fig 4.5). There was a positive association between RL expression and acute infection isolates (Fisher test $p = 0.0008$), which suggests that RL play an active role during acute infections, and that this assay can determine this from culture supernatant.
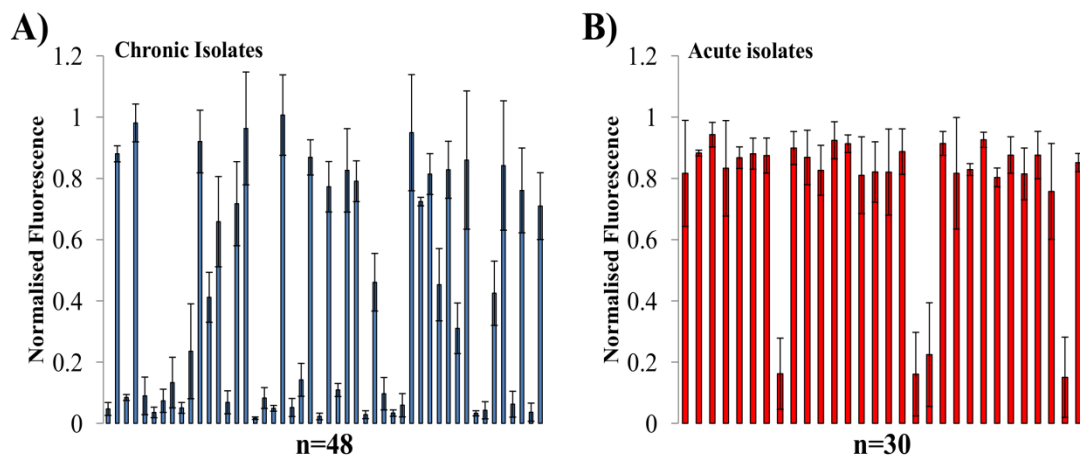
Figure 4.5: Positive association between rhamnolipid expression and isolates from acute infections. Vesicle-supernatant assay exploring the rhamnolipid producing capacity of clinical *P. aeruginosa* isolates derived from either a **A)** chronic (blue; n=48) or **B)** acute infection (red; n=30). A positive association between rhamnolipid production and isolates from acute infections was observed, using an exact fisher test, *p*=0.0008. Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

4.4 Conclusions

The vesicle-lysis assay is responsive to QS regulated factors, which are expressed during the transition from late exponential to early stationary phase of growth (Fig 4.2). *P. aeruginosa* expresses a wide spectrum of exofactors, some of which are important in membrane damage. Using isogenic mutants in key regulatory QS genes and virulence determinants we investigated which factor was important in the observed lysis. Due to their hydrophobic nature, PQS molecules induce the formation of MVs through an interaction with lipopolysaccharide on the bacterial outer membrane facilitating cell-to-cell communication, and this is dependent on the expression of the *pqs* operon (Mashburn and Whiteley 2005). Interestingly , it has also been reported that exogenously added PQS molecules can also induce membrane curvature in erythrocytes, lacking any of the receptors important for MV formation, leading to haemolysis in a concentration dependent manner (535). Additionally, after the blebbing of MVs from the bacterial cell envelope these structures are then able to fuse with recipient cells, transferring their cargo in an elegant transport mechanism (535). Considering these observations we investigated

whether the PQS signalling system was influencing lysis of vesicles used in this study. Genetic inactivation of *pqsA*, *pqsE* or *pqsH* conferred no reduction in vesicle lysis (Fig 4.3a), suggesting no role for the PQS system in disruption of these vesicles.

Surprisingly, the phospholipase C enzyme (PlcH), a key component in the degradation of lipids due to their affinity for phosphatidylcholine head groups, did not play a role in lipid vesicle lysis as no reduction in fluorescence was observed in the *plcH* mutant or heat treated supernatants (Fig 4.3b). Work on phospholipase 2 (PLA) has shown that the degree of saturation and most importantly, the acyl chain length of the phospholipids impact on the ability of this lipase to lyse lipid bilayers (542). The structure of the vesicles in this study are composed of DPPC and DPPE, which are saturated phospholipids containing 16 carbon chain length fatty acids which may not be efficient substrates for PLC activity.

Inactivation of the *rhl* QS system leads to a reduction in vesicle lysis (Fig 4.3a). RL expression is under the control of *rhlABC* operon, where *rhlA*, a gene which encodes a rhamnosyltransferase which catalyses the transfer of L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkonic acid (HAA) and is required for subsequent RL formation, is regulated by the *rhl* system. RL are also heat-resistant molecules, and our results show heat-treatment was insufficient to inhibit the lytic function of bacterial supernatants derived from *P. aeruginosa* strain PAO1 (Fig 4.3b). Finally, deletion of the *rhlA* gene confers a non-lytic phenotype, consistent with TLC results showing no RL production in strains which do not lyse vesicles (Fig 4.3c-d), confirming RL as the sole vesicle lytic agent from *P. aeruginosa*, and fulfilling one of the objectives of the Bacteriosafe project.

As a result of these experiments, we sought to develop a new method to quantify RL based on the lysis of carboxyfluorescein-encapsulated phospholipid vesicles. Here we illustrate that this assay is more rapid, sensitive and easier to perform than current methods. The vesicle-lysis assay requires no extraction procedure and therefore is not susceptible to contamination or sample loss. We have shown that this assay is semi-quantitative and can estimate the amount of RL present in culture

supernatants (Fig 4.4). We observe that our results are in contrast to the values generated using the orcinol assay but are consistent with TLC and dry weight analysis. However, the orcinol test relies on the extraction of RL from the supernatant, the measurement of the rhamnose content by use of orcinol and concentrated sulphuric acid and applying these absorbance values to a standard curve (526). This procedure can be susceptible to contamination from other rhamnose containing molecules such as lipopolysaccharides or from un-extracted media components and can also give erroneous results due to differences in incubation temperature and sample loss (540).

RL production also had a positive association with isolates derived from acute infections; however this link is somewhat tenuous. It is difficult to determine any association between an infection and a specific bacterial factor, particularly when comparing between such different infections. A more informative study should include strains derived from different time periods during the length of specific chronic infection (e.g infection in cystic fibrosis patient) to evaluate whether a specific time point exists that leads to the reduction of RL expression. Equally, a more informative clinical history of the isolate derived from acute infections is necessary in order to make an accurate judgement on the above association. However the superficial association that is illustrated in figure 4.5 is used only as an example of how this novel phenotypic assay may be employed. Another important consideration would to use genotyped strains to examine any polymorphisms that may result in an alteration in RL expression, in an analogous fashion to the experiemtns designed in chapter 7. It is known that genetic changes occur during chronic infections, which can lead to the down regulation of extracellular virulence factors (84) with loss of function mutations in the central regulator *lasR* being most frequent in chronic infections, but other QS mutants involving the *rhl* system are evident (543-545). Since *las* and *rhl* system are intricately linked, with the *las* system controlling the *rhl* system at a transcriptional and post-translational level, it was conceivable that mutations in *lasR* were causing this RL negative-phenotype in certain clinical strains. However, in PAO1 we did not see any statistically significant difference in lysis potential between *lasR* mutant and WT strains. This leads us to

believe that mutations in either the *rhl* system or the *rhlABC* operon are the most likely reasons for RL negative phenotype. Novel point mutations in quorum regulators genes and mutations in the multitude of quorum regulators may also be responsible for the RL negative phenotype and work into elucidating this in ongoing.

Following from these observations, we envisage that this vesicle lysis test may be applied as a rapid phenotypic assay, useful for screening large numbers of clinical strains in an effort to determine novel mutations that may affect the expression of this important virulence factor. Given the specificity of this assay it could be an important tool in analysing the highly complex and interconnected QS systems of *P. aeruginosa*. The effects of mutation on candidate putative regulatory genes or single base pair mutations of known regulators could be assayed quickly to in order to determine their effect on a key QS-regulated virulence factors.

Determining whether an isolate is a strong- or weak-RL producer maybe important considering that specific concentration of RL are required to lyse important immune cells and disrupt the cellular barrier which prevent invasion and dissemination (519, 523). One limitation of this assay is that it is susceptible to other microbial surfactants, notably the phenol-soluble modulin (PSM) peptide toxins of *S. aureus* (546). Therefore, isolation of pure *P. aeruginosa* isolates is required before this assay can be used to detect and quantify RL production.

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Investigating the effects of Toxic Shock Syndrome Toxin-1 on global exoprotein expression in *Staphylococcus aureus*

5.1 Abstract

The main etiologic agent of toxic shock syndrome (TSS) is the toxic shock syndrome toxin-1 (TSST-1) protein secreted by *Staphylococcus aureus*. Diagnosis of TSS is difficult and is significantly under-reported in young children with burns, due to the nonspecific presentation coupled with a rapid deterioration in patient condition. Previous reports have observed a downregulation of exotoxin production mediated by the expression of the TSST-1 protein using a lab strain, RN4282. To investigate this further, the lytic and cytolytic activity of a number of clinical and laboratory TSST-1 positive strains of methicillin-susceptible *S. aureus* (MSSA) (101, 253, 279 and RN4282) were tested *in vitro* using the phospholipid vesicle and T cell toxicity assay. In addition, the activity of lytic exotoxins such as the δ-haemolysin/ PSM peptide toxins, β-haemolysin, α-haemolysin and non-lytic TSST-1 toxin was measured over the 20-hour growth of RN4282 and the isogenic *tst* negative strain, RN6938, with both strains presenting toxic phenotypes. It is important to note that the clinical TSST-1 positive methicillin-susceptible *S. aureus* strains exhibited lytic exotoxin production as well as TSST-1 expression as confirmed by Western blot. We suggest that there is no correlation between the expression of TSST-1 and lack of exotoxin production which has been previously described. We also suggest that purulence in an *S. aureus* infected burn wound in a child should not be used to rule out toxic shock syndrome.

5.2 Introduction

Toxic shock syndrome (TSS) is a serious complication of burn infections, primarily affecting children less than 4 years of age due to a lower level of antibody-mediated protection (547-549). TSS presents clinically as a range of symptoms including shock, pyrexia, an erythematous rash, gastrointestinal disturbance and central nervous system signs including lethargy or irritability (550). TSS is often observed in infants with smaller partial thickness scalds, possibly due to more conservative treatment and the absence of surgical debridement (547). The TSS clinical picture is of a young child with a small burn who deteriorates and rapidly becomes moribund within hours unless treatment is started promptly. Untreated mortality rates are around 50% (551). A study by Guggenheim *et al* showed that the majority of children with burn infections had *Staphylococcus aureus* as the primary infective agent (73). Various strains of *S. aureus* are known to secrete the small 22 kDa toxic shock syndrome toxin-1(TSST-1) protein, which is highly associated with menstrual TSS (mTSS) and, along with staphylococcal enterotoxin (SE) B and SEC, with non-mTSS which can develop during burn wound infection (283, 552, 553).

The *tst* gene (which encodes TSST-1) can be found on one of three mobile staphylococcal pathogenicity islands (SaPIs) that can integrate close to *tyrB* gene (SaPI1), or within *trp* locus (SaPI2) (554). A third *tst* gene is found on SaPIbov, a pathogenicity island associated with certain bovine *S. aureus* strains (555). Approximately 20% of *S. aureus* strains carry a *tst*-containing pathogenicity island (554) however, TSS is fortunately rarely seen, occurring in approximately 0.6 cases per 100,000 people (556). This is most likely due to the tight control and complex regulation of this toxin. Multiple environmental factors and molecular mechanisms all play a role in modulating *tst* expression. Environmental stimuli such as elevated carbon dioxide and oxygen are associated with high levels of toxin production, possibly through the SsrAB two component system, which is hypothesised to differentially regulate certain virulence genes under aerobic and anaerobic conditions (349, 557) (see section 1.3.4.2). TSST-1 production is also inhibited by glucose

(558), and this has been demonstrated to be dependent on the binding of the catabolite control protein CcpA, with catabolite responsive elements (cre) situated in all known *tst* promoter regions (559). Other factors such as NaCl and magnesium concentration and the relative pH all play a role in altering production of this toxin (560-562), factors which were all implicated in the correlation observed between tampon use and mTSS. Furthermore, the role of vaginal mircobiota has been highlighted in the induction and suppression of TSST-1 production (563) and cyclic dipeptides produced by the human vaginal isolate, *Lactobacillus reuteri* RC-14, has been shown to interfere with the accessory gene regulator (Agr) quorum sensing system, resulting in reduced TSST-1 expression (564). Importantly, the most direct evidence of *tst* regulation is via the SarA DNA-binding protein, with SarA directly binding to at least one site within the *tst* promoter (565). Moreover, deletion of *sarA* leads to a more deleterious *tst* transcription than deletion of the effector molecule of the Agr system, RNAIII (565), highlighting the central role of SarA in *tst* regulation.

TSST-1 is part of a class of proteins known as super antigens (SAg), due to their ability to massively and non-specifically over activate T cells. SAg can bridge the major histocompatibility complex (MHC) class II expressed on antigen presenting cells and surface attached T cell receptors on CD4+ T cells without the requirement of a specific antigen (Fig 5.1). This action stimulates a much larger and disproportional release of cytokines including tumour necrosis factor, interleukins and gamma-interferon. The downstream effect is the activation of up to 30% of the T cell population and interruption of normal immune surveillance (566). Recent studies have shown that a related staphylococcal enterotoxin, SEB, interacts with the CD28 co-stimulatory molecule, which is required for full T cell activation and cytokine production (567). Such nonspecific cytokine release leads to rapid clinical deterioration which can result in capillary leakage leading to hypotension, shock and multiple organ failure. This activation of T cells and resulting 'cytokine storms' occurs only after induction of cytokine production from mucosal epithelial cells, a process known as 'outside-in' signalling. Pro-inflammatory cytokine and chemokine production is stimulated at the mucosal epithelial layers causing increased mucosal

permeability, allowing SAg penetration through these layers and subsequent recruitment of T cells and distortion of the immune system (568).
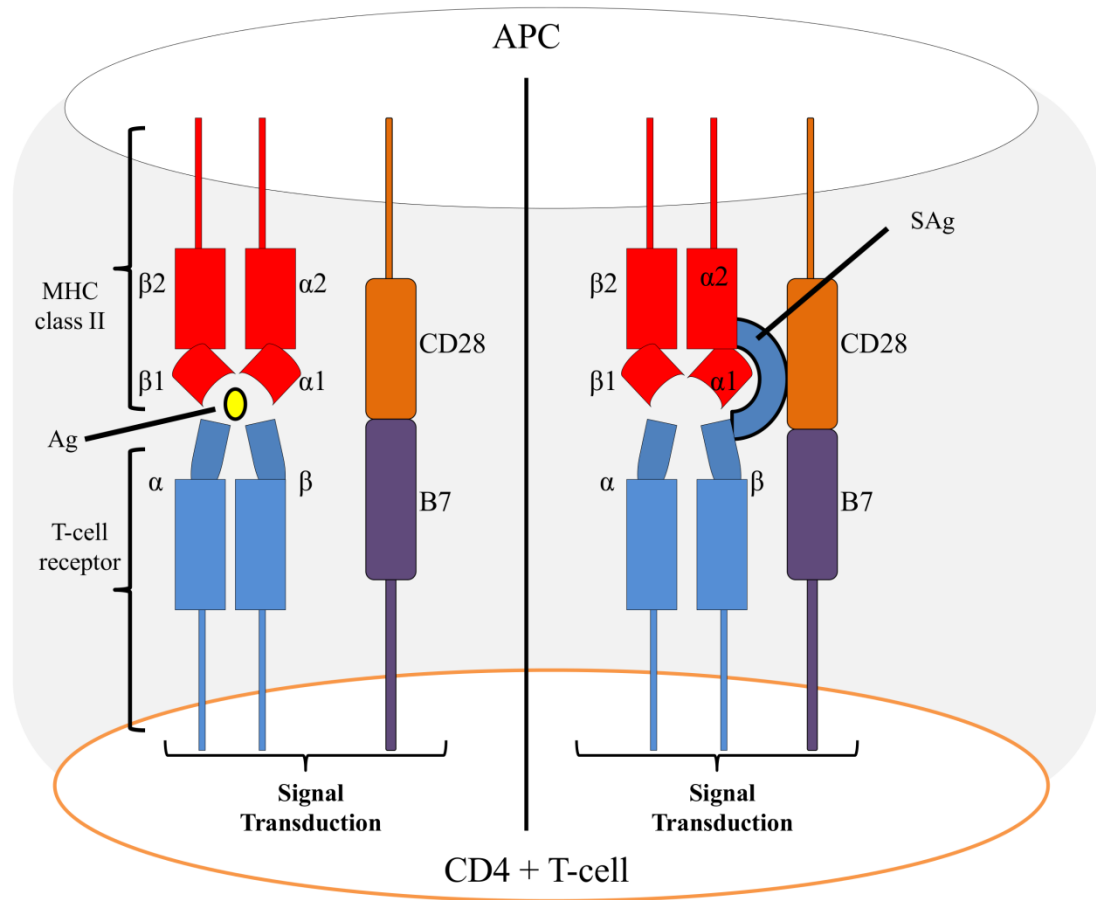
Figure 5.1: Mode of action of superantigen TSST-1. The TSST-1 protein (SAg) generates powerful immune responses by binding to both the MHC II on the antigen presenting cell (APC) and TCR on the CD4+ T cell and is hypothesised to interact with the co stimulatory molecule CD28, which normally ligates with the B7 molecule. SAg mediated bridging bypass normal antigen (Ag) presentation (left) and results in the activation of downstream signalling cascades, leading to the massive stimulation and non-specific proliferation of T cells and secretion of cytokines. Figure adapted from reference (568).

The work described in this chapter is important regarding the aims of the *Bacteriosafe* project; designing a bacterial sensor mechanism dependent on the expression of bacterial membrane damaging factors. As part of this project, TSST-1producing *S. aureus* were studied to investigate whether exotoxins from such bacteria would lyse phospholipid vesicles. This was in relation to a proposed method of *tst* regulation described by Vojtov *et al* demonstrating that TSST-1 undergoes auto

repressive regulation and strongly represses the expression of virtually all exoproteins (457). As a consequence, we investigated the toxin production and vesicle lysis capabilities of the TSST-1 producing *S. aureus* strain, RN4282 and the isogenic *tst* deletion mutant, RN6938, which were used in the original study performed by Vojtov *et al*. Additionally, we also investigate the toxic activity of three other TSST-1 positive clinical *S. aureus* strains (community-acquired methicillin sensitive *S. aureus* strains 101, 253 and 279) while simultaneously analysing TSST-1 expression through western blotting.

5.3 Results and Discussion

One of the primary modes of pathogenicity of bacteria such as *S. aureus* is via the action of their secreted exotoxins. Exotoxins such as δ-haemolysin target the eukaryotic cell membrane by adsorbing to target membranes and disrupting membrane integrity leading to osmotic shock and cell death. As described previously, the phospholipid vesicles used in this work were designed to roughly mimic the eukaryotic cell membrane and be susceptible to such toxins.

5.3.1 Vesicle response to bacterial strains

The dilution of self-quenched and 'switch on' of carboxylfluorescein (CF) is a simple way to follow vesicle membrane damage by toxins, as release and dilution of encapsulated dye switches on fluorescence, resulting in an easily observable / measureable response to vesicle lysis. Figure 5.2 shows the fluorescence response of vesicles to Triton X100 and HEPES buffer (positive and negative control respectively); and the strains under investigation as described earlier. RN6390B (Agr +) and RN6911 (Agr -) strains where used as positive and negative toxin producers as the Agr quorum sensing system plays a central role in general toxin expression and activates the expression of δ-haemolysin and phenol-soluble modulin (PSM) toxins, known to lyse the vesicles used in this study (Chapter 3). We investigated the potential of the TSST-1 secreting strain, RN4282, and the isogenic *tst* knockout

strain, RN6938, to lyse vesicles, in order to examine any influence that TSST-1 expression may have on toxin mediated vesicle lysis. Finally, three clinical methicillin sensitive *S aureus* (MSSA) strains which were annotated as *tst* positive by PCR (427) were also used to examine their vesicle lytic potential. BothRN4282 and RN6938 and the three TSST-1 positive strains caused lysis of vesicles equal to that of the positive control, suggesting that this protein did not downregulate the toxins that are required to lyse vesicles.
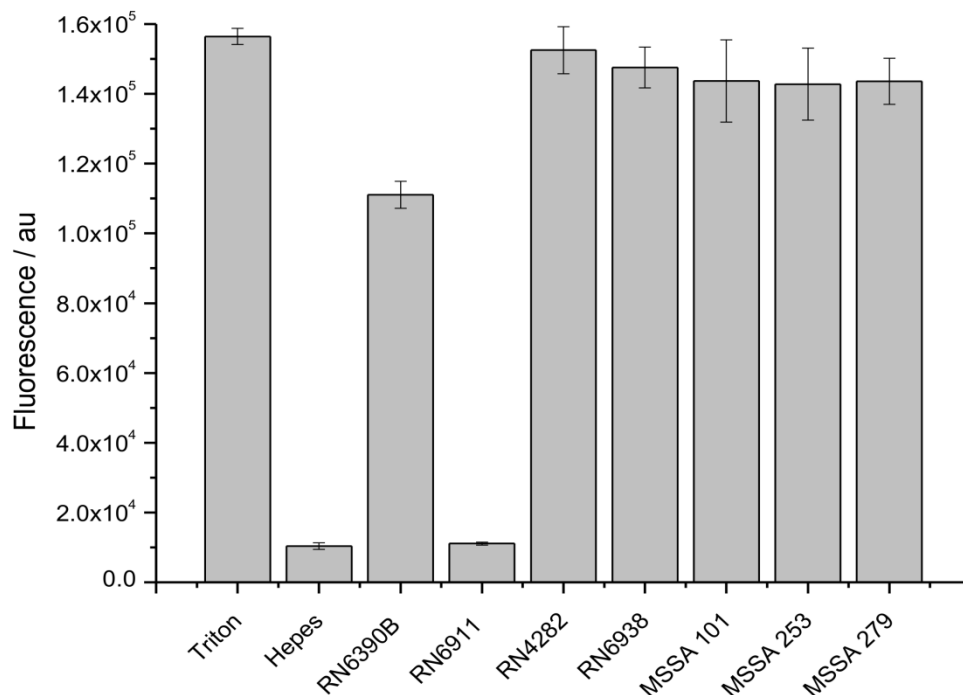
Figure 5.2: Fluorescence response as a result of vesicle lysis by bacterial exotoxins after exposure to overnight bacterial culture supernatant. All strains, except the Agr deletion strain (RN6911) caused lysis of vesicles. Triton X100 and HEPES buffer were used as positive and negative controls respectively. Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

5.3.2 T cell cytotoxicity assay

As discussed later (Chapter 6), the T cells used in the cytotoxicity assay are susceptible to a suite of toxins including the α-, β-, γ- and δ-haemolysin and PSMs. Using this assay we wanted to investigate any deleterious effect on these toxins

associated with the presence/expression of the *tst* gene. T cell survival thus negatively correlates with bacterial pathogenicity / exotoxin production. Hence, the histogram shown in figure 5.3 is almost a mirror image of figure 5.2, with RN6390B, RN4282 and the clinical MSSA strains being toxic to T cells, whereas RN6911 caused no T cell death and was comparable to the HEPES value.
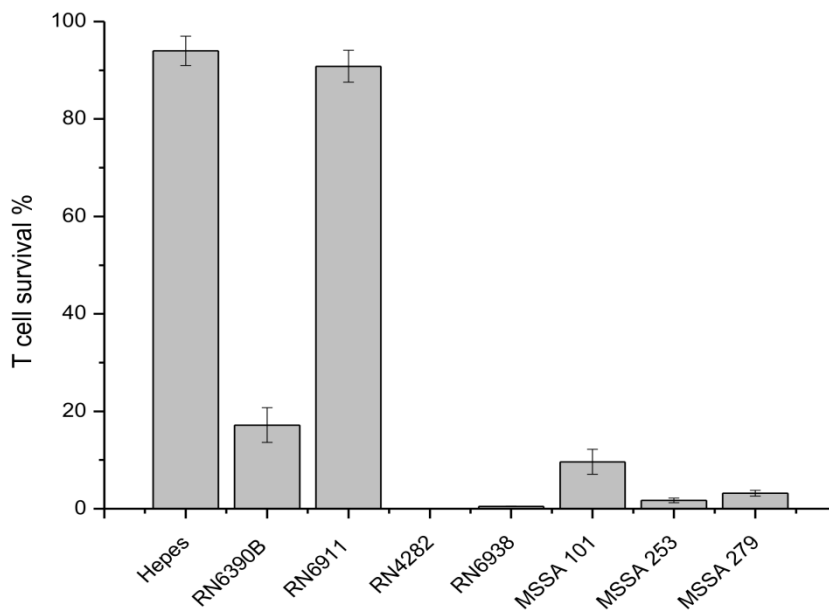
Figure 5.3: T cell toxicity assay. Bacterial supernatant mediated lysis of T cells illustrating toxic phenotype of all strains except RN6911 (Agr negative). Importantly, both the *tst* positive and negative strains show T cell lysis. Experimetns were performed in duplicate three times with error bars representing the 95% confidence interval

5.3.3 Whole cell lysate and exoprotein analysis of RN4282 and RN6938

The previous results have contradicted the role of TSST-1 in repressing global exoprotein secretion, as supernatants derived from both RN4282 and RN6938 caused vesicle and T cell lysis. An investigation into the effects of *tst* deletion on protein profiles was performed in these lab strains to understand if any specific toxins were repressed, as vesicle lysis and T cell death could have been caused by a single toxin and therefore this assay would not accurately reflect global exoprotein analysis. Prior

to further protein analysis, the growth curves of both strains were compared and no deviation in growth was observed (Fig 5.4a). Western blot analysis on TSST-1 expression was also performed (Fig 5.4b), confirming expression of TSST-1 under these experimental culture conditions, as toxin expression can be affected by growth in different media (569). Whole cell lysate (Fig 5.4c) and tricholoroacetic acid (TCA) precipitated supernatant protein analysis (Fig 5.4d) was performed to investigate any global deleterious effects caused by TSST-1 expression. These results clearly show no decrease in exoprotein secretion associated with effect of TSST-1. Additionally, the potential effects of *tst* on specific toxin expression was examined by use of butanol-1 peptide extraction of peptide toxins (δ-haemolysin and PSMs), western blot analysis of the hepatameric pore-forming α-haemolysin and the detection of beta haemolysin on sheep blood agar through the 'hot-cold lysis' method (Fig 5.4 e-g). Again, no deleterious effect was observed in the TSST-1 producing strain as both strains were positive for the above toxin phenotypes.
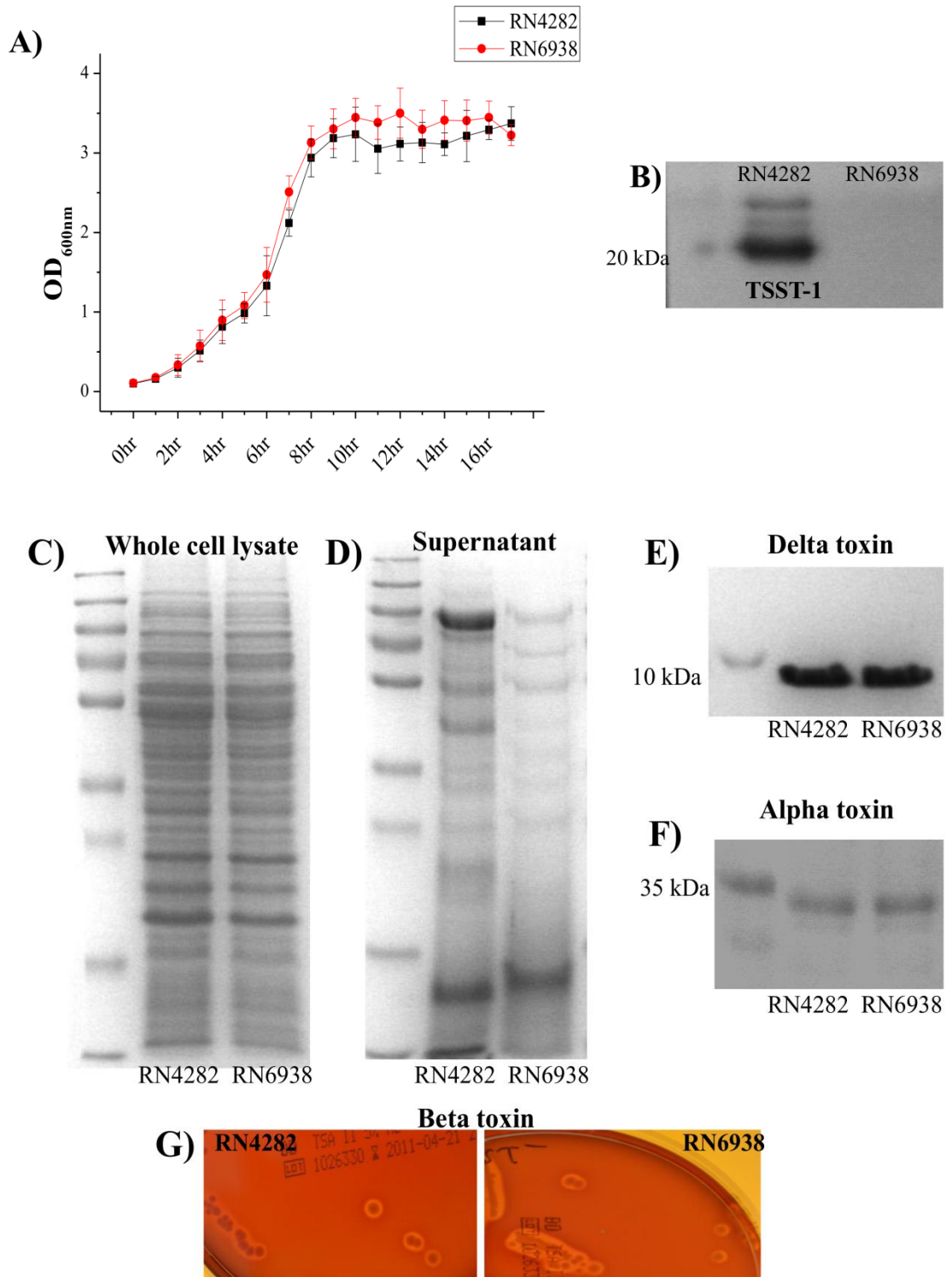
Figure 5.4: Protein analysis of RN4282 (*tst* positive) and RN6938 (*tst* negative). A) Growth curves, **B)** western blot analysis highlighting TSST-1 expression from the *tst* positive RN4282, **C)** whole cell lysate protein preparations and **D)** supernatant proteins analysis of RN4282 and RN6938. **E), F)** and **G)** illustrate both strains are positive for delta-lysin, α-haemolysin and beta-toxin respectively.

5.3.4 Real-time analysis of *S. aureus* clinical isolates: Growth rates, exotoxin production and TSST-1 secretion

The high degree of toxicity to T cells, the response of the phospholipid vesicles to the TSST-1positive *S. aureus* RN4282, and the positive expression of three exotoxins confirms that this strain, as well as producing the super-antigen TSST-1, also expresses and secretes lytic membrane-damaging agents. Although RN4282 was isolated from the clinic, it has been a laboratory strain since 1983 (462) and therefore maybe harbouring mutations that affect its virulence regulation. To fully confirm that the TSST-1 protein had no measurable effect on global exotoxin synthesis, we switched from the laboratory strain (RN4282) to clinical *tst* positive strains (*S. aureus* 101, 253 and 279). Further measurements were carried out, analysing the correlation between growth rate, vesicle lysis as a function of fluorescence and detection of TSST-1 (Fig 5.5). These results show that the lytic exotoxin secretion (not TSST-1) commences early in the bacterial growth phase, with a clear change in fluorescence being measureable (and observable by eye) within 6-8 hours from an initial starting inoculum of 10^5 CFU / mL. In all three strains (Fig 5.5a-c), vesicle lysis is observed during the post-exponential phase of growth, approximately corresponding to 10^8 CFU/mL. TSST-1 secretion does not commence until later on in the growth phase, at approximately 7–11 hours (Fig 5.5a-c). The Western blot analysis therefore confirms not only that the three clinical strains tested here do secrete TSST-1, but are also highly lytic to vesicles and that in an *in vitro* experiment, the putative detection system will signal before or at the onset of TSST-1 expression. We also observed α-, β- and δ-haemolysin expression and explored the respective supernatant protein profiles of these three clinical strains, all illustrating no exotoxin repression (data not shown). This has important implications for the clinical arena and potential early diagnosis of infection via a putative smart dressing that incorporates such vesicles, which is currently being developed (165, 570).
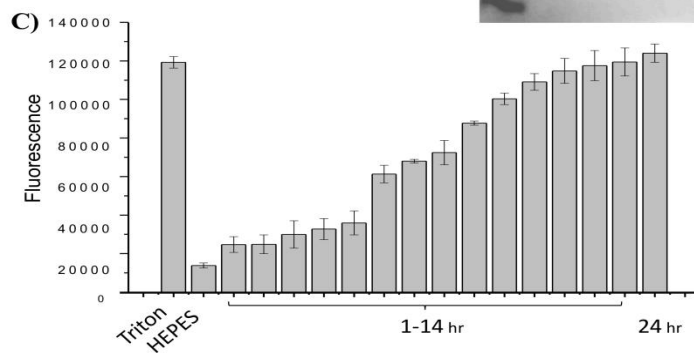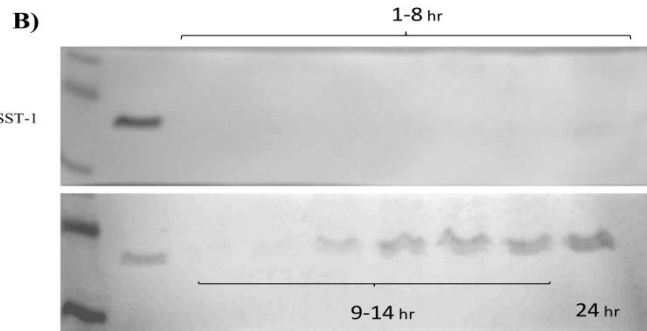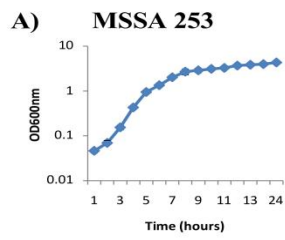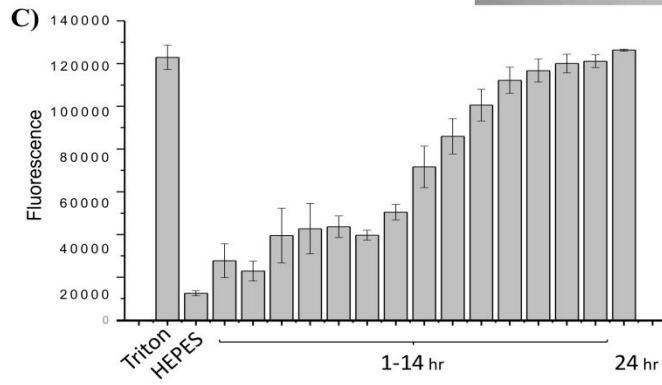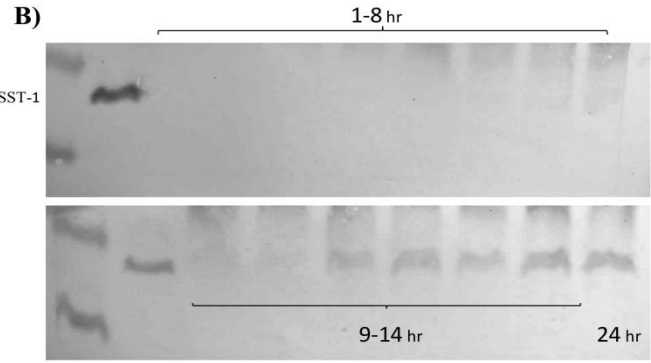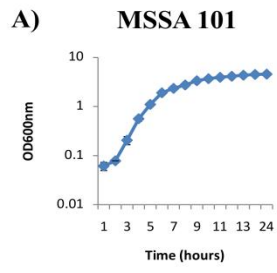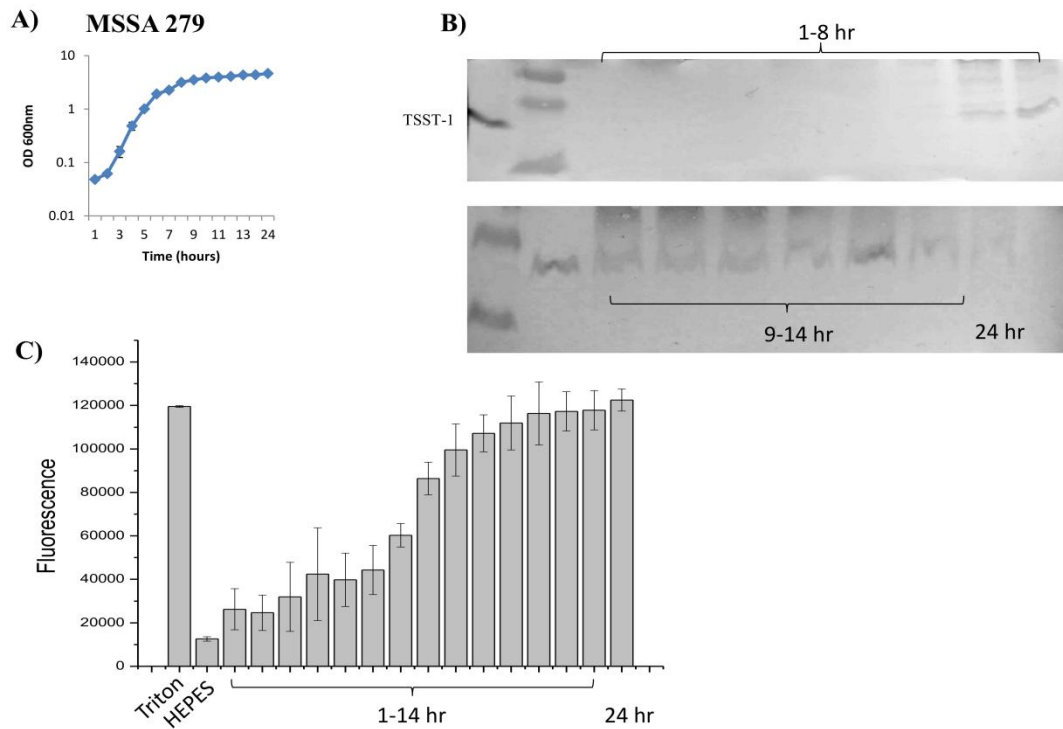
Figure 5.5: Real time analysis of TSST-1 positive clinical strains. Strains MSSA 101, 253 and 279 were analysed based on the association between TSST-1 production (B) and vesicle lysis (C) as a measure of growth rate (A). All three strains grew in a characteristic fashion and secreted TSST-1 at approximately 7-11 hr of growth. Vesicle lysis was significantly apparent between 6-8 hrs of growth. Experimetns were performed in triplicate three times with error bars representing the 95% confidence interval.

5.4 Conclusions

The results of this chapter suggest that TSST-1 expression does not have a negative or global repressive effect on overall exotoxin synthesis in *S. aureus*. Importantly, we see no significant difference in exotoxin profile between the TSST-1 positive and negative strains, which was substantial in the Vojitov *et al* study (457). It was noted previously that TSS causing clinical isolates had lower production of haemolysin, lipase and nuclease, than strains which were not producing the TSST-1 protein (formally known as pyrogenic exotoxin C) (571). However, these virulence genes are regulated by the Agr regulon, the two component SaeRS system and SarA DNA binding protein (329) . Therefore, any deleterious mutation in these regions could impact on the levels of virulence expression, which may have been misrepresented as

TSST-1 global regulatory effects. In this study we show that the presence of the *tst* gene has no repressive effect in the production of three main virulence genes; α-, β-haemolysin and the δ-haemolysin/PSM peptides. Three more clinical strains were also tested (Fig 5.5a-c) with no indication of repressive effect by this SAg. These results are consistent with studies designed to assess the contribution of SAg's in modulating the secretome expression in *Streptococcus pyogenes* (572). This study concluded that neither the streptococcal pyrogenic exotoxin A (SPEA) nor streptococcal mitogenic exotoxin Z (SMEZ) had any regulatory effect on protein expression (572).

An important element in the design of the smart dressings outlined in section 1.4.1 is the role of toxins in damaging dye loaded vesicles for use in signalling responses. Considering that the overall aim was the development of a prototype for use in burn care, with a particular focus on paediatric burn patients, coupled with the fact that TSS can be potentially fatal in young children, highlighted the need for an investigation into whether these putative signalling system could respond to TSST-1 positive *S. aureus* strains, at least *in vitro*. One important factor in disease progression is the speed at which the patient's health declines. Children with TSS deteriorate rapidly (1‑2 hours) and are unresponsive to antibiotics alone. In Figure 5.5, vesicle lysis occurs after 6-8 hours of growth whereas TSST-1 production begins after 7-11 hours and is strain dependent. Therefore, in this *in vitro* model, these vesicles could be used to signalling infection before or at the same time as TSST-1 production begin. We are concerned that over interpretation of the article by Vojtov *et al* could lead to a potentially incorrect differential diagnosis of TSS because the authors suggest that *S. aureus* RN4282 downregulates exotoxin production, in contradiction to findings presented here. The *in vitro* data presented suggests that clinicians should not discount the possibility of toxic shock in a child with evidence of lytic exotoxin production/cytotoxic infection including direct tissue damage, inflammation and purulence (Dr. Amber Young MB, ChB personal communication).

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Oxacillin Alters the Toxin Expression Profile of Community-Associated Methicillin-Resistant *Staphylococcus aureus*

6.1 Abstract

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is an emerging global concern. CA-MRSA strains are known to be highly virulent due to increased expression of core-genome-encoded and phage-encoded toxin genes. It has been shown previously than hospital-acquired (HA)-MRSA strains have high-level expression of the alternative penicillin-binding protein, PBP2a, encoded by the *mecA* gene on type II staphylococcal cassette chromosome *mec* (SCC*mec*) elements. This high PBP2a expression caused a reduction in toxicity by interfering with the Agr quorum sensing system. CA-MRSA strains express significantly lower levels of PBP2a than other MRSA types, which is believed, in part, to explain their relatively high toxicity. We hypothesized that since oxacillin increases *mecA* expression, use of this antibiotic could attenuate virulence gene expression by interfering with global regulatory systems. This chapter illustrates the use of a subinhibitory concentration of oxacillin which induced PBP2a expression while repressing RNAIII expression. Consequently, this repression caused a decrease in phenol-soluble modulin (PSM) secretion, but increased α-haemolysin and Panton-Valentine Leukocidin (PVL) expression consistent with other studies. As oxacillin treatment had both positive and negative effects on toxin expression, the net effect of these changes on three different cell types were examined. Where PSM and α-haemolysin are important, oxacillin reduced overall lysis, but where PVL is important oxacillin increased lysis. We also examined the effect of oxacillin on bacterial binding to extracellular matrix proteins specifically fibrinogen and fibronectin, invasive capacity in an endothelial cell invasion assay and on the surface expression of the multifunctional protein A protein.

Where we found no difference in invasion capacity, a positive association between subinhibitory oxacillin treatment and binding to fibrinogen in strain LAC and decreased protein A expression in both CA-MRSA strains was recorded, demonstrating the pleiotropic effect of oxacillin on virulence gene expression in CA-MRSA.

6.2 Introduction

Since the mid-1940s, consecutive waves of antibiotic-resistant *Staphylococcus aureus* strains have posed difficult challenges to healthcare professionals (289). Infections caused by methicillin-resistant *S. aureus* (MRSA) have reached epidemic proportions globally and incidence rates are increasing, both in the health care and community environments (282, 573, 574). Historically, MRSA infections were associated with predisposed patients in a nosocomial setting, however, the recent emergence of MRSA infecting patients lacking any contact with hospitalisation or any healthcare individual/facility, lead to the introduction of the term, community-associated MRSA (CA-MRSA) (575, 576). There are specific factors which discriminate between these two MRSA groups which involve the acquisition of certain mobile genetic elements and virulence determinants (292, 296, 301) (see section 1.3.3.1 for more details). In the United States, the CA-MRSA USA300 clone (ST-8, PVL$^+$, type IV SCC*mec*) has caused the most severe outbreak, in terms of frequency and infection severity (577, 578). CA-MRSA strains are considered hyper-virulent, possessing extra virulence genes encoded on staphylococcal pathogenicity islands (SaPIs), such as the phage-encoded Panton-Valentine Leukocidin (PVL) and having increased expression of core-genome encoded toxins genes, notably α-haemolysin and Phenol-soluble Modulin peptide toxins (PSMs) (313).

All MRSA strains possess staphylococcal cassette chromosome *mec* (SCC*mec*) elements (reviewed in section 1.3.1.2), which confer resistance to β-lactam antibiotics (579, 580). These elements, vary in size (20 to 70 kb), genetic

content and metabolic burden (581-583), but share common features, namely the *mecA* gene, which encodes the alternative penicillin binding protein, PBP2a (584). Also contained within the *SCCmec* elements are recombinases (*ccrA* and *ccrB*) which permit correct integration and excision at *orf*X on the staphylococcal chromosome, other mobile genetic elements integrated within, which contribute to resistance to other antibiotics and heavy metals, and repressor (MecI) and signal transduction (MecR) proteins involved in *mecA* regulation and expression (585, 586). Based on their protein homology with BlaI and BlaR1 and experimental data, MecI and MecR are believed to regulate inducible transcription of *mecA* (587). MecR can detect and respond to the presence of β-lactam antibiotics through the penicillin-binding domain, activating the cytoplasmic protease domain by autocatalytic cleavage, leading to the cleavage of the mecI repressor and inducing *mecA* transduction (588). The BlaRI *S. aureus* signal/transduction system directly acts against β-lactams, through the induction of β-lactamase expression (589) as shown in figure 6.1. The BlaR1 protein has three domains; C-terminal sensor domain, a trans-membrane domain and a cytoplasmic domain which is hypothesised to possess a zinc-dependent protease (590). In a similar fashion to the *mecA* complex, in the presence of a β-lactam antibiotic, the surface domain of the BlaR1 protein interacts with the antibiotic, inducing a conformational change which activates a downstream signalling cascade, culminating in the induction of a zinc protease, relieving the action of the repressor and subsequent BlaZ activation. Interestingly, this system also causes the expression of *mecA* independently of a functional *mecR*, highlighting the importance of *S. aureus* response to β-lactams (591). This dual activation is extremely important as CA-MRSA strains possess a type IV SCC*mec* element, containing a class B *mec* gene complex, which has a non functional, truncated MecR gene (209).
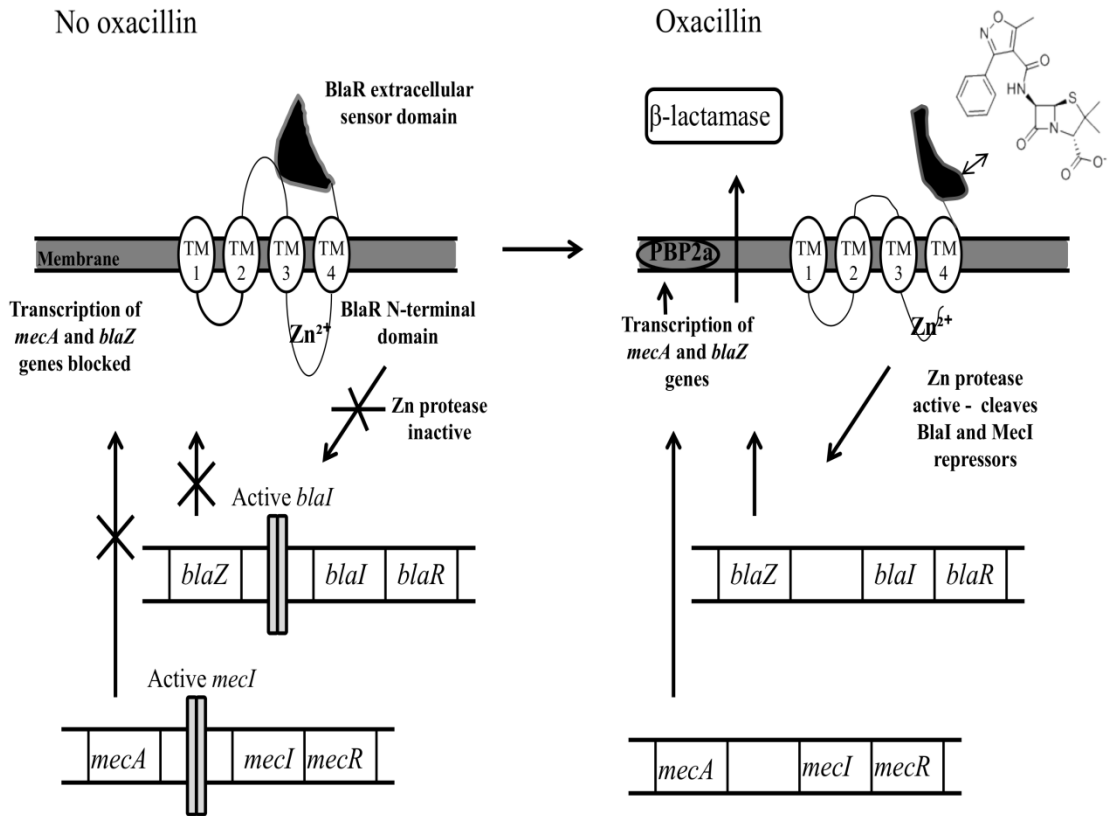
Figure 6.1: Sensing of β-lactam antibiotics in *S. aureus*. In the absence of β-lactam antibiotics (shown here as oxacillin) the repressor proteins, BlaI and MecI, are blocking the transcription of *blaZ* and *mecA* respectively. Oxacillin enters the environment and interacts with the sensor domains in the MecR and BlaR proteins, causing a signal transduction event, leading to the activation of proteases and degradation of the repressors and expression of *blaZ* and *mecA*.

It has been observed by us and others that HA-MRSA strains express higher levels of PBP2a than do CA-MRSA strains in the absence of β-lactam antibiotics (238, 592). This lab has also shown that high levels of PBP2a expression in HA-MRSA strains caused a significant downregulation in toxicity (238). CA-MRSA strains also express PBP2a, but this expression does not affect their toxicity, which was shown to be due to relatively lower basal levels of expression (238). As shown in figure 6.2, PBP2a expression can be induced by the presence of oxacillin (585, 586, 589, 591) and high PBP2a expression can attenuate toxicity by interrupting Agr signalling (238), we hypothesized that subinhibitory concentrations of oxacillin (determined to be 0.5 µg/mL (593)) could stimulate PBP2a expression, thus reducing toxicity and therefore the severity of CA-MRSA infections. However, a number of studies have

investigated the effects of subinhibitory oxacillin on toxin gene transcripts, where an increase, rather than a decrease in activity of α-haemolysin and PVL was observed (594-596). In this chapter, we sought to test our hypothesis by examining the lytic effect of bacterial supernatants after growth in medium containing subinhibitory concentrations of oxacillin (shown not to have any deleterious effect on growth (593)), on three important cell types. We also investigated the expression of an important cell surface protein, protein A, under these conditions, and whether any differences were observed in solid-phase binding assay and endothelial invasion assays. We found that the expression of PSMs can be repressed by inducing higher levels of PBP2a following subinhibitory oxacillin exposure. However, this treatment also induced an overall increase in exoprotein expression levels and specifically an increase in α-haemolysin and PVL expression. In our cytotoxicity assay, we found that where PSM and α-haemolysin are known to be important, oxacillin reduced cell lysis, but where PVL is important, it increased cell lysis. No difference was seen in cell invasion assays, but an increase in binding to fibrinogen during exponential phase binding with LAC was observed. Subinhibitory oxacillin also reduced the levels of protein A expression significantly, illustrating the pleiotropic effect of oxacillin of protein synthesis.
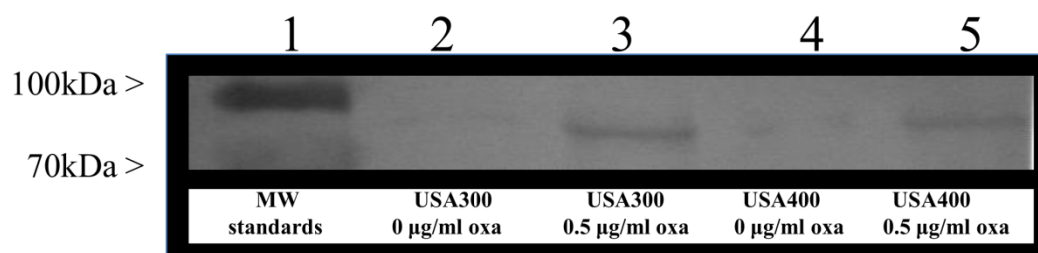
Figure 6.2: Oxacillin-induced expression of PBP2a in CA-MRSA strain LAC (USA300) and MW2 (USA400). Western blot of whole-cell lysates illustrating PBP2a expression in CA-MRSA strains. Lane 1, Molecular weight ladder; Lane 2, LAC after growth in antibiotic-free medium; Lane 3, LAC after growth in medium containing 0.5 µg/mL oxacillin; Lane 4, MW2 after growth in antibiotic-free medium; Lane 5, MW2 after growth in medium containing 0.5 µg/mL oxacillin. Figure taken from reference (593).

6.3 Results and Discussion

Previous results have shown that oxacillin-induced expression of PBP2a caused a decrease in toxicity in T cell survival experiments (593). The mechanism behind this reduced toxicity was shown to be dependent on Agr with quantitative real-time PCR experiments illustrating a decrease in RNAIII expression, the effector molecule of the Agr system, in LAC and MW2 grown in subinhibitory oxacillin (593). This chapter investigated the effects of subinhibitory oxacillin on secreted proteins, namely α-haemolysin and PVL toxin, and how this affects the outcome of cell lysis and on the *S. aureus* surface proteins, which are known to be important in adhering to matrix protein, evading the immune system and invading cells. The strains used in this study can be found in Table 3.2.

6.3.1 T cells are susceptible to specific staphylococcal toxins

As stated previously, a statistically significant decrease in lytic activity was observed after treatment with oxacillin in a subset of type IV CA-MRSA strains (593). The immortalised T2 cell line was used in this study, so we therefore wanted to first analyse which staphylococcal toxins were important in T cell lysis using a variety of mutants and synthetic peptide toxins as shown in figure 6.3 a and b.

The T cells were shown to be sensitive to α-, β-, γ-, and δ-haemolysin as well as highly susceptible to PSMα1, PSMα2 and PSMα3, but not susceptible to PVL, or the leukocidins, LukAB or LukED (Fig 6a). It has been shown recently that PVL requires the expression of human complement receptors C5aR and C5L2 for cell toxicity (308). Additionally, the LukAB and LukED cytotoxins require other specific host leucocytes surface receptors such as the CD11b subunit of the Mac-1 integrin for LukAB (443) and the CCR5 receptor for LukED (441) and it is possible that none of these receptors are expressed on T2 cell surface.

Interestingly, the importance of specific toxins were shown to be strain specific; in the 8325-4 background, α-haemolysin was shown to be significant in T

cell lysis, as the *hla* mutant caused significantly lower lysis in all but the 0.125 dilution factor. However, when *hla* was deleted in the Newman background, there was no significant difference in lysis potential across any of the dilutions used (Fig 6.3a). To understand this discrepancy, western blot analysis on α-haemolysin was performed on both 8325-4 and Newman, and it was observed that strain 8325-4 expresses 4.1 fold higher α-haemolysin than strain Newman under our growth conditions, possibly highlighting why α-haemolysin is important for strain 8325-4 lysis (Fig 6.4c).

Despite this, strain Newman has a higher lytic capacity than strain 8325-4, as shown by decrease in T cell viability resulting from differing supernatant concentrations (Fig 6.3a). To investigate whether other small peptides such as the PSMs are involved, hydrophobic peptide extractions were performed which demonstrated a 1.7 and 1.3 fold higher concentration of δ-haemolysin/PSM peptides from strain Newman than from 8325-4 and LAC respectively (Fig 6.4d), which may in part explain the highly lytic potential of strain Newman, despite the lower levels of α-haemolysin secretion. PSM peptides have been shown previously to be highly lytic to a variety of cells and artificial membranes (312, 316, 546) and have been shown here to efficiently lyse T cells, in a lytic pattern consistent with other cell types with the PSMα1-3 and delta being lytic, while PSMα4 is lytic only at high concentrations, while the PSMβ1 and 2 peptides have no lytic capability.

A)
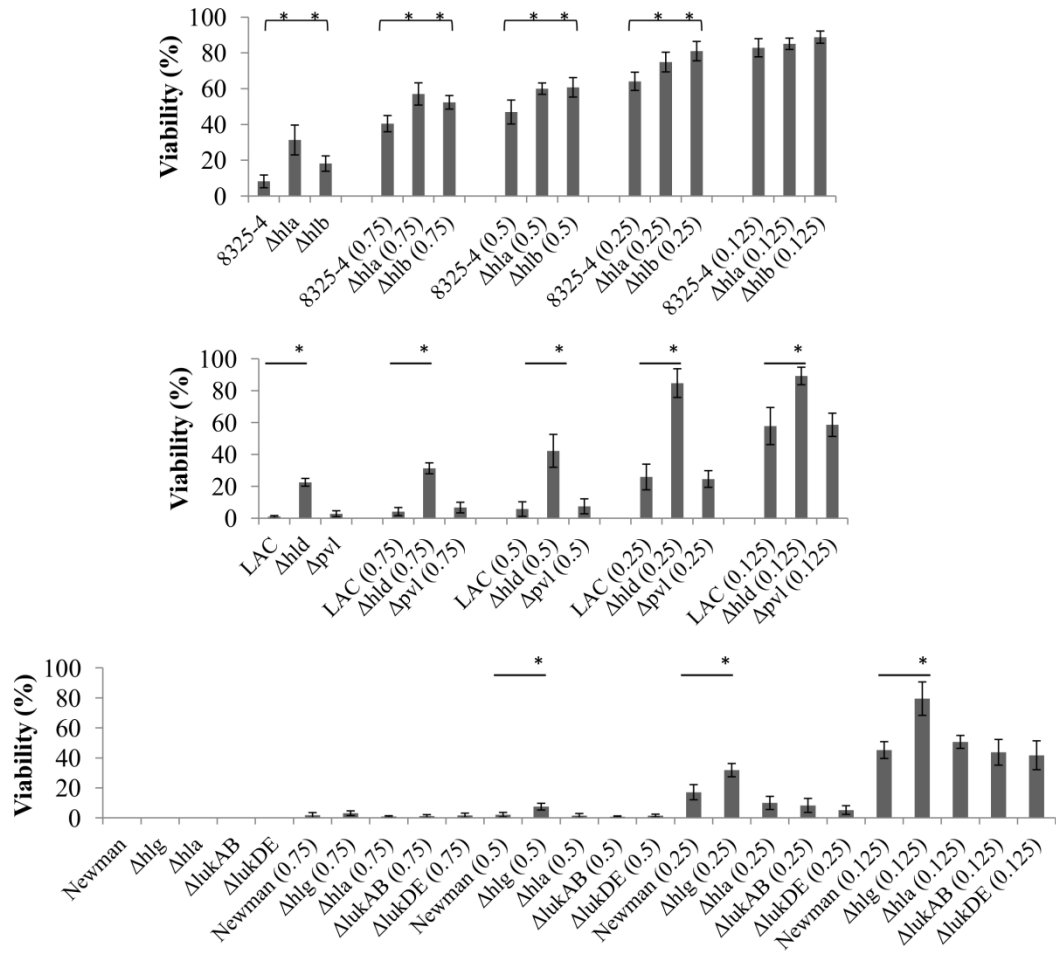
B)
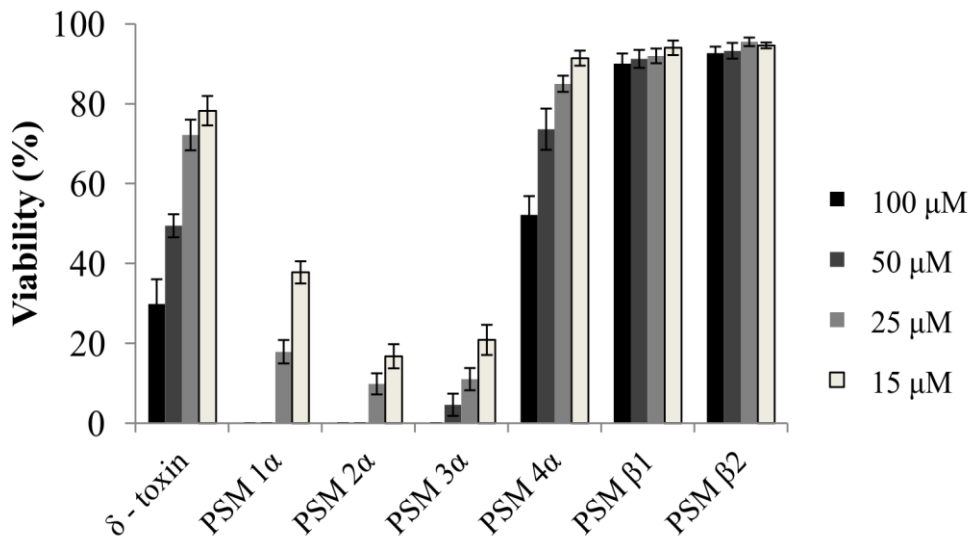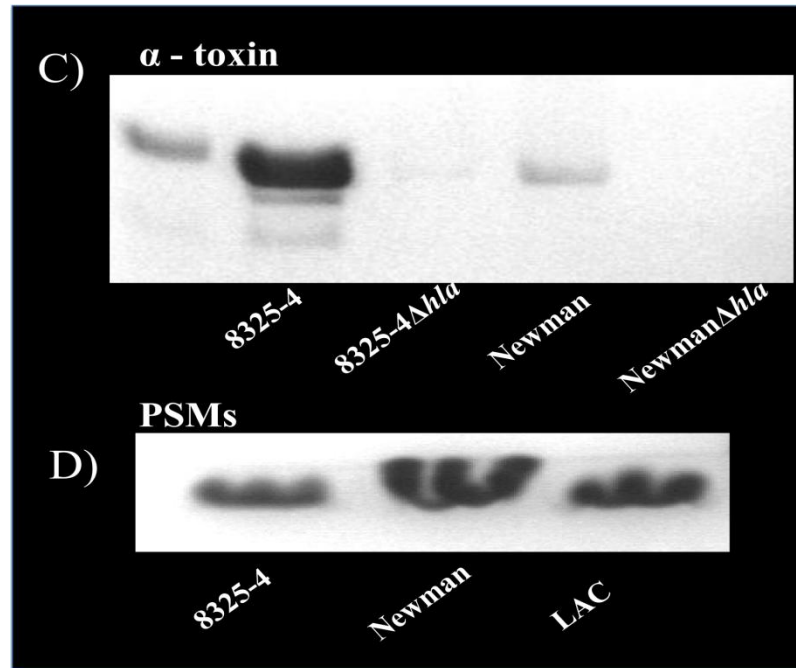
Figure 6.3a-d: Specific toxins cause T cell lysis. A) Using isogenic mutants from three different genetic backgrounds allowed for the evaluation of staphylococcal toxin lytic capacity against the immortalised T2 cell line. We show that deletion of α-, β-, γ-, and δ-haemolysin results in a statistically significant decrease in T cell viability (all p values are lower than 0.05). **B)** Synthetic PSM peptides were used and illustrate the highly lytic capacity of the PSMα1-3 toxins and δ-haemolysin to T cells **C)** Higher expression of α-toxin in 8325-4 than in Newman and **D)** Higher expression of δ-haemolysin and PSMs from Newman than 8325-4 and LAC, may explain the strain-specific roles of some toxins in T cell lysis. T-cell experiments were done in duplicate three times with error bars representing the 95% confidence interval. * denotes a stastically significant difference using students t test $P<0.05$.

6.3.2 Oxacillin alters the toxin expression profile of CA-MRSA

The overall effect of treatment with subinhibitory oxacillin on the exoprotein expression profile of two clinically important CA-MRSA strains, LAC (USA300 clone) and MW2 (USA400 clone), was determined. The supernatants of these strains grown with or without 0.5 μg/mL oxacillin were precipitated using trichloroacetic acid and further analysed. Despite the decrease in RNAIII expression (593) there was an overall increase in the expression levels of exoproteins by LAC and MW2 following growth in subinhibitory oxacillin (Fig 6.4a). We next investigated the effects of oxacillin on specific toxins, known to be important in infection, namely the α-haemolysin and PVL toxin. Previous publications have reported increases in

transcript levels of important cytolytic toxins, specifically, α-haemolysin and PVL in response to subinhibitory oxacillin treatment (594-596). Western blots of these CA-MRSA supernatant extracts demonstrated that although there was an increase in α-haemolysin for both strains, this was not statistically significant (1.8- and 1.4- fold for LAC and MW2 respectively) as illustrated in figure 6.5b and table 6.1. However the level of PVL expression had increased significantly for both LAC and MW2 (3.01- and 4.1- fold respectively) as shown in figure 6.5c and table 6.1. This complemented the results of previous qRT-PCR directed against *lukS*, where a significant increase in *lukS* transcript taken at 10 and 20 h growth correlated with oxacillin treatment (593).

PSMs are extremely important virulence factors, which are intimately associated with CA-MRSA strains (296, 301). Therefore we wanted to investigate the effect of subinhibitory oxacillin on PSM secretion qualitatively using a butanol-extraction process for hydrophobic peptides (546). These peptide toxin range in size from 2.3-4.5 kDa and therefore cannot be separated from each other and migrate as a signal band during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); however a clear reduction in band intensity can be seen upon exposure of the bacterial cultures to subinhibitory oxacillin concentrations (Fig 6.5d). To fully examine and quantify these changes, supernatants were sent for HPLC/ mass spectrometry analysis (performed by Hwang-Soo Joo and Micheal Otto, NIAID, NIH, Bethesda, Maryland, USA), which showed a statistically significant reduction in the secretion of PSMα type peptide and δ-haemolysin in both LAC and MW2 (Fig 6.4e), confirming the down-regulatory effect that subinhibitory oxacillin had on the Agr operon and associated peptide toxins regulated by this system.
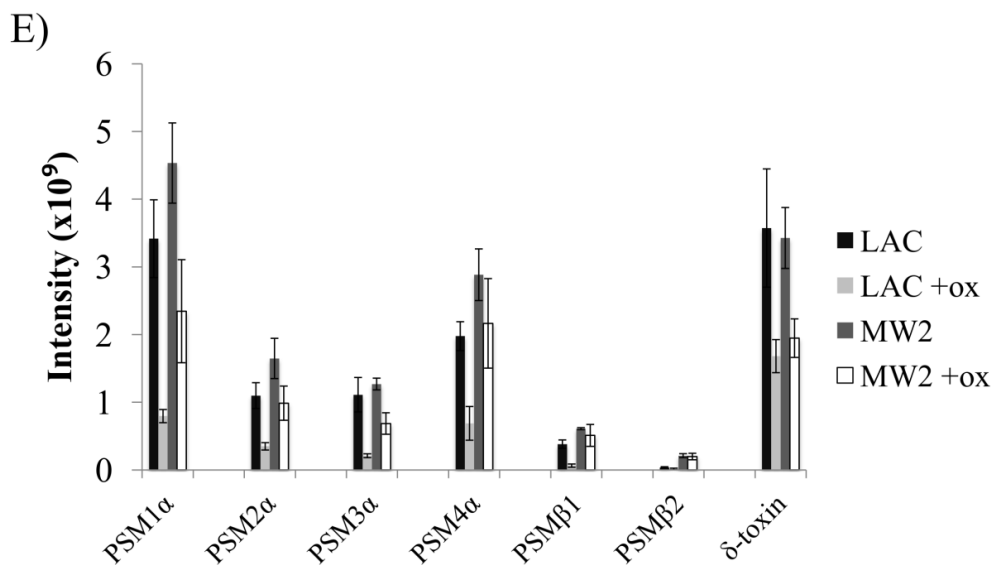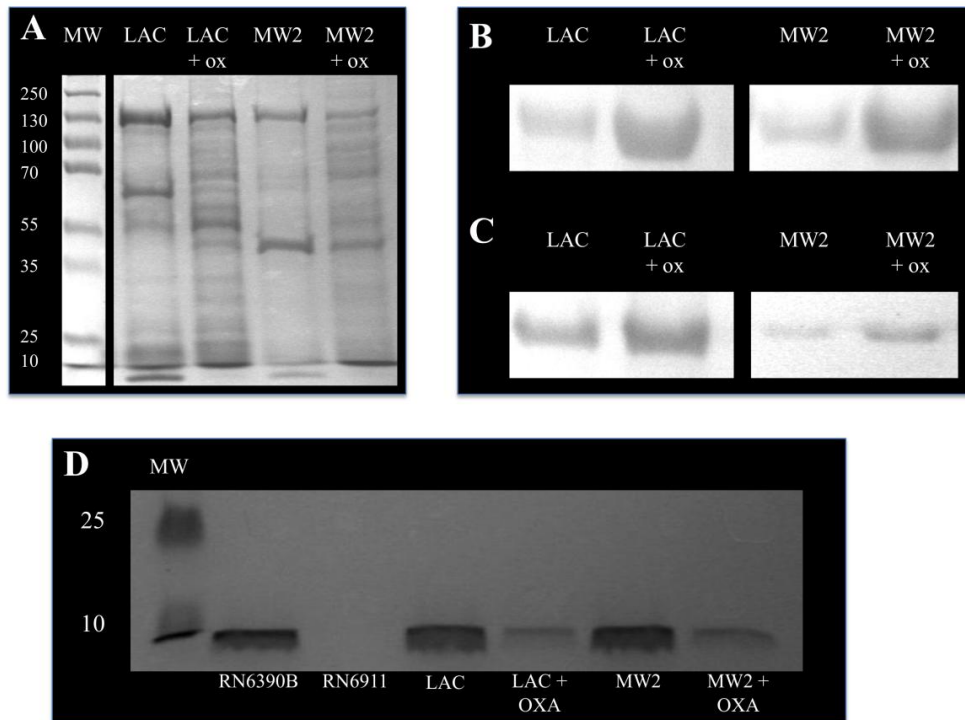
Figure 6.4a-e: Oxacillin alters the toxin expression profile of CA-MRSA. Bacterial cultures were grown in the presence or absence of 0.5 μg/mL oxacillin as indicated. **A)** Exoprotein profile, Western blot using **B)** anti-LukS antibodies or **C)** anti-α-haemolysin antibodies. **D)** SDS-PAGE of butanol extracted PSM. **E)** Results of HPLC/MS signal intensities of each of the PSMs from LAC and MW2 culture filtrates with and without oxacillin (error bars represent the 95% confidence). Western blot analysis was done three times and band intensity analysed by using Image J software.

Isolate, locus	Fold change (+ox vs –ox)	Significance (*P*)
LAC, *hla*	↑ 1.8	0.19
MW2, *hla*	↑ 1.4	0.29
LAC, *lukS*	↑3.01	0.008*
MW2, *lukS*	↑4.1	0.011*
LAC, *spa* (Stationary phase)	↓2.6	0.024*
MW2, *spa* (Stationary phase)	↓2.1	0.036*

Table 6.1 Protein expression level changes in response to subinhibitory oxacillin treatment through western blot and band intensity analysis using Image J software. Arrows indicate the direction of the fold change in response to oxacillin (ox). * Indicates statistically significant values using student *t* test, *P*<0.05

6.3.3 The effects of oxacillin on the cytolytic potential of bacterial supernatants are cell-specific

S. aureus expresses a vast array of membrane-damaging toxins, a formidable arsenal which has been central to its success as a globally important pathogen. *S aureus* has evolved to employ an 'insurance policy' with regards toxins expression, where the activity of a subset of toxins is enough to lysis most human cells. Therefore, in order to understand the overall cytolytic effect, bacterial cultures, treated with and without oxacillin, were harvested and supernatants tested against a set of three cell types: the T cells, included here as a reference, human red blood corpuscles (RBCs), sensitive to α-haemolysin and PSMs and human polymorphonuclear leucocytes (PMNs) which are sensitive PVL and the PSMs.

The effects of oxacillin on the lytic potential of both LAC and MW2 were compared across these cell types (Fig 6.5 a-f). PVL has been known to be overexpressed when grown in casein hydrolysate and yeast-extract containing medium (CCY) (458, 597) and here we show that growth in CYY causes differential exoprotein expression and higher PVL expression (Fig 6.5g-h). Therefore the effect of oxacillin treatment when LAC and MW2 were cultured in this medium was also compared in these assays. Subinhibitory oxacillin increased T cell survival in both LAC and MW2 and when grown in both culture media (Fig 6.5a-b). Despite an

increase in α-haemolysin expression, oxacillin decreased the lysis of RBC significantly in both TSB (LAC, *P*=0.0172 and MW2, *P*=0.0169 and CYY (LAC, *P*=0.0361 and MW2, *P*=0.0336). Therefore, with regards RBC lysis, the decrease in PSM secretion had more of a dominant effect than the increase in α-haemolysin. Contrarily, although human PMNs are sensitive to both PSMs and PVL, when LAC and MW2 were grown in CYY, the increase in the expression of PVL had more of a dominant effect, with PMN survival decreasing upon oxacillin exposure and statistically significant for LAC (*P*=0.0197) and MW2 (*P*=0.042) (Fig 6.5f). However, this was not the case when either strain was grown in TSB (Fig 6.5e), where an increase in survival was observed for both LAC (*P*=0.022) and MW2 (0.01). These results illustrate that where PVL expression is important, oxacillin causes a decrease in PMN viability, but as shown here, specific conditions are required for maximum PVL expression, and how this relates to *in vivo* conditions remains to be determined.
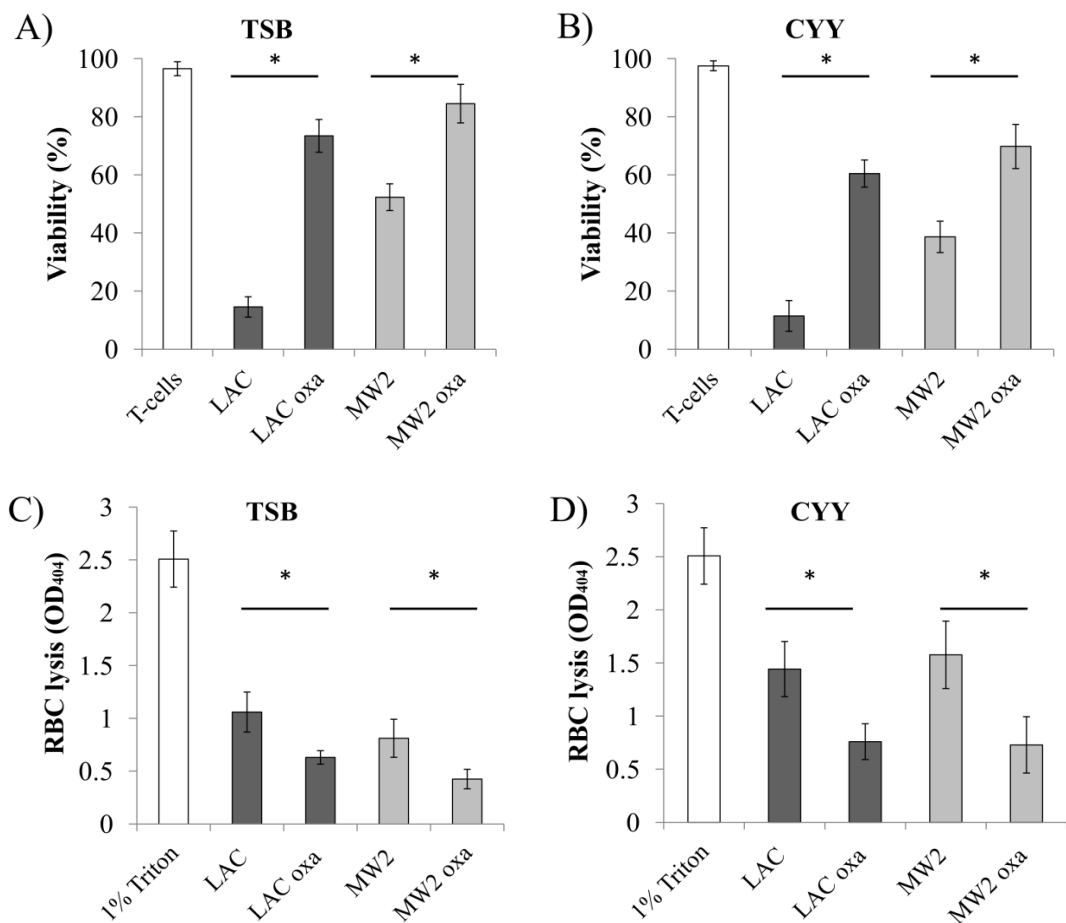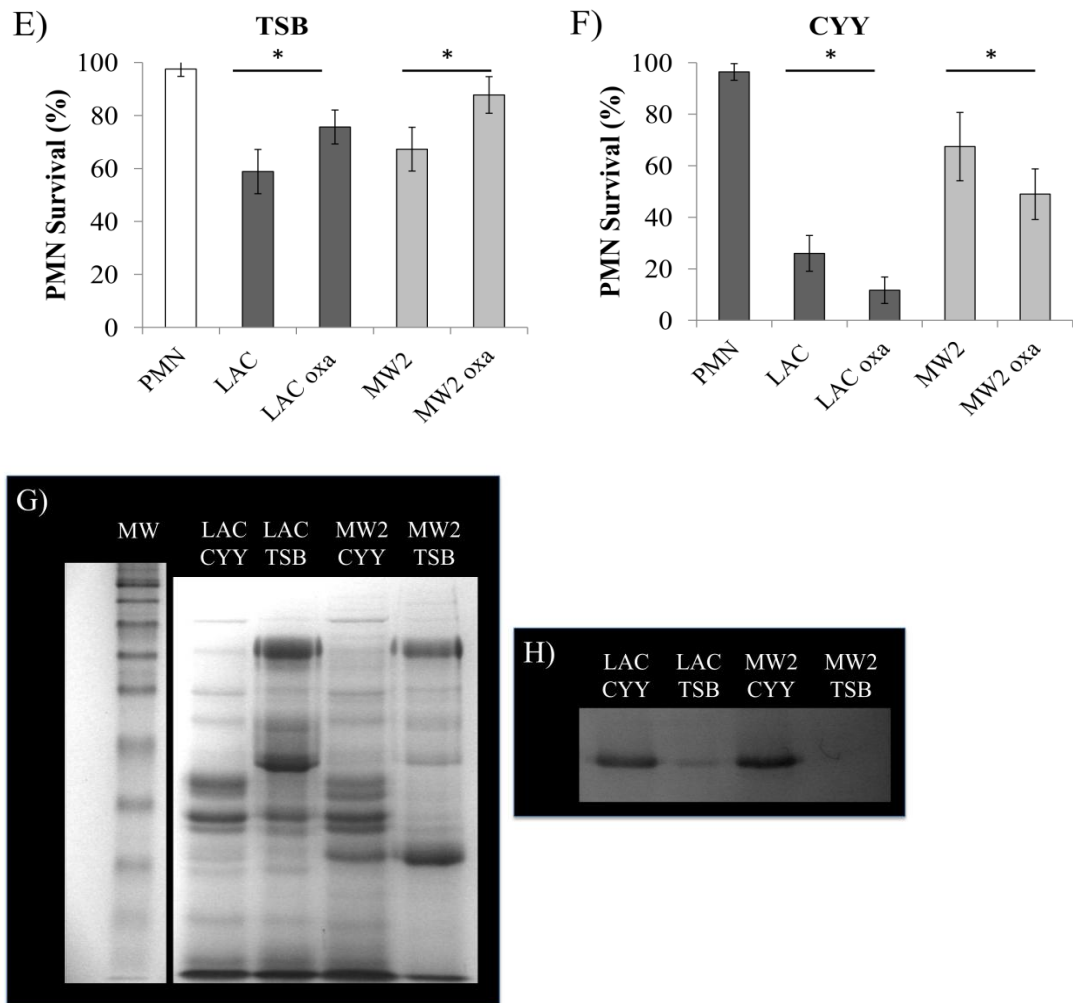
Figure 6.5a-h. Oxacillin differentially alters the sensitivity of cells to lysis. Oxacillin decreased the ability of CA-MRSA to lyse immortalized T cells when strains were grown in **A)** TSB or **B)** CYY. Oxacillin decreased the ability of CA-MRSA to lyse fresh human RBCs when strains were grown in **C)** TSB or **D)** CYY. Oxacillin decreased the ability of CA-MRSA to lyse fresh human PMNs when grown in **E)** TSB, but increased the ability of CA-MRSA to lyse fresh human PMNs when grown in **F)** CYY. **G)** Comparison of exoprotein profile of LAC and MW2 grown in TSB or CYY. **H)** Higher PVL expression of LAC and MW2 grown in CYY rather than MW2. T-cell experiments were done in duplicate three times with error bars representing the 95% confidence interval. * denotes a statically significant difference using students *t* test *P*<0.05.

6.3.4 Oxacillin modulates surface protein expression

A major part of *S. aureus* pathogenesis is the expression of surface proteins, collectively designated as microbial surface component recognising adhesive matrix molecules (MSCRAMMs). These proteins are intimately involved in initial host colonisation, attachment to extracellular matrix proteins and cell uptake/invasion.

Although the Agr system plays a major role in downregulating the expression of these factors in the transition from late exponential to early stationary phase of growth, further complex regulatory networks involving other two-component systems, DNA-binding proteins and non-coding regulatory RNAs are also important in regulation, many of which may be induced or repressed upon external stimuli, such as subinhibitory antibiotics.

Here the effect of oxacillin treatment on the expression of a major surface protein protein A (SpA) was investigated. SpA is present in over 90% of *S. aureus strains*, (598) and is an important multifactorial virulence factor. SpA binds to a variety of ligands, including the Fc domain of IgG (599), von Willebrand factor (426) and tumour necrosis factor receptor-1 (TNFR-1) (600). This protein also allows the adherence to specific cells such as osteoblasts (601) and is an important component in disrupting opsonisation (423) (see section 1.3.5.1.3 for more details).

Examining the overall surface protein profile of these two important CA-MRSA strains reveals that LAC is affected quite substantially with many proteins being overexpressed when grown in subinhibitory oxacillin (Fig 6.6a). However, protein A expression was reduced significantly, as shown by the western blot in Figure 6.6b and Table 6.1. A 2.6- and 2.1- fold reduction was observed in LAC and MW2 respectively. Protein A expression is growth phase dependent and controlled by complex regulatory networks acting at the transcriptional, translational and post-translational level (355, 370, 372, 389, 598, 602, 603). Previous studies have shown the reduction of *spa* mRNA after subinhibitory antibiotic treatment with clindamycin and linezolid (604, 605). Both antibiotics inhibit protein synthesis, albeit through different mechanisms, which may result in the differential inhibition of one or more regulatory proteins.
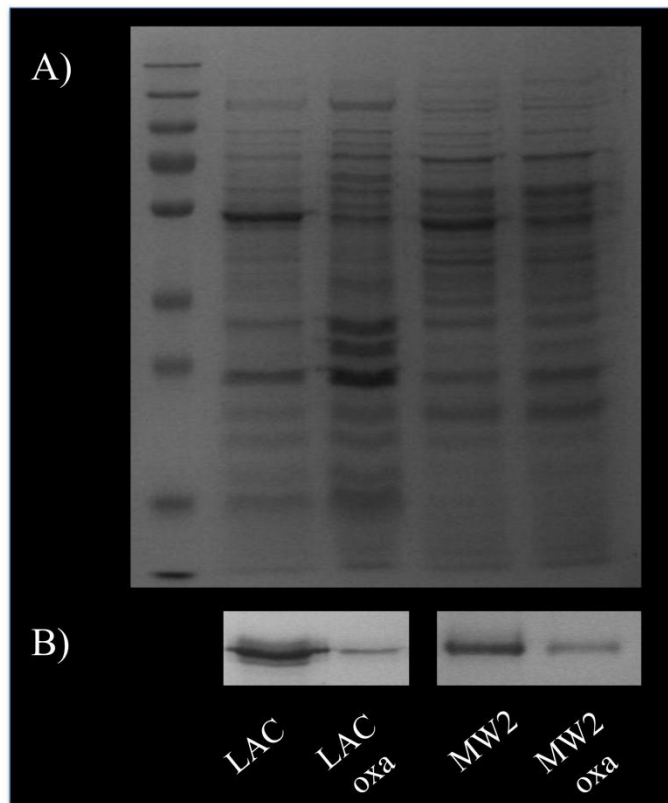
Figure 6.6a-b: Subinhibitory oxacillin reduces protein A expression in CA-MRSA strains. A) Oxacillin modulates surface protein expression in LAC and MW2 strain shown by SDS-PAGE of surface proteins isolated from raffinose mediated spheroplasts. **B)** Western blot of protein A showing decreased expression in both LAC and MW2 following growth in subinhibitory oxacillin.

As subinhibitory oxacillin causes a reduction in RNAIII expression it was hypothesised that these strains would become hyper-adhesive after oxacillin exposure as RNA III expression is in part responsible for the down regulation of surface adhesins (329). To gain a greater understanding of this, a solid phase binding assay was performed using two important matrix proteins, fibrinogen (Fb) and fibronectin (Fn). The ability of *S. aureus* to bind to both of these molecules have been studied in depth, requiring primarily, clumping factor A (ClfA) for Fg (407, 408) and the fibronectin binding proteins (FnBPs) FnBPA and FnBPB which can interact with both Fb and Fn (405, 409). The results of the binding assay complemented the pattern of overexpression of surface proteins in LAC, as a statistically significant increase in binding to fibrinogen was observed with LAC (*P*=0.016) but not with MW2 during exponential phase (Fig 6.7 a).
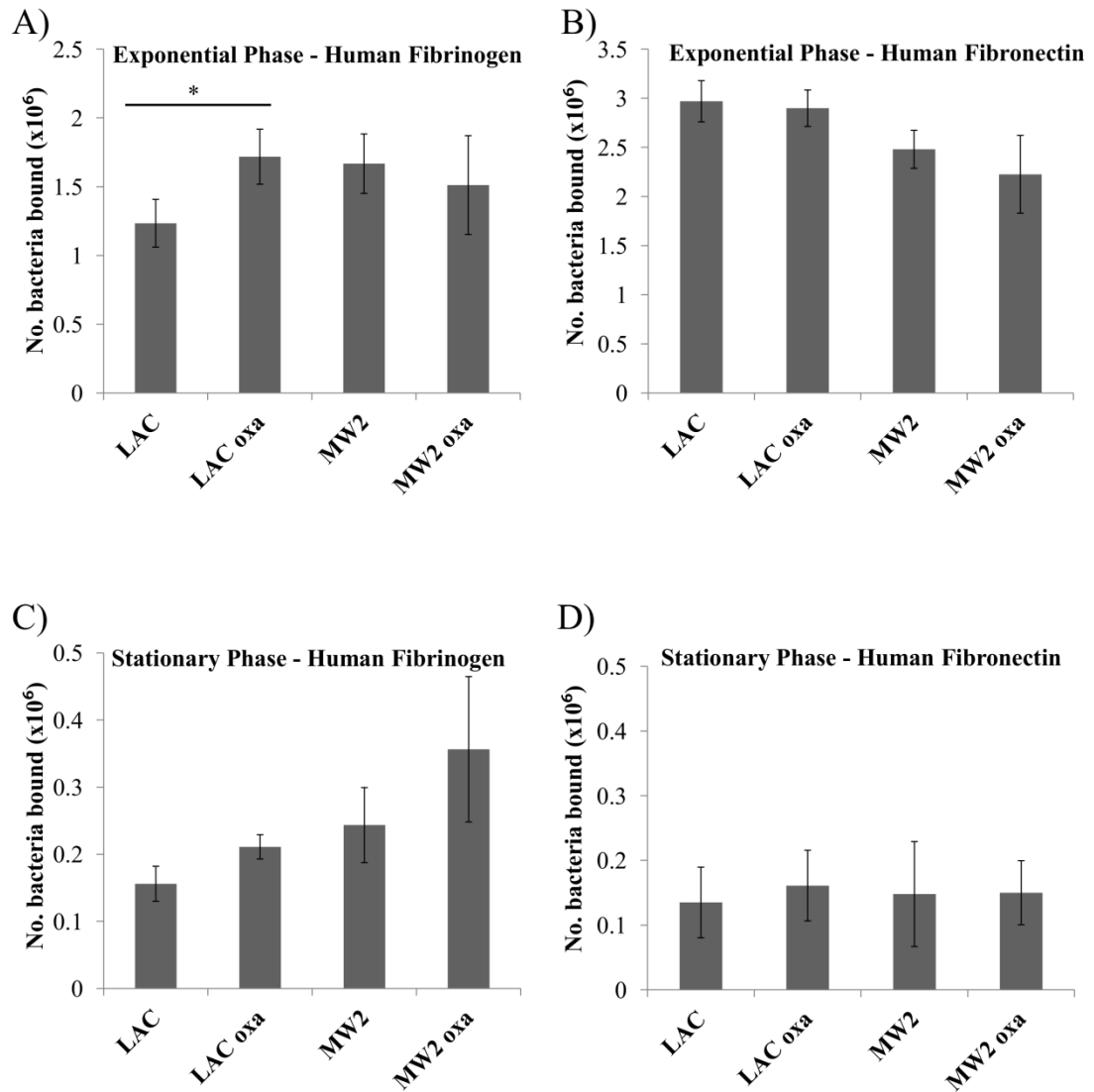
Figure 6.7a-d: Effect of oxacillin on solid-phase binding of LAC and MW2 to fibrinogen (Fg) and fibronectin (Fn). A) Exponential phase binding to Fg and **B)** Fn showing a statistically significant increase in binding capacity of LAC grown with oxacillin. No difference in binding was observed for MW2 grown with or without oxacillin. Stationary phase binding to **C)** Fb and **D)** Fn illustrating no statistically significant difference in binding potential for LAC or MW2 with and without oxacillin. Solid-phase binding assays were done in duplicate three times with error bars representing the 95% confidence interval. * denotes a stastically significant difference using students *t* test *P*<0.05.

Interestingly, no difference was observed in the binding affinity to fibronectin by either of these strains, which suggests that the FnBPs are not overexpressed, but further experimentation is required to confirm this. These results are in contradiction to a previous study which observed a hyper-adhesive phenotype with subinhibitory

oxacillin (606), however these strains were not CA-MRSA and cultures were grown in different media and using a different sub-MIC. During stationary phase of growth, no difference was observed in the binding potential of LAC or MW2 grown with and without oxacillin (Fig 8 c-d), suggesting an induction of surface proteins seen in LAC is relieved during stationary phase of growth.

S. aureus is well equipped to invade cells, relying heavily on the expression of FnBPs. Through their interaction with Fn they initiate integrin-mediated intracellular uptake/invasion by non-professional phagocytes such as keratinocytes, osteoblasts and endothelial cells (607-609). Due to the importance of invasion in *S. aureus* pathogenicity, the invasive capacity of LAC and MW2 with and without oxacillin over three time points (15, 30 and 60 min) was investigated (Fig 6.8a-c) using an endothelial cell invasions assay. Consistent with a previous publication which observed no difference in invasion of osetoblasts (606), no difference was observed over the three time points between untreated control and oxacillin treated culture. As a side observation, after 60 min incubation with endothelial cells, LAC grown with and without oxacillin displayed a statistically higher invasive capacity than MW2 under the same conditions (P=0.016), highlighting differences in invasiveness between these two CA-MRSA strains.
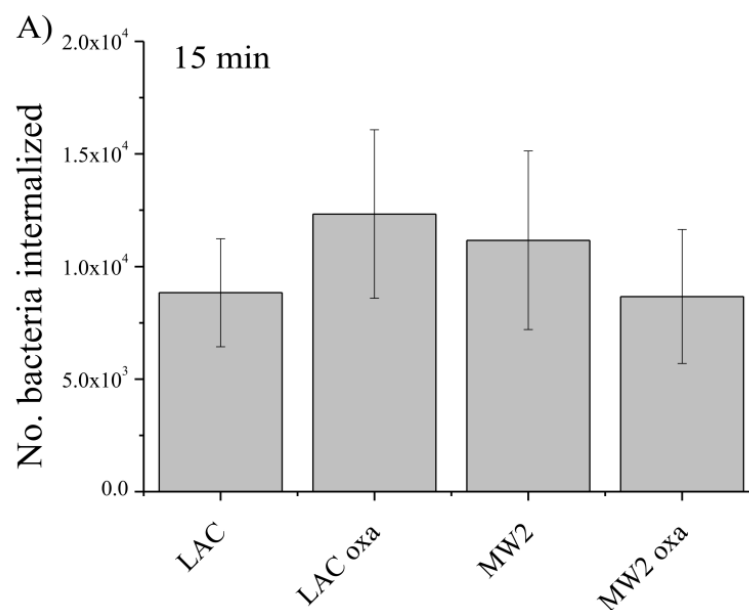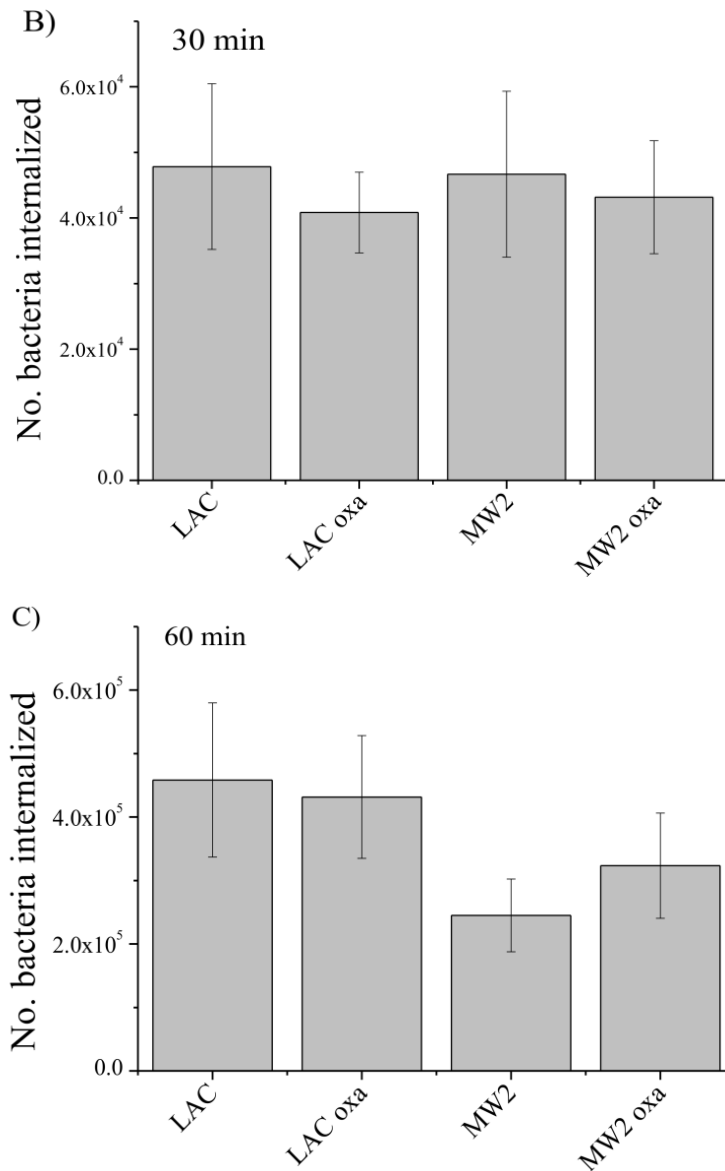
Figure 6.8a-c: Effect of oxacillin on invasion of endothelial cells. LAC and MW2 were grown with and without oxacillin and their invasive capacity assessed using an endothelial cell line. No difference in invasion was observed over the three time points **A)** 15min, **B)** 30min and **C)** 60 min. Cell invasion experiments were done in duplicate three times with error bars representing the 95% confidence interval. * denotes a stastically significant difference using students *t* test *P*<0.05.

6.4 Conclusions

Previous work in this group demonstrated that increased PBP2a expression reduced disease severity *in vivo* by downregulating cytolytic toxin expression (238). We hypothesized that as this β-lactam antibiotic induced the expression of PBP2a, shown by RT-PCR (593) and western blotting (Fig 6.2), it could be used as an anti-virulence agent in conjunction with other antibiotics to attenuated virulence and therefore decrease disease severity in infected patients. The main findings show that although subinhibitory oxacillin interrupts and decreases Agr activity and PSM secretion in strains that represent two prominent CA-MRSA clones, it also up-regulates other cytolytic toxins.

One of the most widely studied and best characterised virulence factors of *S. aureus* is the heptameric β-barrel pore-forming α-haemolysin (314). At high concentrations it causes pore-formation depending on critical membrane domains and receptors (428), whereas at lower, sub-lytic concentrations, it has been shown to alter the cell signalling pathways that govern cell proliferation, inflammatory responses, cytokine secretion and cell-cell interactions (430). In this chapter, we show that in concordance with other studies, subinhibitory oxacillin increased the level of α-haemolysin expression; however this was not statistically significant when densitometry of the bands were analysed. Human red blood cells are the classical cell type used to study α-haemolysin, but previous studies have shown that the small, amphipathic alpha-helical PSM peptide toxins also lysis RBC (316). LAC/MW2 supernatant-RBC lysis assays revealed that the overall effect on lysis of this type of cell decreased upon exposure to oxacillin, as the decrease in PSM secretion had a greater overall effect than the small increase in α-haemolysin. Like α-haemolysin, the PSMs have been shown to contribute to many infection types including skin and soft tissue infections (SSTI), bacteraemia, and osteomyelitis (610). PSMs are unique in that they do not require a proteinaceous receptor, but are only limited in their lytic ability based on the lipid membrane composition, permitting lysis on a broader range of cell types. The downregulating effect of oxacillin on PSMα1 and -β1 and δ-haemolyin secretion by USA300 strain LAC was shown previously (611) and here

we show that it also affects the expression of PSMα2, -α3, and -α4 and PSMβ2 for LAC and MW2.

PVL is a member of the bi-component class of *S. aureus* leukocidins, consisting of two proteins synthesised and secreted separately which are inactive individually but act synergistically to efficiently damage white blood cells (612). This toxin has been the subject of debate concerning its role in CA-MRSA infections, but it is believed to contribute to necrotic and recurrent SSTIs, necrotizing pneumonia and bone and joint infections (296, 301). PMNs are one of the primary lines of defence against bacterial infections. The fact *S. aureus* employs both PVL and PSMs to lyse these cells is unsurprising given the importance for bacteria to survive this arm of host cell-mediated immunity. Oxacillin had opposite effects on the expression of these toxins, and as such, it was important to test which change had the more dominant effect on PMN killing. When bacterial cultures were grown in TSB, this lead to weak or no expression of PVL and therefore oxacillin caused a reduction of PMN death in both LAC and MW2. However, growth in CYY induces PVL expression (Fig 6.5h), and in this condition oxacillin stimulates higher expression of PVL and thus there was an increased killing of PMNs. However, the translation of these findings to infection models that are sensitive to α-haemolysin, PSMs, and PVL is critical to test the gross effect that oxacillin has on toxicity and virulence.

Full pathogenesis of *S. aureus* depends on the expression of a wide range of cell wall associated proteins which promote colonisation of host tissue and evasion of the immune system (98). Of these surface proteins, the FnBPs, ClfA and SpA are arguably the most important. FnBPs contain an A domain required for Fb binding and multiple non-identical repeat regions with differing affinities for Fn, which is required for host invasion and sepsis in a murine model (410). The ClfA protein binds to Fb, and mediates bacterial attachment to platelets and plasma clots, which is believed to be an important mechanism for initiating endocardial infection (613, 614). SpA, like FnBPs and ClfA, is a cell wall LPXTG anchored protein, and has multiple domains, important in binding IgG and adhering to susceptible cells. We

investigated the effects of oxacillin of the surface protein expression profile, and specifically on SpA. In both LAC and MW2, oxacillin caused a statistically significant reduction of SpA (Fig 6.6b). This was the first time that a reduction in SpA was observed after subinhibitory oxacillin treatment and as SpA is anti-phagocytic, it would be interesting to assess the effects of subinhibitory oxacillin in a phagocytosis assay, which may reveal an important anti-virulence effect. The ability of LAC and MW2 to bind Fb and Fn was assessed and observed that, during exponential phase, LAC bound to Fb a statistically higher capacity when grown in oxacillin then without (Fig 6.7 a). No difference was recorded for MW2 under the same conditions. In contrast to previous publications, no difference in binding to Fn was observed (606). To further investigate the effects of oxacillin on surface expression, an endothelial cell invasion assay was performed, however growth in subinhibitory oxacillin confirmed no benefit or loss in the invasion capacity of LAC or MW2 (Fig 6.8a-c).

S. aureus virulence gene expression is regulated by specific elements clustered into complex networks driving specific interactions. Important within this regulatory network are the two-component regulatory systems, composed of a sensor domain and response regulator, which are sensitive to environmental signals, such as the *agr* (335), autolysis-related locus *arlRS* (353), *S. aureus* exoprotein expression *saeRS* (343) and staphylococcal respiratory response *srrAB* (348) systems (see section 1.3.4 for more details). DNA-binding proteins, such as SarA (615) and its homologues SarR (369), Rot (616), SarS (372), SarT (617) and SarU (375) also regulate virulence factor expression. Subinhibitory oxacillin reduces toxicity directly by down regulating RNAIII and δ-haemolysin expression by an as yet unidentified mechanism, but it is proposed to affect the cell wall peptidoglycan and interfere with auto-inducing peptide signalling (238). The PSMs and δ-haemolysin are regulated directly by the Agr system in an RNA-dependent and independent fashion (215). Although Agr is important for regulating toxicity and adhesins, many of the other factors may be involved.

Previous studies analysing the effects of subinhibitory antibiotics observed an increase in alpha and PVL toxins (594-596). Others have shown that the induction of the *saeRS* system after subinhibitory β-lactam exposure caused an increase in α-haemolysin at the transcript level using *hla::blaZ* promoter fusions (618). *saeRS* differentially regulates many virulence genes but also modulates an additional 22 genes including *sarA* and *sarT*, resulting in reduced transcript levels. Accordingly using this model, *saeRS* induction after subinhibitory oxacillin exposure could account for the increase in α-haemolysin and PVL toxin expression, even in the context of RNAIII reduction. The effect of reducing *sarT* transcript levels may also increase α-haemolysin expression, as *sarT* is a repressor of this haemolysin (617). The potential decrease in SarA/T may also explain the reduced SpA levels, as SarT induces the expression of another SarA homologue, SarS, which directly binds to the *spa* promoter stimulating SpA expression (372). Work by Dumitrescu *et al*, observed an induction of SarA and a reduction in Rot mRNA levels which conferred an increase in PVL expression when grown with imipenem and oxacillin (596). In terms of regulatory elements effecting PVL expression, they observed no relevant contribution of the *saeRS* system as opposed to Kuorda *et al* (618). However different strains, β-lactams antibiotics and MIC were used which may trigger different signalling pathways affecting different regulatory elements. To understand the exact mechanisms causing this oxacillin-mediated pleiotropic effect, specific regulatory elements must be assessed in this context under these conditions. These experiments using subinhibitory antibiotics highlighted how bacteria differentially regulated important virulence genes in response to their environment. These changes may help us understand how the complex regulatory network which governs *S. aureus* virulence interacts upon external stimuli.

Exposure of bacteria to sub-lethal levels of β-lactam antibiotics can also lead to the activation of several stress responses, most notably the SOS-response (619). The SOS system is a global response to DNA damage and is classically governed by two major genes: *lexA* and *recA* (620). The LexA protein acts as a repressor binding to specific operator sites of SOS-regulating genes, inhibiting their expression. In response to DNA damage, the RecA protein becomes activated and acts a protease,

enabling full cleavage of the LexA protein and this inducing the expression of some SOS-regulated genes. The affinity of LexA to each SOS-regualted gene promoter is different leading to a temporal expression of genes, where some genes are expressed, partially expressed or only expressed under conditions of high or persistent DNA damage (621). Once DNA damage has been repaired, RecA and LexA restore repression to the system. Previous work has highlighted that induction of the SOS-response by sub-inhibitory antibiotics can result in the expression of virulence genes for example the adhesins, FnBPB (622), or can trigger the induction of staphylococcal prophage carrying virulence determinants such as TSST-1 (623). Recently it has been shown that sub-inhibitory levels of oxacillin (and other antibiotics which target PBP-1) triggers the SOS-response in a *lexA*/*recA* dependent fashion which lead to an increased mutation rate and selection of homogenous high level resistance (624). Therefore it would be interesting to investigate the effects of sub-inhibitory oxacilin in a recA mutant to determine whether this SOS-response plays a role in modulating toxicity in *S. aureus*.

Future work to evaluate the potential of other cell-wall-active antibiotics that can mediate PBP2a-dependent *agr* repression will be of interest in the context of virulence attenuation, but ultimately, the potential of beta-lactams and related antimicrobials as anti-virulence drugs may depend on the site of infection, the *S. aureus* strain, and the relative contribution of individual toxins to specific infections.

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Identifying virulence associated SNPs in the Genome of Methicillin Resistant *Staphylococcus aureus*

7.1 Abstract

Staphylococcus aureus employs hundreds of genes which are dedicated to the regulation and expression of virulence factors. Virulence is a complex and often multifactorial process. This chapter attempts to investigate genetic polymorphisms which are associated with a specific virulent phenotype. Toxicity, the ability to destroy host cell membranes, and adhesion, the ability to adhere to human tissues, are the major virulence factors of many bacterial pathogens, including *S. aureus*. Here, we assayed the toxicity and adhesiveness of 90 closely related MRSA (methicillin resistant *S. aureus*) ST239 isolates derived from four hospitals in Turkey. We found that while there was remarkably little variation in adhesion, toxicity varied by over an order of magnitude between these isolates, suggesting that different evolutionary selection pressures acts on these two traits and that significant variation exists within specific *S. aureus* lineages. To understand the genetic factors responsible for this observed variation a genome wide association study was performed on the genomes of these 90 clinical isolates. 124 single nucleotide polymorphism (SNPs) and insertion/deletions (InDels) were identified to be associated with toxicity. By using a transposon mutagenesis library we highlight four novel genetic loci associated with a decrease in toxicity, underlying the applicability of this approach in determining genetic polymorphism associated with toxicity.

7.2 Introduction

*S*taphylococcus aureus is a major human pathogen, the treatment of which has been complicated by the worldwide emergence of multiple lineages that have acquired resistance to methicillin (275, 276, 296). Its virulence is conferred by the activity of many effector molecules (reviewed in section 1.3.5) which can be broadly grouped into being either toxins – factors that cause specific tissue damage in the host, or adhesins – factors that facilitate adherence to and invasion of host tissues. A complex network of regulatory proteins controls the expression of many individual toxins (reviewed in section 1.3.4) such that various sites on the *S. aureus* chromosome contribute to the overall toxicity of an individual isolate. The ability of *S. aureus* cells to bind human glycoproteins, such as fibrinogen and fibronectin, is another critical determinant in disease outcome. It facilitates attachment to and damage of host tissues, host cell invasion, and systemic dissemination (404). Several genes encode fibronectin- and fibrinogen-binding proteins (e.g., *fnbA*, *fnbB*, *clfA*, *clfB*, *eap*, i*sdA*, *emp*, *ebh*, see table 1.1.), whose expression is again controlled by a complex regulatory network (96). Similar to toxicity, many sites on the chromosome can therefore contribute to the overall adhesiveness of *S. aureus*, with many regulators common to both adhesion and toxicity (96).

A key factor affecting the severity and outcome of any infection is the virulence potential of the infecting organism. Since the first whole genome sequence of a free-living organism, *Haemophilus influenzae*, was published (625) sequencing technology has advanced to a stage where a bacterial genome can be sequenced in a matter of hours (479, 626). This has led to an explosion of genomic data that has revolutionised molecular epidemiology allowing the close monitoring of outbreaks in hospitals (224, 225, 627), tracking strains transitioning from carrier to invasive status (627), and performing detailed epidemiological studies to understand aspects of pathogen biology (180, 241, 628, 629). This huge advancement in sequencing technology and what can be done with the ensuing enormity of genomic data provides the motivation behind this chapter.

If the virulence phenotype could be determined directly from its genome sequence, next generation sequencing technology would provide for the first time an opportunity to make predictions of virulence at an early stage of infection. While there has been some success in predicting the antimicrobial resistance from the genome (241, 630) complex phenotypes such as virulence, involving the contribution of several genes, as discussed above, has not yet been possible. The success of epidemic MRSA clones such as USA300 and ST239 may be attributed to the variation in the expression of their toxin or adhesion genes, presumably through modification of the main virulence regulatory genes. The ability to identify virulence associate genetic polymorphisms may help firstly in unravelling the complex genetic regulatory network, highlighting which regulators and specifically which residues are important in cross regulation, but could also be implemented in a predictive model, which may infer virulence directly from the genome sequence.

Here we addressed this by first adopting a direct approach analysing the sequence variation in virulence regulators from a diverse collection of sequence types (STs) in an attempt to explain why strains from different STs are more virulent than others. We investigated the impact of SNPs on the SarS virulence regulator, known to be important in the regulation of protein A. The second approach used a more general approach, measuring the lytic and adhesive potential of a collection of 90 closely related ST239 MRSA clinical isolates derived from four hospitals in Turkey. ST239 contains a type III SCC*mec* element and is a highly transmissible, highly antibiotic resistant MRSA clone, which is associated with a strong biofilm phenotype and enhanced ability to adhere and invade airway epithelial cells (631). Epidemiological studies suggest that at least 90% of the cases of HA-MRSA within a geographical region encompassing >60% of the world's population can be attributed to this single clonal subgroup (234, 632, 633). ST239 has evolved through a large scale recombination event, involving the construction of a mosaic chromosome composed of a majority CC8 genetic background while approximately 20% of the genome is derived from a CC30 lineage (634). To investigate genetic polymorphisms associated with virulence we adopted a genome-wide association study (GWAS)

which highlighted novel loci which were associated with either an increase or decrease in toxicity. Using this information we were able to functionally verify four novel loci which when inactivated resulted in a decrease in toxicity.

7.3 Results and Discussion

7.3.1 Investigating the effect of SNPs in the virulence regulatory activity of SarS

A bioinformatics approach addressing the sequence variability within the virulence regulatory genes across ten *S. aureus* strains that represented a diverse collection of sequence types highlighted two strains (MRSA252 and TW20) which contained two SNPs in the virulence regulator SarS (96). These SNPs conferred an asparagine-to-aspartic acid substitution at positions 221 and 243. Additionally, mapping of the SNPs onto the crystal structure of the SarS protein confirmed that these SNPs were localised within a region predicted to interact with RNA polymerase (96). Given the change in local charge associated with the above N221D and N243D mutations, an investigation into the effect of these polymorphisms on SarS regulatory activity was examined. As described in section 1.3.4.3, SarS is a DNA binding regulatory protein which induces the expression of protein A by directly binding and activating the *spa* promoter (372). Therefore, we investigated the regulatory potential of WT SarS and SarS containing the above SNPs using protein A expression as an indication of SarS activity (Fig 7.1). Both genes were cloned into the inducible plasmid, pRMC2, and electroporated into a *sarS* knockout strain, ALC1927, creating ALCML1 (WT SarS) and ALCML2 (N221D & N243D SarS) (Table 7.1). Probing for protein A expression as an end-stage measure of SarS activity illustrated no detectable difference via western blot analysis (Fig 7.1). A series of concentrations of the inducible chemical (10-100 nM; anhydrous tetracycline) was used to investigate any minor changes which may have been masked due to high expression of SarS from the plasmid resulting in high protein A expression, but again no differences were

observed, suggesting that these two SNP changes did not affect the ability of SarS to induce protein A expression.
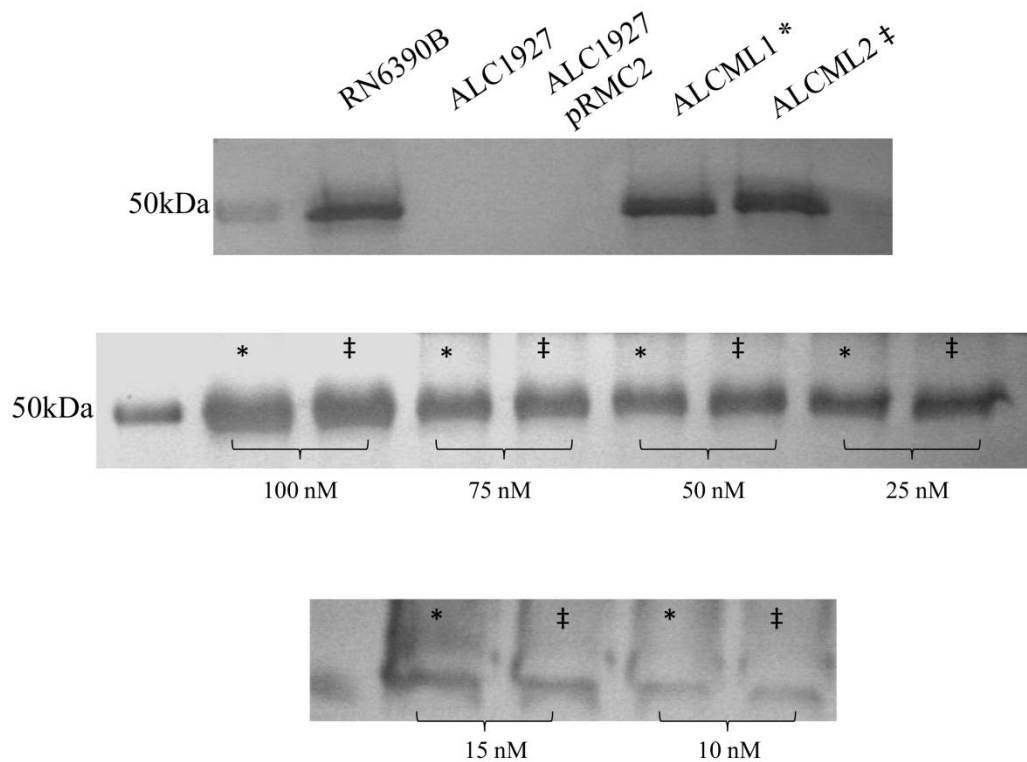
Figure 7.1: Investigating the effect of SNPs in the virulence regulatory activity of SarS by analysing protein A expression via western blotting. WT SarS and SarS containing the N221D & N243D SNPs were cloned into the pRMC2 plasmid and transformed into the *sarS* knockout ALC1927, generating ALCML1 and ALCML2 respectively. Analysis of protein A expression using a series of concentrations of the inducible chemical revealed no difference in the protein A expression. ALCML1 is designated * and ALCML2 is designated ‡.

7.3.2 Analysis of toxicity and adhesiveness between closely related ST239 strains

As the direct approach in identifying SNPs important in virulence did not yield positive results we opted to look more broadly for the effect of SNPs on virulence across the whole genome. In order to investigate this, we chose to look within a single clone as there would have been too much variation across many different clones. The strain collection chosen was previously described by Castillo-Ramiriez et al and comprises of 90 clinically derived ST239 strains, isolated from one of four

hospitals in Turkey. These strain have been previously sequenced using an Illumina Genome Analyser with 75 base paired-end reads and mapped onto the chromosome of S. aureus TW20 (180).

To investigate SNP association with virulence, we first assayed the ability of 90 closely related clinically derived ST239 strains to bind two important matrix proteins, fibrinogen (Fb) and fibronectin (Fn) during both the exponential and stationary phase of growth (Fig 7.2 a-d). As expected the adhesiveness was higher in the exponential phase of growth. However, across the 90 isolates only two were significant from the others (DEU16 and HU2) with strain HU2 retaining the adhesive phenotype during stationary phase. The limited variability of this virulence phenotype suggests it may be under strong stabilizing selection and would provide limited information on which to base a prediction of disease severity. Comparing the core genomes of DEU16 and HU2 with the reference strain, TW20, highlighted no mutation in any of the known virulence regulators, adhesin genes or proteases which may account for the highly adhesive phenotype. Results of gene association studies were inconclusive in providing statistically significant SNPs associated with high or low phenotypes, possibly due to the limited variability.
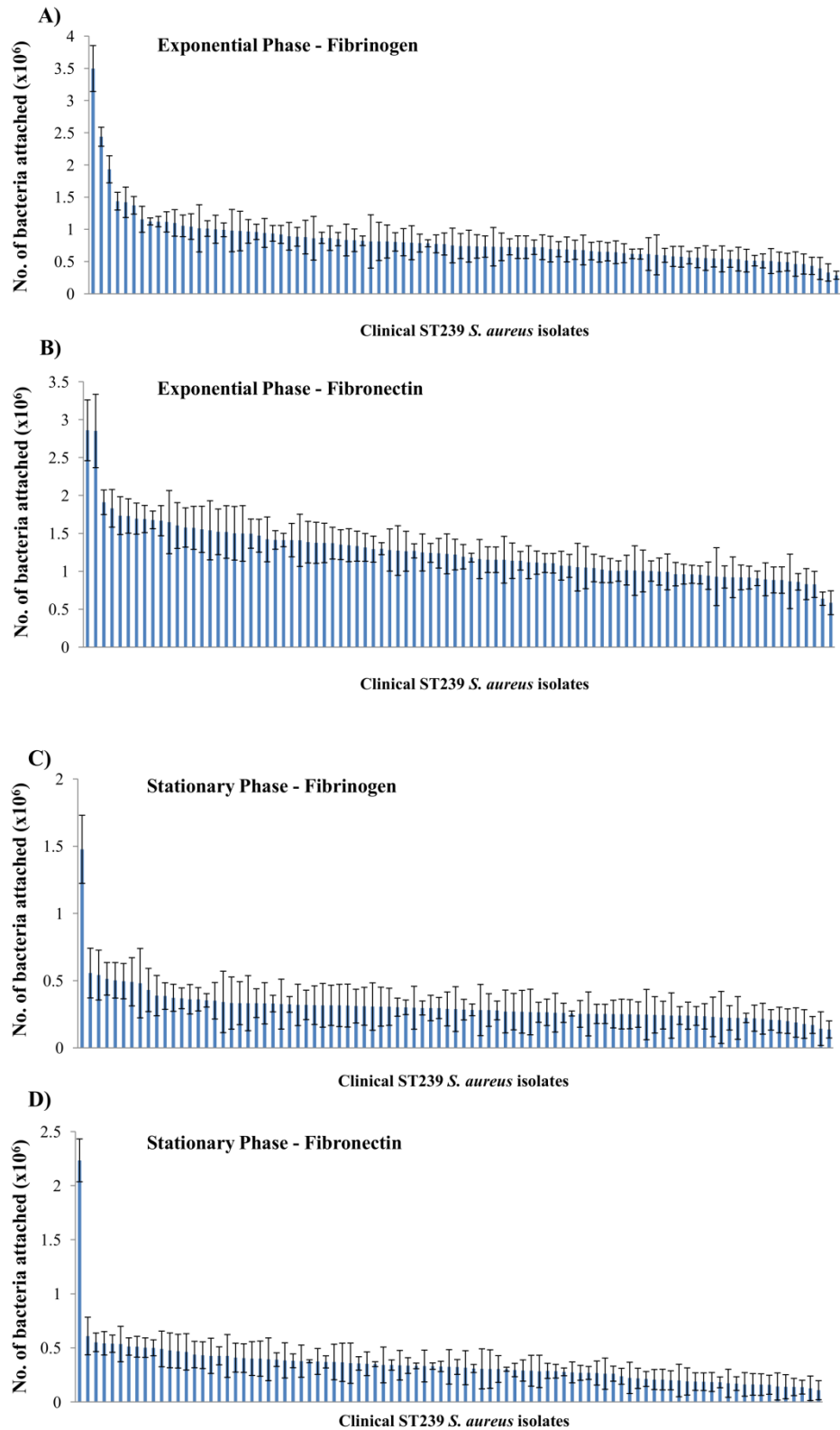
Figure 7.2: Adhesive activity of clinical ST239 isolates. **(a)** adherence of 90 ST239 isolates to fibrinogen in exponential phase of growth. **(b)** adherence of 90 ST239 isolates to fibronectin in exponential phase of growth. **(c)** adherence of 90 ST239 isolates to fibrinogen in stationary phase of growth. **(d)** adherence of 90 ST239 isolates to fibronectin in stationary phase of growth.

Secondly, the gross lytic activity of the 90 ST239 isolates was measured using the T cell assay and vesicle lysis test (VLT) described previously. As illustrated in the previous chapters, the T cell assay is sensitive to α- β-, γ-, δ-haemolysins and PSMα1-3 peptides whereas the vesicles are sensitive to the δ-haemolysin and PSMα1-3 toxins. No differences were observed between the two assays, illustrated in figure 7.3a, where the data from each assay from a representative sample of seven high and seven low toxic isolates is presented. This suggests the effect is either largely PSM driven for the ST239 clone, or that the toxins assayed here are co-regulated. Due to the important role α-haemolysin plays in *in vivo* pathogenesis we also investigated the expression of this toxin from this set of clinical strains; however no α-haemolysin was detected, illustrated here by a western blot negative phenotype in a subset of strains both of high and low toxic background (Fig 7.3b).
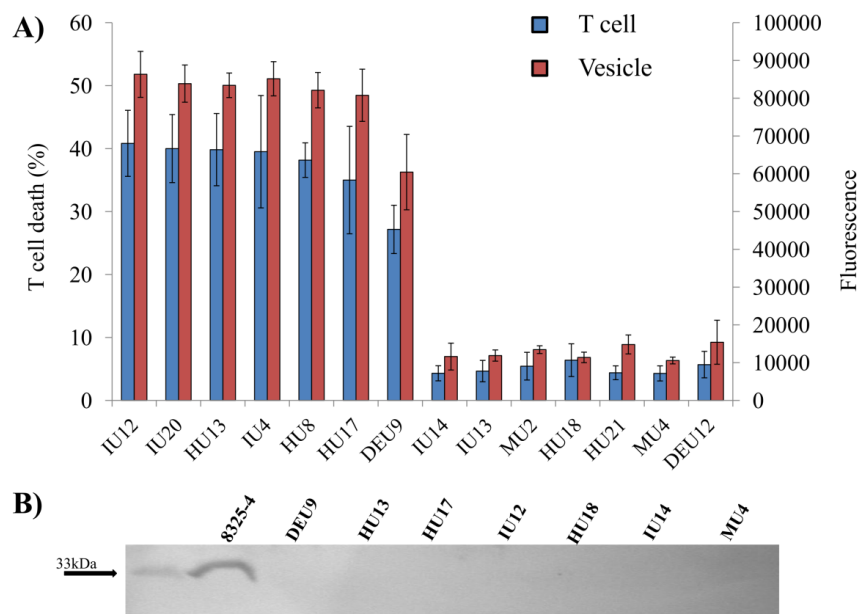
Figure 7.3: ST239 supernatants lyse T cell and vesicles equivalently and do not express α-haemolysin. A) The ST239 MRSA isolates used in this study lysed T cells and lipid vesicles equivalently. A subset of 14 isolates is shown for illustrative purposes. Their ability to lyse T cells is represented in blue and on the left hand Y axis, and their ability to lyse the lipid vesicles is represented in red and on the right hand Y axis. **B)** The ST239 isolates used in this study do not express detectable amounts of α-haemolysin. Western blots using anti-α-haemolysin antibodies were performed on TCA precipitated 18h bacterial supernatants of all 90 isolates. A subset of seven isolates is shown for illustrative purposes. As a positive control the laboratory strains 8325-4 was included as it produces detectable amount of α-haemolysin.

The full set of toxicity data from the vesicle assay is presented in figure 7.4a. Despite the lack of α-haemolysin expression, many isolates were highly lytic and the combined activity of the other toxins varied widely between the 90 isolates, with an 18-fold difference between the most and least toxic isolates. Interestingly, both the highly adhesive isolates identified above expressed low level toxicity. (NB: This clone does not contain the Panton-Valentine leukocidin [PVL] containing phage (180)).

To understand how differences in toxicity are distributed across the genetic variability that exists within this collection of isolates we divided the data into three classes, scoring isolates as expressing either high (red: levels of >63,000 units), medium (amber: levels of 30,000:63,000 units) or low (green: levels of <30,000 units) toxicity. These three data ranges were selected so that a mid-toxicity range was included to account for possible cumulative effects of genetic polymorphisms. This was mapped onto a maximum likelihood tree based on the genome sequences of these isolates, showing a broad distribution of toxicity phenotypes across the genotypes as well as some clustering (Fig 7.4b; this figure was constructed by Dr. Daniel Wilson, University of Oxford, United Kingdom).

B)
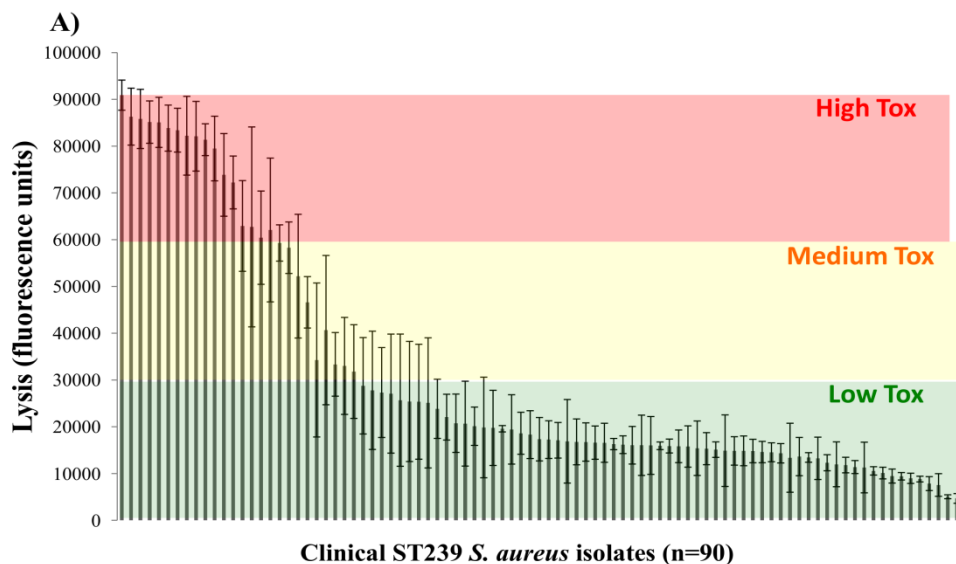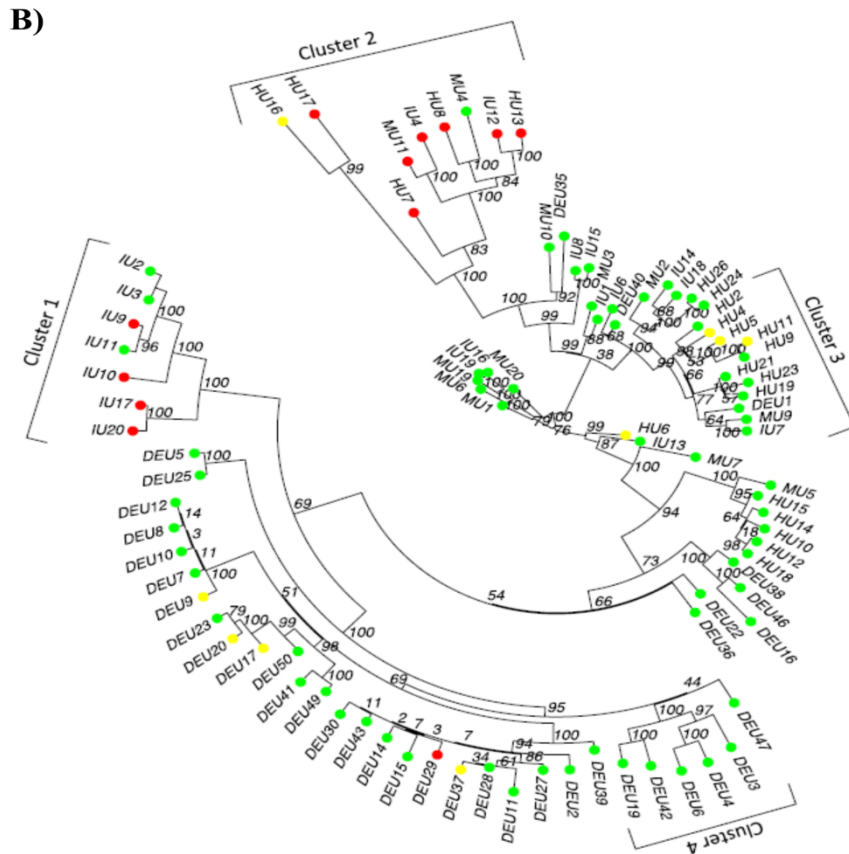
Figure 7.4: **Toxic activity of clinical ST239 isolates.** **(A)** The toxic activity of 90 ST239 isolates was assayed by incubating their supernatants with lipid vesicles containing a fluorescent dye. Dye release due to toxin-mediated vesicle lysis is determined using a fluorometer. **(B)** A maximum likelihood tree based on whole-genome sequences of the 90 isolates illustrating the distribution of the toxic activities of each isolate. Toxicity has been colour-coded (red for highly lytic, yellow/amber for moderately lytic and green for low level lysis). Clusters 1–4 are indicated for use in the stringent GWAS analysis.

7.3.3 Toxicity correlates with disease severity *in vivo*

To verify that toxicity correlated with disease severity, two isolates shown to have the highest and the lowest levels of toxicity in vitro (HU13 and MU9, respectively) were selected and their *in vivo* pathogenicity compared in a model of invasive infection (635). Mice were injected intravenously with two different inoculum sizes; and murine survival, the development of septic arthritis, and weight loss were monitored over two weeks as a measure of disease severity (Animal experiments were carried out by Dr. E. Josefsson, University of Gothenburg, Sweden). Uninfected control mice did not die, did not develop septic arthritis, and did not lose

weight over the duration of this experiment. In each aspect of disease measured here, the highly toxic HU13 isolate caused the most severe disease symptoms (Fig 7.5a-f). It led to more deaths at both doses, although this was not statistically significant, caused significantly more severe arthritis at both doses at day 4, and resulted in significantly greater weight loss at both doses across many time points.

The isolates tested here are from the same sequence type but are not isogenic, and so other virulence-related traits may have played a role in the disease outcome. However, as toxicity is well established to affect disease severity, its variability even within this closely related group of isolates suggests that the ability to predict toxicity at an early stage of infection would be valuable clinical information.
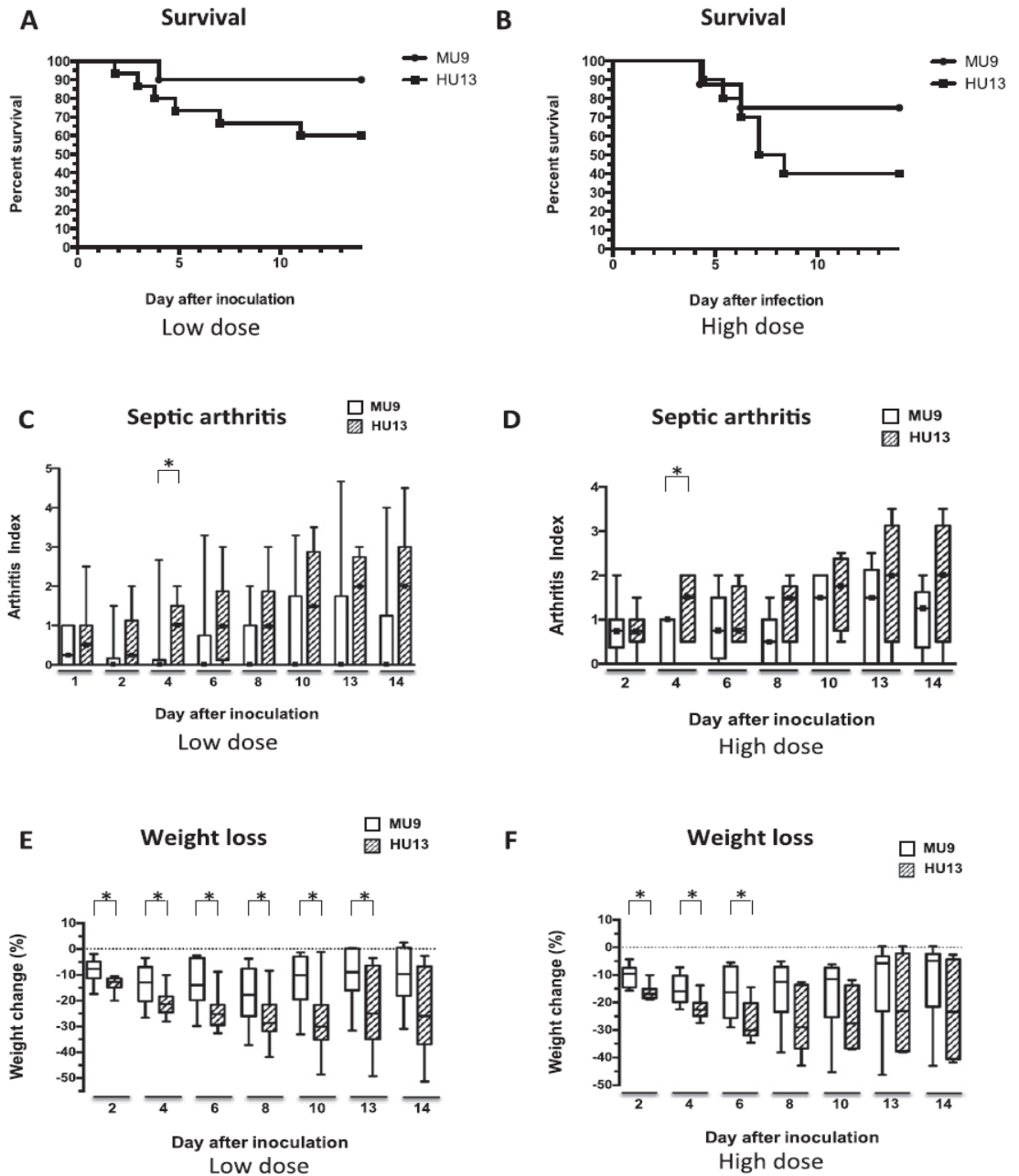
Figure 7.5: Predicted toxicity correlates with disease severity *in vivo*. Using high and low doses (7.8–8.0 and 3.7–4.1 x 10^7 CFU, respectively), mice were inoculated intravenously with the high and low toxic isolates (HU13 and MU9, respectively), and survival of the mice, the development of septic arthritis, and weight loss were recorded as indications of disease severity. In each case the highly toxic HU13 isolate caused the most severe disease symptoms. (A) n = 10–15. (B) n = 8–10. (C) n = 10–20. (D) n = 10. (E) n = 10–19. (F) n = 10. Significant *P*-values (<0.05) are indicated (*).

7.3.4 Identifying virulence associated loci

To identify the genetic polymorphisms that may be associated with the toxic phenotype, a genome-wide association study (GWAS) was adopted on the genomes of the 90 *S. aureus* isolates using the genome association tool *Plink* (467). Out of a total of 3060 SNPs, we identified 102 that associated significantly with toxicity (with *P* <0.05 after Bonferroni correction) as shown in figure 7.6 and listed in table 7.1 (polymorphisms associated with an increase in toxicity) and table 7.2 (polymorphisms associated with a decrease in toxicity), using a frequency cut off for the occurrence of a polymorphism across the population of >90% and a minor allele frequency of >5%. We further identified 22 toxicity associated InDels, using the same cut offs for quality control. These SNPs and InDels were distributed across the genome amongst mobile genetic elements, genes involved in metabolism and regulation, in hypothetical genes, and in intergenic regions (Table 7.1 and 7.2). Two genes previously shown to affect the expression of toxins contained significantly associated SNPs: *mecA* (238) and *agrC* (636, 637) which provided some proof of principle for the validity of our approach. Mobile genetic elements, such as the *S. aureus* pathogenicity Island I (SaPI1) (196) and the β-haemolytic converting phage (638) also contained several associated genetic changes, implying that variability in many diverse regions of the genome contributes to the toxicity of a given isolate. Some of the polymorphisms appeared to be in linkage disequilibrium which will increase the rate of false positive associations, but many were uniquely occurring (i.e., unique patterns of polymorphisms across isolates).

This GWAS approach only requires an excess association between a SNP and the phenotype in question, and as such is likely to produce false positives with linkage disequilibrium and phylogenetic structure affecting the outcome. We therefore performed a second, more stringent approach, similar to those described in other recent work (630, 639) which instead requires repeatable independent evolution of a marker to be associated with the phenotype (toxicity). Although this approach should have a lower false positive rate, it is likely to produce a higher false negative rate. We focused on four clusters of isolates (indicated on Fig 7.4b): cluster 1

(isolates IU20–IU2), cluster 2 (isolates HU16–HU13), cluster 3 (isolates MU2–IU7), and cluster 4 (isolates DEU3– DEU19). Clusters 1 and 2 contained the majority of the highly toxic isolates in this study, whereas clusters 3 and 4 represent the closest related clusters of low toxicity isolates to clusters 1 and 2. When toxicity-associated polymorphisms are found in both clusters 1 and 2 but are absent from clusters 3 and 4 suggest that they have arisen independently. As such they are likely to be causative as they are independent of phylogeny. Of the 124 polymorphic sites that associated significantly with toxicity, only four were found in both high toxicity clusters (1 and 2) but not in their sister, low-toxicity clusters (3 and 4). All four of these polymorphisms (SNPs 78396, 2128192 and InDels 2111134 and 2147199, see Tables 7.2 and 7.3) reside on mobile genetic elements, suggesting they may have been acquired horizontally. Of these four polymorphic loci, the *mecA* gene (in which SNP78396 resides) confers methicillin resistance and has previously been shown to affect toxin expression (238).
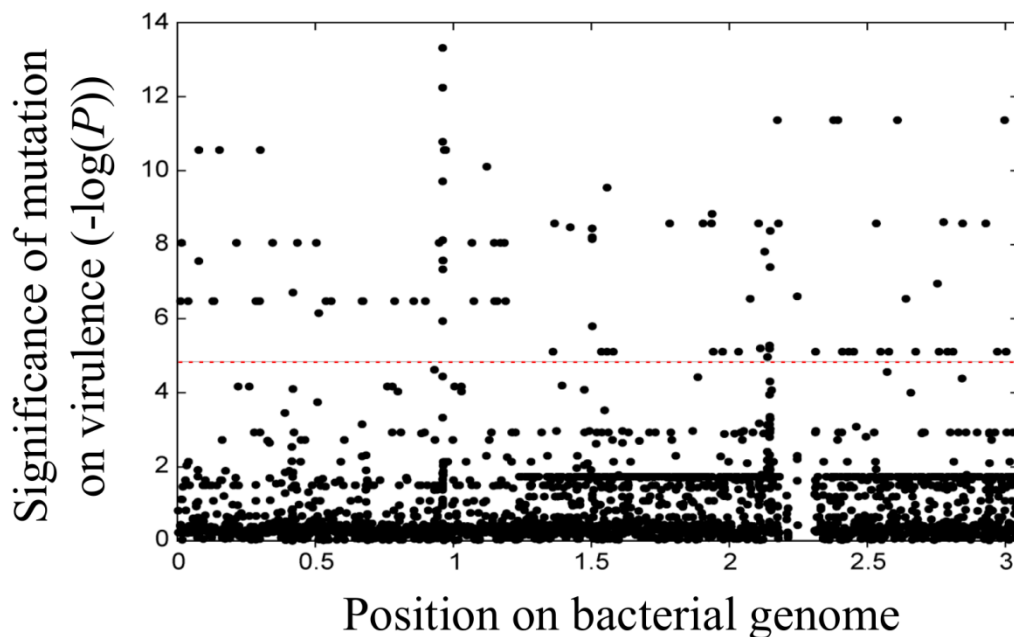
Figure 7.6: Identification of SNPs significantly associated with toxicity. GWAS was used to identify toxicity associated SNPs; shown here on the X axis which represents the *S. aureus* genome with 0 representing the origin of replication and the Y axis representing the statistical significance of each SNP associating with increased or decreased toxicity. Using a cut-off of –log (p) of 5 (illustrated by red dots representing a *p*<0.05 after Bonferroni correction) left 104 SNPs and 20 InDels significantly associated with toxicity.

Polymorphic site	nt change	gene/locus	(-LogP)	syn/non-syn
11760	T to G	SATW20_00080 *hutH*	3.077430231	S290R
39575	C to T	SATW20_00320 hypothetical protein	3.077430231	P185L
78119	C to T	SATW20_00740 *mecA*	4.159204101	E239K
78396	T to A	SATW20_00740 *mecA*	7.161465729	K147N
125512	+1	SATW20_01180 *norC*	6.049	out of frame from V246 onwards
128956	G to A	SATW20_01200 putative myosin-crossreactive antigen	3.077430231	Syn
133041	G to C	SATW20_01220 *lldP1*	3.077430231	A517P
202813	+3	intergenic between SATW20_01840 and SATW20_01850	3.86106606	n/a
220518	G to T	SATW20_01950 putative ornithine aminotransferase precursor	0.773657913	N136K
260556	G to A	SATW20_02250 putative lipoprotein	0.773657913	Syn
285239	A to G	SATW20_02430 *ldh1*	3.077430231	Syn
295473	C to G	SATW20_02520 putative zinc-binding dehydrogenase	3.077430231	P88A
298644	T to C	SATW20_02550 *tarK*	3.077430231	F36L
318013	-1	SATW20_02690 *blgA*	3.86106606	out of frame from Y278 onwards
345590	C to T	SATW20_02920 hypothetical protein	4.655411257	Q250Stop
390199	A to G	SATW20_03490 phage protein	0.058488567	I62V
435458	G to T	SATW20_04040 *glpT*	4.655411257	T248K
508254	C to T	intergenic between SATW20_04840 and SATW20_04850	0.349498205	n/a
512441	A to G	intergenic between SATW20_04840 and SATW20_04850	2.756961951	n/a
538580	G to A	SATW20_05110 hypothetical protein	3.077430231	E141K
557945	A to G	SATW20_05290 putative Cys/Met metabolism PLP-dependenten	3.077430231	T176A
669521	-82	SATW20_06190 putative peptidase	3.86106606	out of frame from F191 onwards
669532	G to C	SATW20_06190 putative peptidase	3.077430231	S188R
669533	C to G	SATW20_06190 putative peptidase	3.077430231	S188T
672721	C to A	intergenic between SATW20_06210 and SATW20_06220	3.077430231	n/a
761112	T to C	intergenic between SATW20_07050 and SATW20_07060	0.773657913	n/a
779318	G to A	SATW20_07230 *fhuB*	0.773657913	Syn
787629	T to C	intergenic between SATW20_07310 and SATW20_07320	3.077430231	n/a
799276	C to T	intergenic between SATW20_07410 and SATW20_07420	0.638461029	n/a
824672	-6	intergenic between SATW20_07690 and SATW20_07700	3.86106606	n/a
856804	T to C	SATW20_07990 putative aminotransferase	3.077430231	Syn
898669	A to G	SATW20_08340 *uvrA*	3.077430231	T777A
958718	-1	SATW20_08990 on SaPI1	3.86106606	out of frame from V161 onwards
961636	+1	intergenic between SATW20_-9020 and SATW20_09030 on SaP	5.105130343	n/a
1004808	A to G	SATW20_09520 *mnhA*	0.773657913	N56K
1029240	A to G	SATW20_09700 coenzyme A disulfide reductase	0.773657913	Syn
1029745	T to C	intergenic between SATW20_09700 SATW20_09710	0.637517525	n/a
1074386	A to G	SATW20_10100 sodium:alanine symporter family protein	3.077430231	H334R
1121594	+1	intergenic between SATW20_10520 and SATW20_10530	3.86106606	n/a
1149765	C to T	SATW20_10790 *ptsI*	3.077430231	H142Y
1158979	A to C	SATW20_10870 putative lipoprotein	3.077430231	D140A
1189274	G to T	SATW20_11160 hypothetical protein	3.077430231	E44D
1360889	C to G	SATW20_12700 FtsK/SpoIIIE family	1.711303739	Syn
1394050	T to C	SATW20_13000 *Alu-2*	0.799149502	Y272H
1474596	-77	SATW20_13760 haloacid dehalogenase-like hydrolase superfam	3.86106606	out of frame from P223 onwards
1474673	A to T	SATW20_13760 haloacid dehalogenase-like hydrolase superfam	0.684239509	K249N
1536348	T to C	SATW20_14350 *ebh*	1.711303739	K8461E
1548040	A to G	SATW20_14350 *ebh*	0.131590697	Syn
1557275	C to A	SATW20_14350 ebh	1.711303739	S1485I
1579791	G to A	SATW20_14490 *pbp2*	1.711303739	Syn
1886160	A to G	SATW20_17320 hypothetical protein	1.023879382	V94A
1941832	G to A	SATW20_17820 hypothetical protein	1.711303739	P233L
1975607	A to G	SATW20_18180 lantibiotic biosynthesis protein	1.711303739	V153A
2033565	C to T	SATW20_18600 ABC transporter ATP-binding protein	1.711303739	E129K
2075672	G to A	SATW20_19010 pcrB	3.143996546	R198Stop
2111134	+1	intergenic between *sak* and *scn*	1.842543232	n/a
2128192	G to A	SATW20_19530 β-haemolytic converting phage protein	4.414652089	P30L
2145772	A to G	SATW20_19860 on b-haemolytic converting phage antirepressor	4.894112574	Syn
2147172	-32	SATW20_19900 on b-haemolytic converting phage antirepressor	5.655411257	out of frame from I9 onwards

2147199	+4	SATW20_19320 b-haemolytic converting phage protein	1.842543232	out of frame from M1 onwards
2312226	G to A	SATW20_21870 ABC transporter ATP-binding protein	1.711303739	A618T
2409540	C to T	SATW20_22780 hypothetical protein	1.711303739	Syn
2432219	G to A	SATW20_22950 *fmtB* or *mrp*	1.711303739	A2016V
2450342	A to G	intergenic between SATW20_r160 and SATW20_23010	1.711303739	n/a
2548323	C to T	SATW20_24090 *modC*	1.711303739	D158N
2571739	A to T	intergenic between SATW20_24320 abd SATW20_24330	1.167108055	n/a
2578126	T to C	SATW20_24400 putative bifunctional protein	1.711303739	D798G
2583090	+1	Intergenic between SATW20_24420 SATW20_24430	3.86106606	n/a
2639747	T to C	SATW20_24980 *lldP2*	3.143996546	Syn
2640325	+1	intergenic between SATW20_24980 and SATW20_24990	3.86106606	n/a
2657438	G to C	intergenic between SATW20_25130 and SATW20_25140	0.60502328	n/a
2674904	T to C	SATW20_25300 *nasD*	1.711303739	I737V
2753734	G to A	intergenic between SATW20_25980 and SATW20_25990	3.553462833	n/a
2759775	T to C	SATW20_26050 putative short chain dehydrogenase	1.711303739	Syn
2790429	T to C	SATW20_26280 hypothetical protein	1.711303739	E12G
2810368	A to G	intergenic between SATW20_26460 and SATW20_26470	1.711303739	n/a
2843286	C to T	SATW20_26760 aldehyde dehydrogenase family protein	0.988852639	G161S
2970902	T to C	SATW20_27860 hypothetical protein	1.711303739	K26E
3002241	A to G	SATW20_28120 *hisH*	1.711303739	Syn
3002845	T to C	SATW20_28130 *hisB*	1.711303739	T126A

Table 7.2 Polymorphisms associated with an increase in toxicity

SNP site	nt change	gene/locus	(-LogP)	syn/non-syn
15605	G to A	SATW20_00110 hypothetical protein	4.655411257	Syn
129380	-1	SATW20_01200 putative myosin-crossreactive antigen	5.438898616	out of frame from K342 onwards
152937	C to T	SATW20_01390 putative non-ribosomal peptide	7.161465729	T35I
214537	G to A	SATW20_01890 putative non-ribosomal peptide	4.655411257	Syn
301089	A to G	intergenic between *tarK* and *tarF*	7.161465729	n/a
503698	C to T	intergenic between SATW20_04720 and SATW20_04730	4.655411257	n/a
948986	A to G	SATW20_08840 hypothetical protein	4.655411257	Syn
961622	A to G	intergenic between SATW20_-9020 and SATW20_09030 on SaPI1	8.8467951	n/a
961636	T to C	intergenic between SATW20_-9020 and SATW20_09030 on SaPI1	9.918652692	n/a
961639	+1	intergenic between SATW20_-9020 and SATW20_09030 on SaPI1	2.999132279	n/a
961642	C to T	intergenic between SATW20_-9020 and SATW20_09030 on SaPI1	7.384892013	n/a
961673	C to T	intergenic between SATW20_-9020 and SATW20_09030 on SaPI1	6.315603522	n/a
961675	T to A	intergenic between SATW20_-9020 and SATW20_09030 on SaPI1	6.315603522	n/a
967656	A to G	SATW20_09130 hypothetical protein	7.161465729	Syn
972267	A to G	SATW20_09170 NifU-like protein	7.161465729	M36V
1038124	-1	SATW20_09760 hypothetical protein	5.438898616	out of frame from G292 onwards
1067541	A to G	SATW20_10060 putative divalent cation transport protein	4.655411257	D22G
1121452	A to G	intergenic between SATW20_10520 and SATW20_10530	6.711303739	n/a
1148420	T to C	SATW20_10770 hypothetical protein	4.655411257	I15T
1171002	G to A	intergenic between SATW20_10990 and SATW20_11000	4.655411257	n/a
1184963	C to T	SATW20_11110 *ctaB*	4.655411257	P87L
1366717	A to G	SATW20_12770 *pgsA*	5.176330187	I107V
1423837	T to C	SATW20_13360 *katA*	5.073657553	Syn
1503105	-399	SATW20_14060 pseudogene	5.438898616	n/a
1503110	G to A	SATW20_14060 pseudogene	4.805485658	n/a
1503113	A to T	SATW20_14060 pseudogene	5.041054068	n/a
1557020	G to A	SATW20_14350 *ebh*	6.147764061	T1570I
1783936	T to C	SATW20_16540 *folC*	5.176330187	Y243C
1904373	C to T	SATW20_17460 *sasC*	5.176330187	S832N
1931155	-1	intergenic between SATW20_17700 and SATW20_17710	3.632644079	n/a
1935622	A to G	SATW20_17770 aldo/keto reductase family protein	5.176330187	Syn
1937314	C to T	SATW20_17780 hypothetical protein	5.434979072	A57T
2046299	+1	SATW20_18730 putative aminopeptidase	3.784891419	out of frame from K141 onwards
2106193	T to C	SATW20_19310 *eap*	5.176330187	Syn
2138224	C to G	SATW20_19530 β-haemolytic converting phage protein	1.572027286	Stop81Y
2147902	G to A	SATW20_19910 b-haemolytic converting phage antirepressor protein	4.000304113	Syn
2174068	G to A	SATW20_20210 *agrC*	7.967381239	A343T
2178247	A to G	SATW20_20250 *scrR*	5.176330187	Syn
2377659	G to A	SATW20_22440 *atpH*	7.967381239	Syn
2393426	G to A	SATW20_22620 *murA2*	7.967381239	A37V
2532617	T to C	SATW20_23930 hypothetical protein	5.176330187	L62S
2608746	A to G	SATW20_24680 putative ribose 5-phosphate isomerase	7.967381239	V77A
2642208	+1	intergenic between SATW20_24990 and SATW20_25000	5.438898616	n/a
2845228	G to A	SATW20_26790 *copA*	5.176330187	Syn
2928993	C to T	SATW20_27570 hypothetical protein	5.176330187	A91T
2997008	C to T	SATW20_28080 *gehC*	7.967381239	V631I

Table 7.3 Polymorphisms associated with a decrease in toxicity

7.3.5 Functional verification of effect of polymorphisms on toxicity

Two SNPs (78119 and 78396) which were hypothesised to affect toxicity were present in the *mecA* gene, which codes for the alternative penicillin binding protein 2a. Recently, the deletion of the SCC*mec* element from a HA-MRSA background drastically affected toxicity leading to a statistically significant increase in toxicity measured by the T cell assay (238). Complementation of this strain with the *mecA* gene expressed from an inducible plasmid restored the non-toxic phenotype illustrating that *mecA* negatively affected toxicity. The exact mechanism for this repression is unknown but does involve *agr* interference possibly through peptidoglycan modifications imposed by PBP2a (238). Therefore, we sought to investigate the contribution of these SNPs to toxicity. As described in section 2.5.3, four *mecA* genes were amplified, cloned into the inducible plasmid pRMC2and electroporated into the SCC*mec* deletion mutant BHICCΔSCC*mec*. Four *mecA* constructs were required due to SNP78119 and SNP78396 only occurring in *mecA* genes which already had two SNPs present which differed from the WT, which were SNP78097 and SNP78222. These SNPs were not highlighted as modulating toxicity from the GWAS studies. Nonetheless we constructed pRMC2 containing TW20 WT *mecA* (p*mecA*1; ML122), TW20 *mecA* containing SNPs 78097 & 78222 (p*mecA*2; ML123), *mecA* containing the SNPs of p*mecA*2 and SNP78396 (p*mecA*3; ML124) and *mecA* containing the SNPs of *pmecA3* and SNP78119 (p*mecA*4l ML125). All strains containing the *mecA* plasmid constructs when induced conferred resistance to oxacillin up to128 μg/mL when induced with anhydrous tetracycline and therefore were considered to be expressing the PBP2a, whereas those induced with the empty plasmid (pRMC2; ML121) did not (data not shown). However, toxicity did not differ significantly from strains expressing the 4 different *mecA* alleles suggesting that these two SNPs were not involved in increasing toxicity (Fig 7.7). Interestingly, unlike previous work in the laboratory where the BH1CC *mecA* gene was used to restore methicillin resistance, none of these *mecA* genes when used to complement the SCC*mec* mutant restored the non toxic phenotype. The sequence of *mecA* from TW20 differs from *mecA* derived from BH1cc (CC8) in three positions (K146N, K204N and E246G, whereby the first amino acid is found in TW20 at the indicated

position and the second amino acid is present in BH1cc). This suggests that mecA from different SCCmec backgrounds may differentially regulate toxicity.
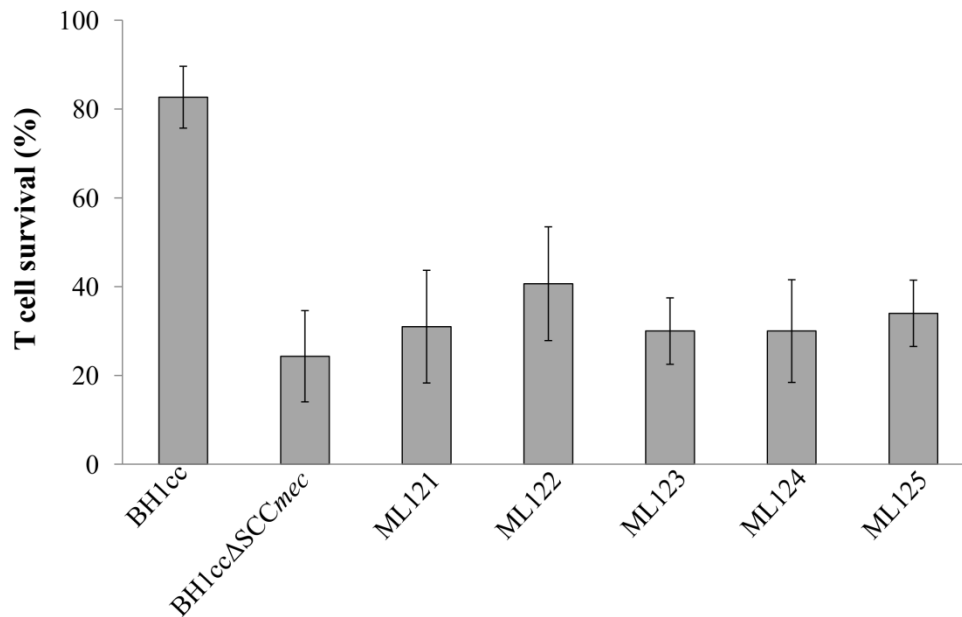
Figure 7.7: Effect of *mecA* SNPs on toxicity. p*mecA* constructs were electroporated into a BH1cc∆SCC*mec* background to assess the affect of *mecA* SNPs on toxicity using the T cell assay. Deletion of SCC*mec* leads to a decrease in T cell survival (an increase in toxicity). Complementing this strain with 4 different *mecA* alleles did not restore a non-toxic phenotype nor illustrate a difference between the *mec*A alleles.

With the initial GWAS approach likely to produce a high number of false positive associations, we sought to obtain an estimate of this by determining the functional effect of a subset of these polymorphisms. We focused on 14 of the intergenic polymorphisms that could either affect the transcription of neighbouring genes, or encode novel regulatory RNA molecules. We obtained transposons insertions in these intergenic polymorphic loci, ranging from 10 to 304 bp distal to the polymorphic site from the Nebraska Transposon Mutant library (640), and determined the effect of this insertion on the toxicity of the mutant. The transposon (Tn) library was constructed in the USA300 LAC background cured of plasmids (JE2 strain) using a mini-*mariner bursa aurealis* plasmid containing a transposon region, generating random mutations in *S. aureus*. This delivery vector contains and erythromycin resistance gene (*erm*B) for selection and AciI sites used for cleavage of DNA isolated form Tn clones. Cleavage of AciI sites closest to the insertion sites

occurs and DNA is circularized and amplified by inverse PCR using primers complementary to regions on the Tn insertion site. Sequencing of the genomic regions identifies site of disruption (640).

Five of the 14 insertions affected toxicity (Fig 7.8a) verifying that these loci contain toxicity-regulating activity. The SNP at position 301,089 (represented by the transposon insertion in strain 95E07 in Fig 7.8) is in between the *tarK* and *tarF* genes that are involved in the synthesis of wall teichoic acids (641). The SNP at position 1,121,452 (represented by the transposon insertion in strain 207A03 in Fig 7.8) is between a hypothetical gene and *fmt*, which is involved in methicillin resistance and autolysis (642) both activities known to contribute to staphylococcal virulence. The SNP at position 1,503,110 (represented by the transposon insertion in strain 90D01 in Fig 7.8) is in a locus annotated as a pseudogene in TW20, but as intergenic between genes encoding a TelA-like protein and a putative branched-chain amino acid transporter protein in FPR3757. The SNP at position 2,532,617 (represented by the transposon insertion in strain 108B09 and 184F11 in Fig 7.8) is annotated in FPR3757 as intergenic between a hypothetical and an AcrB/AcrD/AcrF family protein-encoding gene; however, in TW20 it has been annotated as a hypothetical gene. To determine more specifically the effect these transposons have on specific expression of toxins we examined their protein expression profiles. As shown in figure 7.8 b-d, the protein profiles, α-haemolysin and PSM expression are significantly affected in two strains with more subtle differences in the others. The two with significant effect were108B09 and 184F11 are in the same locus and further molecular characterization is underway to determine the activity of these loci, but this work demonstrates that although this approach produces false positive associations, having looked at only 13 polymorphisms it has identified four novel toxicity-affecting loci.
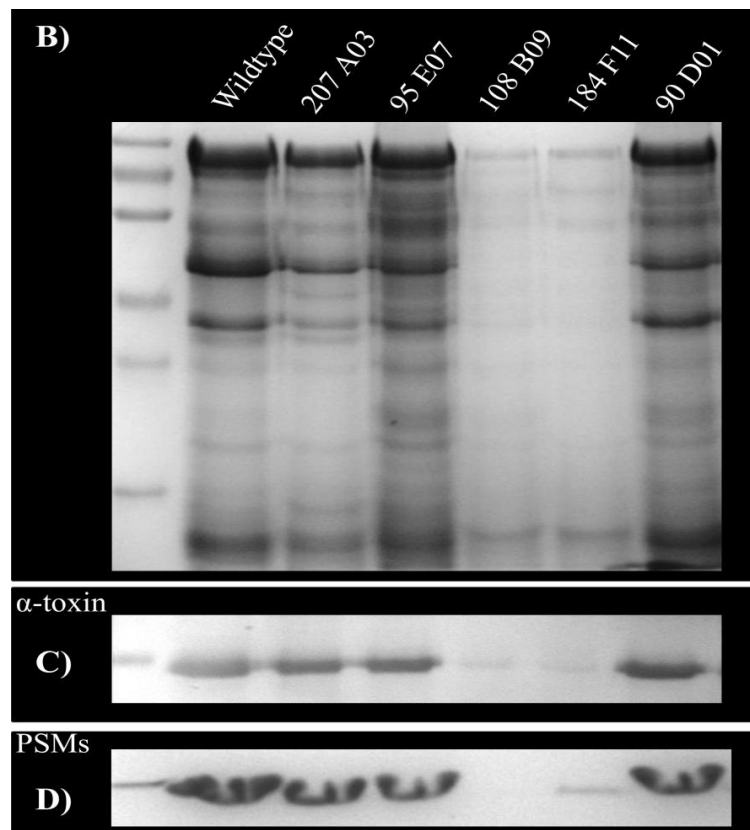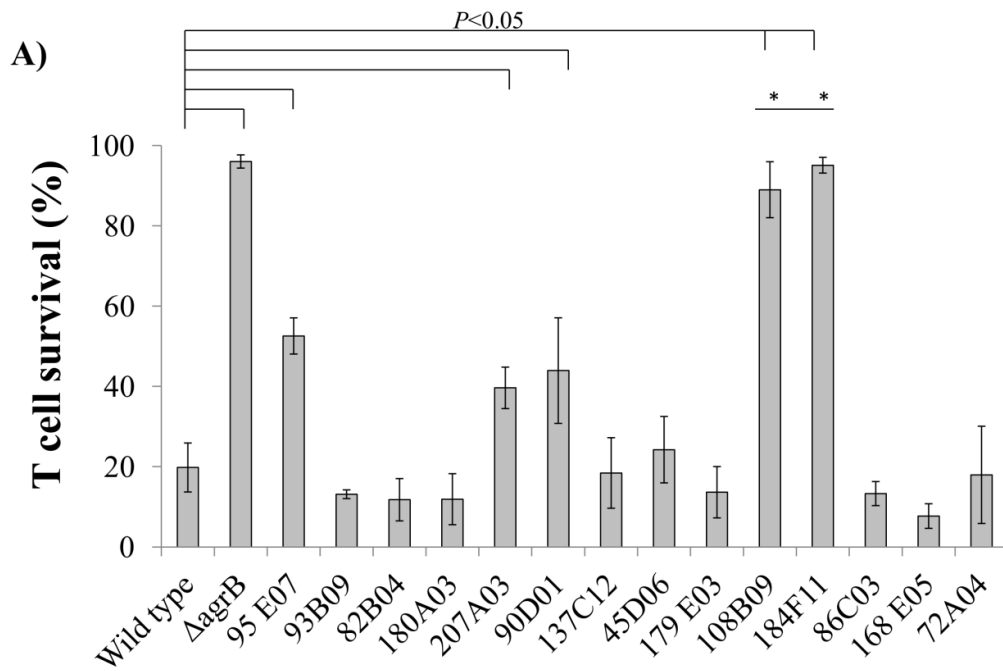
Figure 7.8: Functional verification using transposon mutagenesis. A) Mutated S. aureus isolates with transposon insertions in 15 of the 124 toxicity associated loci were isolated (all in intergenic loci). Four of the 15 transposon insertions affected the toxicity of the isolate. The bars represent the mean % T cell survival following incubation with bacterial supernatant, and the error bars the 95% confident intervals. Wild type represents the unmutated parent isolate, ΔagrB is a negative control,

and the following are the transposon insertion mutants and their associated polymorphism: 95E07: 301089; 93B09: 761112; 82B04: 787629; 180A03: 799276; 207A03: 1121452; 90D01: 1503110; 137C12: 1931155; 45D06: 2027204; 179E03: 211134; 108B09: 2532617; 113D01: 2571739; 86C03: 2640325; 168E05: 2657438; 72A04: 2753734; 64A09: 2810368. **B), C)** and **D)** represent the exoprotein profile, α-haemolysin and PSM expression respectively of the wild type and toxicity deficient strains highlighted from the T cell assay. * highlights that these strains contain a Tn insertion in close proximity to each other.

As the more stringent approach described above yielded a shortlist of only four toxicity-affecting polymorphisms, we also sought to determine whether this approach, while reducing the false positive rate, would inadvertently dismiss potentially important loci. For example, a SNP in the *agrC* gene was identified by the initial approach as significantly associated with toxicity, but dismissed by the secondary more stringent approach. This protein forms part of a critical toxin regulatory system (reviewed in section 1.3.4.1), and the SNP results in an A343T change to the amino acid sequence of the protein. The particular nucleotide change described here had not been identified previously, although other polymorphisms in the *agrC* gene have been shown to delay activation of the Agr system and as a consequence reduced the toxicity (475). Using a reporter system the impact of SNP2174068 on the function of AgrC was evaluated with respect to activation by exogenous AIP 315 (643) (This *agr* reporter system experiment was constructed by Dr. Tim Sloan at the University of Nottingham, United Kingdom). The response of AgrC from the ST239 isolate TW20 was compared with the AgrC encoded by the SNP2174068 containing *agrC* variant, by determining the half maximal effective concentration (EC_{50}) of exogenous synthetic AIP-1 for both (Fig 7.9). The EC50 for the TW20 allele was 17.4 ±3.5 nM, but almost twice as much AIP (29.5 ±3.1 nM) was needed for the SNP2174068 containing AgrC variant, which suggests that, like previously identified polymorphisms in *agrC*, SNP2174068 delays the activation of the Agr system and as a consequence reduces toxicity. This work functionally verified the contribution of this particular polymorphism to the toxic phenotype, which would have been disregarded by the more stringent approach.
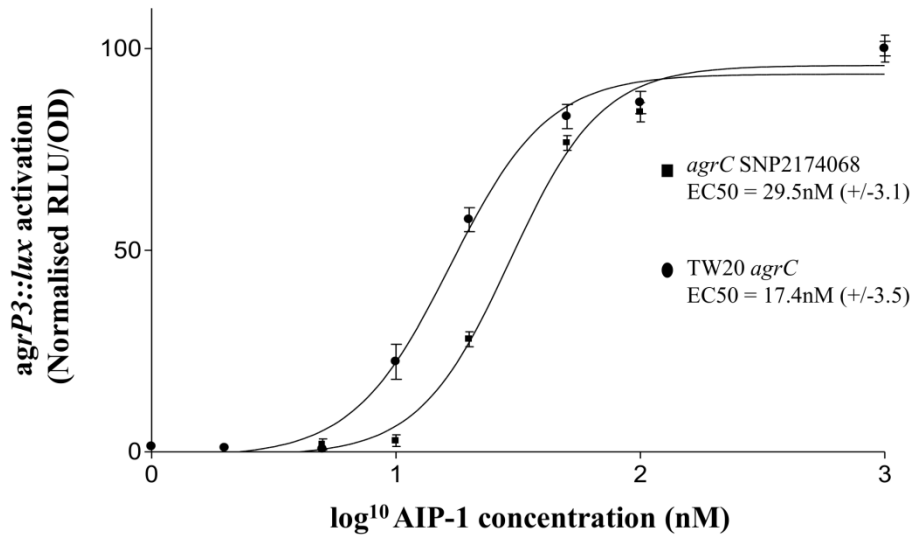
Figure 7.9: SNP2174068 has a major impact on the response of AgrC to AIP and hence toxicity. Dose-response curves for the activation of the *lux* based *agr*P3 reporter via AIP-1 by the TW20 *agr*C allele (circles) compared with the SNP2174068 variant (squares).

7.4 Conclusions

Antibiotic-resistant bacteria are a major global threat to human health. The rate at which these organisms acquire resistance to new drugs is such that it is clear that novel methods to control disease are required. A greater understanding of bacterial virulence is needed to develop such novel control strategies. While new antimicrobial compounds are being developed, the introduction of novel drugs into the hospital population will undoubtedly select for resistance, highlighting the need for clinicians to be able to tailor treatment to the specific requirements of the infection. Currently, there is progress in the development of identifying antibiotic resistance genes from the microbial genome; however there are no methods in place to understand the virulence potential of an infecting strain.

The production of toxins and the ability to adhere to host proteins are major contributors to microbial virulence. Initially, in an effort to understand how genetic polymorphisms affect virulence and how this information could be used in developing new ways to understand and control disease, experiments were designed

to assess the role of SNPs in known virulence regulators. Here we investigated the effect of mutations in SarS, an important transcription factor involved in regulating protein A. In these experiments we found no difference in the ability of wild type SarS and SarS containing the SNPs of interest to activate protein A expression. In a separate, more general approach we sought to determine the toxicity and adhesive activity in a set of 90 clinical ST239 strains. Although little variation in adhesiveness was observed, toxicity varied substantially and to our knowledge this was the first time such variation was observed within a single clone of *S. aureus*, thus, understanding the genetic basis of this variation became the main focus of this chapter.

GWAS has been used extensively to identify disease loci associated with the five most common cancer types (644). Although phylogenetic structure may affect the application of this to a prokaryotic system, GWAS is still a useful tool to identify candidate virulence affecting loci. Bacteria possess a single chromosome and have high mutation rates enabling the phenotypic effects of these mutations to be detected immediately. Furthermore, bacteria readily undergo horizontal gene transfer which is independent of phylogeny. Taking these considerations into account we used GWAS to investigate the association of genetic polymorphisms with toxicity resulting in 124 polymorphisms (102 SNPS and 22 InDels) significantly associated. Initially we anticipated that the majority of the polymorphisms associated with a change in toxicity would be present in the known virulence regulators. However, only one SNP (2174068) associated and verified with a decrease in toxicity was found in the *agrC* gene, a histidine kinase known to be a central component in toxin regulation (329). Surprisingly, SNPs associated with toxicity variation were found in diverse regions on the chromosome, with many of them never been associated with toxicity or virulence before. A more stringent analysis was also used and reduced the number of SNPs to a more manageable number, highlighting only four candidate loci. While this is an easier number to functionally verify, at least one functional locus (*agrC* SNP) was lost by this method. Although one method produced a high rate of false positives and the other dismissed potentially important loci, both have proven to be informative. When we attempted to functionally verify a subset of loci (n = 13) from

the long list of 121 by testing transposon insertion mutants in these regions, four proved to have toxicity regulating activity. This provided an indication of the false positive rate associated with the GWAS approach, and demonstrated that it is an effective means of prioritizing candidate genes for further functional characterization.

The results of this chapter have shown that virulence regulation, particularly in toxin expression, is highly complex. In addition to the already intricate virulence regulatory system discussed in section 1.3.4 the results of this chapter suggest that there is an added layer of virulence regulation yet to be defined. Transposon insertion within the intergenic regions outlined in section 7.3.5 caused a statistically significant reduction in toxicity. Based on the location of this region and preliminary analysis of the sequence of this site illustrating a high level of secondary structure in the single stranded form, we hypothesise that the Tn insertions lead to inactivation of small noncoding RNA molecules (sRNAs) which are playing a role in modulating global toxicity. Generally sRNAs regulate gene expression by either base-pairing with target mRNAs in an anti-sense mechanism preventing translation and assisting degradation of the target RNA or interact with proteins and modify their activities (394). This represents a rapid method of gene regulation and future work into unravelling these structures is underway.

The identification of SNPs and InDels associated with variation in toxicity has been used in the development of predictive models designed to assess the toxic phenotype of clinical isolates solely from the genomic sequence (645). Here a machine learning algorithm has been used to run a series of decision trees based on the presence of significant polymorphisms associated with toxicity to generate a predication on the likely phenotypic outcome. The results are promising with the majority of isolates predicted correctly. This approach of toxicity prediction is an attractive control strategy, as knowledge of the virulence potential of an infecting organism allows for the patient to be isolated, administered with virulence-modulating antibiotics and monitored more stringently for potential complications. In summary, the identification of SNPs associated with phenotypic variability can be

used in identifying new virulence regulatory circuits and developing new control strategies, with future work in fully characterising these genetic polymorphisms and identifying new mutations associated with other phenotypes.

8

Conclusions

Burn injury represents one of the most frequent causes of hospitalisation. Globally in 2004, approximately 11 million people required medical attention due to burn trauma (646). Infection complications following burn injury account for 75% of burn-related deaths (3, 4). Two major factors contribute to the predisposition of burn victims to infection: firstly the skin and gastrointestinal epithelium barrier fails, which can lead to bacterial translocation and subsequent burn wound infection derived from the patients' endogenous flora. Secondly, the immune system becomes dysfunctional, expressing anti-inflammatory mediators which can interfere with immune cell (PMNs and macrophages) microbial killing potential. Therefore, the natural barriers to infection in the burn victim are distorted resulting in colonisation by opportunistic microorganisms.

S. aureus and *P. aeruginosa* are regarded as the most frequent burn wound colonisers. Currently, there is no burn wound dressings which can report on the microbiological content of the burn. The *Bacteriosafe* project was established to address this medical requirement. The objective of the *Bacteriosafe* project was to develop a smart wound dressing that would signal infection in a burn wound through the development and immobilisation of novel nanocapsules onto specific wound dressings.

In this thesis the use of phospholipid vesicles (vesicles) as the core nanocapsule central in the smart dressing, is described. Vesicles are produced naturally in the body, consisting of a lipid bilayer membrane trapping a specific solution, generally used for transport within the cells (transport vesicles) or for housing enzymes important in cellular or microbial digestion (lysosomes). Vesicles used in this project are synthesised through sonication and extrusion of a cocktail of synthetic phospholipids, cholesterol and polydiacetylene molecules. These vesicles

are formed in a solution of self-quenchable fluorescent dye which remains non-fluorescent in intact vesicles.

A major contributor to the pathogenic success of *S. aureus* and *P. aeruginosa* is the ability to secrete membrane damaging factors. The active secretion of bacterial cytolytic exofactors is also fundamental in the application of this microbial signalling system. Here, the vesicle represents a mimic of the eukaryotic membrane, susceptible to bacterial mediated lysis culminating in the release and switch on of the fluorescent cargo, which can be observed and measured. The identification of the bacterial factors which resulted in vesicle lysis is critical to the development of a smart dressing. Both *S. aureus* and *P. aeruginosa* secrete a number of factors which could have been important in lysing the vesicle membrane. In this thesis we highlight the role of molecules with surfactant-like properties; the phenol-soluble modulin (PSM) peptides α1-3 and δ-haemolysin secreted from *S. aureus* and the glycolipid molecule, rhamnolipid (RL), from *P. aeruginosa* as the sole agents in rupturing vesicles.

δ-haemolysin is the translatable product of *rnaIII*, the effector molecule of the accessory gene regulator (*agr*) system, which is involved in the up-regulation of toxins/enzymes in *S. aureus*. The importance of Agr in disease progression in animal models is well documented. Therefore, this toxin is a prime candidate in which to act as a trigger for a microbial sensing system (i.e. the smart wound dressing) as expression of *rnaIII* appears to be a conserved feature required for *S. aureus* pathogenesis. This conserved expression of δ-haemolysin is represented by the high percentage of clinical isolates (96.6%) that caused vesicle lysis (Fig 3.4). This toxin is also involved in measuring the activity of *agr* using the CAMP assay. In chapter 3 we highlight the use of the Vesicle Lysis Test (VLT) in measuring *agr* activity. Our results suggest that the CAMP assay is not sensitive enough to fully discriminate between *agr* positive and negative strains and therefore over- represents *agr*-negative strains and thus *agr* dysfunction. These results may have important clinical implications as it has been reported that *agr* dysfunction is associated with increased vancomycin resistance and persistent bacteraemia (474, 476, 477). Many of these studies used the CAMP assay as the sole measure of *agr* activity. For this reason it

would be interesting to see whether the same associations are confirmed using the more sensitive VLT.

Using vesicles to investigate the lytic potential of toxins is well documented. In the second part of chapter 3 we demonstrate that the PSMα and β class of PSMs have different lytic activities. The pattern of lysis observed using vesicles outlined in this thesis is similar to that observed using immortalised T cell line (Figure 6.4b). To investigate why such differences in lysis were observed in such related peptide toxins, we analysed some of the structural parameters of the PSMs and highlighted the alpha helicity of the peptide through circular dichroism measurements as the single most important factor involved in vesicle, and by extension, cell lysis. Recently, we have shown that the percentage of cholesterol in the membrane plays a major role in the ability of PSMs to lyse vesicles (647). We hypothesise that cholesterol shifts the membrane form a liquid disordered to a liquid ordered state, making the lipid membrane more stable and less susceptible to PSM integration and permeablization. The results of these experiments may help to explain why PSMs lyse certain cells more efficiently than others, considering that no proteinaceous receptor is known to be involved.

RL were identified as the only *P. aeruginosa* factor lysing vesicles. Like δ-haemolysin and PSMs, this molecule has a surfactant-like property enabling the rapid solubilisation of membranes. In chapter 4 we highlight the development of a novel assay designed to measure RL directly from the supernatant. As with δ-haemolysin, we wanted to examine the percentage of *P. aeruginosa* clinical strains that expressed RL that was detected by our vesicles system. Interestingly, we show a significant difference in RL expression from strains derived from acute infections (wound and bloodstream) and chronic infections (cystic fibrosis patients) (Fig 4.5). This assay may be useful in quantifying RL expression from strains isolated from different infections to gain an understanding of the role of this virulence factor in specific diseases.

As described previously, two parameters are essential for a working vesicle signalling system: sensitivity and stability. The results reported in this thesis show that the vesicles are more sensitive to bacterial factors than eukaryotic cells. This suggests that in a wound environment if toxins are actively secreted and can diffuse to the signalling system then vesicles would lyse first and would signal infection before the onset of cellular and tissue destruction. These factors (δ-haemolysin/PSMs and RL) are also very important in biofilm formation and maintenance. Current unpublished work form our group has shown that our vesicles also respond to microbial biofilm growth, important as biofilms have been recently implicated in burn infection (102-104). In chapter 5 we demonstrate that the proposed repressive role of toxic shock syndrome toxin-1 (TSST-1) on global exoprotein expression not only has no impact on vesicle lysis but does not appear to down-regulated toxin production. Considering that TSST-1 has been implicated in rapid mortality in young burn victims, our *in vitro* experiments show that TSST-1 expression occurs at the same time or after the expression of toxins suggesting that if this is reflected in a burn wound, vesicles will lyse and respond before or at the same time as TSST-1 is expressed. As a group we have investigated the impact of different environmental conditions on the stability of vesicles. Various temperature, pH and stability in human serum and in the presence of growing skin cells (HaCATs) has been examined extensively with high levels of stability shown (165, 456).

However, they are also limitations associated with this vesicle signalling system which require attention. Although toxicity contributes to pathogenesis, the results shown in chapter 7 highlight that strains within certain *S. aureus* lineages have low or no toxin production. Here we show that 70% of a set of related clinical ST239 strains were shown by the vesicle lysis assay as non-toxic and would not signal infection in a burn wound in which a vesicle-based smart dressing was applied. Future work requires analysing the toxicity associated with burn wounds isolates. It would be interesting to further determine the epidemiology of burn wound isolates, whether a specific *S. aureus* lineage is dominant in burn wounds, to compare the toxicity of strains isolated from burn wounds and other diseases (abscess/bacteraemia/SSTI) and to what extent strains isolated from burn wounds are

MSSA, MSSA, HA- or CA-MRSA. Other burn wound colonisers such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* do not express vesicle lysing agents (data not shown) and therefore would not signal infection in a burn wound using this smart wound dressing, highlighting another limitation. However, in spite of this, vesicle signalling infection of the most common burn wound colonisers is still an advantage over current systems in use. Presently we do not know the diffusion constant of these toxins through a hydrogel; an important consideration as these vesicles will be immobilised within a hydrogel for improved stability and shelf-life. Preliminary *in vitro* experiments show switch on of vesicles within this hydrogel triggered using bacterial supernatant however how this will act within a wound is currently unknown.

Although vesicles are stabile in human serum, their stability in a sterile burn wound environment is unknown. Phospholipases A_2 (PLA_2) molecules are central in inducing inflammation as they lead to the degradation of phospholipids important in stimulating the formation of eicosanoid and related bioactive lipid mediators (648). (PLA_2) attacks the *sn-2* position of phospholipids, including those present in our vesicles. The concentration of (PLA_2) required to lyse vesicles used in this study and that present in a burn wound needs to be measured to investigate whether in a non-infected wound, vesicles will remain stable. In summary, the primary objective of identifying the vesicle lysing agents of *S. aureus* and *P. aeruginosa* has been achieved. An extension of this work saw the development of two novel, highly sensitive assays for measuring *agr* and RL activity (chapters 3 and 4). We also question the validity of the proposed TSST-1 mediated global exoprotein repression as discussed by Vojtov *et al* (457). However there is still much work required to evaluate the role smart wound dressing may play in the future of burn wound management.

In chapter 6 we investigated the use of sub-inhibitory concentrations of oxacillin as a means of anti-virulence treatment in CA-MRSA strains. Previous work in the Massey lab demonstrated that over-expression of the *mecA* gene, encoding the alternative penicillin binding protein PBP2a, decreased toxicity in HA-MRSA

strains. CA-MRSA strains are considered highly toxic but have considerably lower PBP2a expression. Therefore, treatment of CA-MRSA stains with sub-inhibitory oxacillin was hypothesised to induce higher levels of PBP2a expression with the added effect of decreasing toxicity. In this chapter we demonstrate that oxacillin treatment does in fact interfere with *agr* signalling resulting in altered toxin expression in CA-MRSA strains. As *S. aureus* expresses many different cytolytic toxins we assessed the impact of oxacillin treatment on three cell types. Our results demonstrate that oxacillin treatment leads to a decrease in PSM expression but also an increase in PVL expression. Therefore, where PSMs are important in cell lysis, such as with T cells and erythrocytes overall cell lysis is reduced, however where PVL is important such as lysing PMNs, overall cell lysis is increased with respect to oxacillin treatment. The impact on protein A and bacterial adherence to extracellular matrix proteins and invasion of endothelial cells following oxacillin treatment was also assessed. We show that oxacillin treatment results in a decrease in protein A expression but causes increased binding of the USA300 strain LAC to Fb in response to sub-inhibitory oxacillin treatment. No difference in the invasiveness of CA-MRSA strain was observed.

The results presented demonstrate that sub-inhibitory oxacillin modulates global virulence regulation, down regulating Agr and associated *agr*-dependent virulence factors but up-regulating other cytolytic toxins such as PVL. RNAseq analysis should help unravel how the complex regulatory network involved in *S. aureus* toxicity responds to sub-inhibitory antibiotics. Future work in analysing the impact of other cell wall antibiotics which may mediate PBP2a-dependent *agr* repression is ongoing and may facilitate our initial application of using sub-inhibitory antibiotics for virulence attenuation. The mechanism of how overexpression of PBP2a leads to reduced *agr*-specific toxins such as δ-haemolysin and PSMs is unknown. Previous work highlighted that removal of the cell wall by-passed this repression (238). This suggests that PBP2a expression leads to changes in the architecture of the cell wall which inhibits *agr* signalling and/or response. It has been shown before that the methicillin resistance strains produce abnormal peptidogylcan when grown in the presence of β-lactam antibiotics (649). It is possible that subtle

changes in the cell wall as a result of PBP2a expression may lead to a charge difference preventing the AIP from diffusing through the cell wall to reach AgrC. The identification of novel chemically distinct peptide cross-bridges present in MRSA cell walls and the role of the other PBPs may highlight the mechanism of PBP2a mediated *agr* repression.

Keeping with the theme of *S. aureus* toxicity, in chapter 7 we investigate the potential of using the genomes of 90 closely related ST239 strains to identify genetic polymorphisms associated with variation in toxicity, as measured by the VLT and T cell assay. Here we evaluate two methods to identify specific single nucleotide polymorphisms (SNPs) and insertion/deletions (InDels) present in the core genomes that are associated with either an increase or decrease in toxicity. The genome wide association study (GWAS) is a robust method, requiring only an association between polymorphism and phenotype and therefore is likely to result in false positives. The second approach is much more stringent and requires the repeated independent evolution of the polymorphism to be associated toxicity variation. Although the GWAS approach results in false positives the stringent approach failed to identify a SNP known to affect toxicity (SNP identified in AgrC, verified to reduce *agr* activity) and thus produce a higher false negative rate.

Using the GWAS approach we sought to functional verify some of the genetic polymorphisms associated with a decrease in toxicity. Interestingly, we identified and verified four novel genetic loci, which, when mutated through transposons insertion, resulted in a drastic and significant decrease in toxicity. This suggested that there is an added layer of virulence regulation yet to be defined. However, inactivation of the gene does not tell us if the SNP in question caused the predicted decrease in toxicity. Future work is in place to complement mutant strains with wild type and SNP containing gene to fully understand the role of individual SNPs in toxicity variation. The elucidation of the impact the specific SNP plays in modulating toxicity is important in investigating or predicting the virulence potential of a strain directly from its genome sequence. This prediction approach has been

employed by our group and has been shown to be an effective model with the toxicity of the majority of isolates predicted correctly.

In this chapter we show that the GWAS approach is a good method in identifying SNPs responsible for variation in toxicity. In addition to using the Turkish collection of ST239 strains, we have also investigated the toxicity of other ST239 strains from other geographical regions. Preliminary experiments illustrate a large difference in the toxicities of strains derived from the Turkish collection and global collection of ST239 clinical isolates (Figure 8.1). The box plot in figure 8.1 shows that the majority of isolates from the global collection are highly lytic, whereas the opposite is true for the Turkish collection. The line within the box represents the median value, again highlighting the vast difference in toxicity between these related groups within this *S. aureus* lineage.
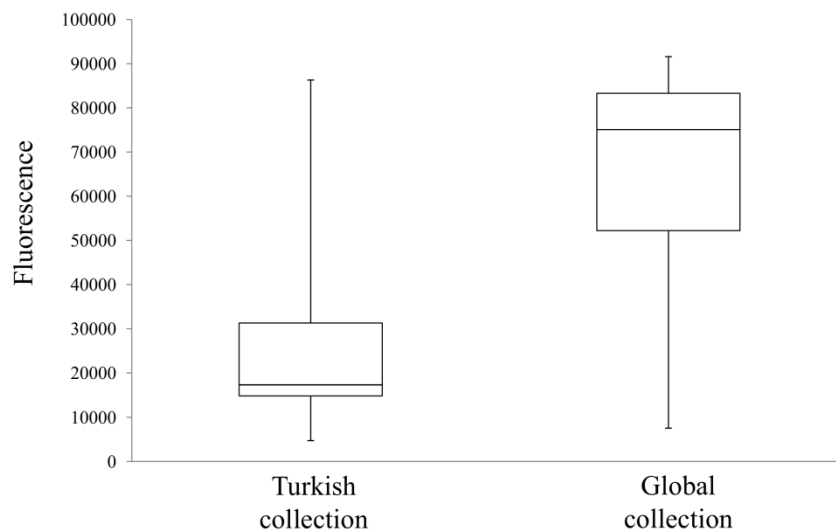
Figure 8.1: Differences in vesicle lysis from strains derived from the Turkish and global collection of ST239 strains. The box plot represents the differences in the lytic activity defined by fluorescence release from vesicles.

Both groups of ST239 strains have been isolated from the hospital environment. The degree of antibiotic resistance, particularly to beta-lactam antibiotics, between the two ST239 groups may determine the observed toxicity as resistance to beta-lactams conferred by the *mecA* gene has been shown to influence toxicity in HA-MRSA

containing the type II SCC*mec* element (238). Differences in resistance to antimicrobial peptides, which has been linked to thicker cell walls (650), may also interfere with *agr* signalling and affect overall toxin output. Another possibility which may explain the large deviation in toxicity profiles from the two ST239 groups may lie within the sequencing technology used. One of the limitations of the genomic data used in these studies is that DNA not found in the reference genome (TW20) is ignored. Therefore, ST239 strains form the Global collection may possess mobile genetic elements which express novel genes conferring the observed highly toxic phenotype. The improvement of sequencing technology and *de novo* assembly of genome sequences will report on the entire sequence of the strains highlighting all genetic differences between strains.

This thesis represents an interdisciplinary approach to investigating bacterial virulence in areas of detection, identification and prevention. Work in this thesis has assisted collaborative work in developing an active wound dressing for burn wound management. In areas of prevention we address the use of sub-inhibitory oxacillin to reduced overall toxicity, highlighting mixed results. Finally, we develop a novel method to identify genetic polymorphism associated with, and verify four novel loci affecting, toxicity. These results have future implications in areas of elucidating novel virulence regulatory circuits and in developing predictive models for assessing virulence from the genomes of *S. aureus*.

Appendix A: Strain Collection

Chapters 3 and 6:

Strain/peptide/primer	Description	Reference
RN6390B	NCTC8325 cured of three prophages	(331)
RN6911	RN6390B Δ*agr*::tetM	(335)
8325-4	Lab strain NCTC8325 cured of three prophages	(651)
DU1090	8325-4 Δ*hla*::Em^r	(652)
DU5719	β- haemolysin - ve Phage 42E lysogen of 8325-4	(652)
LAC (USA300)	Community acquired MRSA strain	(305)
LAC Δ*hld*	LAC *hld* deletion mutant; *hld* start codon changed from ATG to ATT	(317)
MW2 (USA400)	Community acquired MRSA strain	(305)
MW2 Δ*pvl*	MW2 Δ*lukF/S-PV*::spcm	(305)
MSSA 476	Community-acquired invasive MSSA strain	(427)
RN4220	Restriction negative derivative of 8325-4	(462)
Newman	MSSA lab strain	(437)
NewmanΔ*lukAB*	Constructed using pKOR-1 plasmid	(437)
NewmanΔ*lukDE*	Constructed using pKOR-1 plasmid	(437)
NewmanΔ*hlg*	NewmanΔ*hlg*::tetM	(653)

Purified toxins		
Δ-haemolysin	fMAQDIISTIGDLVKWIIDTVNKFTKK	
PSMα1	fMGIIAGIIKVIKSLIEQFTGK	
PSMα2	fMGIIAGIIKFIKGLIEKFTGK	
PSMα3	fMEFVAKLFKFFKDLLGKFLGNN	
PSMα4	fMAIVGTIIKIIKAIIDIFAK	
PSMβ1	fMEGLFNAIKDTVTAAINNDGAKLGTSIVSIVENGVGLLGKLFGF	
PSMβ2	fMTGLAEAIANTVQAAQQHDSVKLGTSIVDIVANGVGLLGKLFGF	

Primers		
*gyr*B Forward	CCAGGTAAATTAGCCGATTGC	
*gyr*B Reverse	AAATCGCCTGCGTTCTAGAG	
RNA III Forward	GAAGGAGTGATTTCAATGGCACAAG	
RNA III Reverse	GAAAGTAATTAATTATTCATCTTATTTTTAGTGAATTTG	

Chapter 4:

Strain	Description	Reference
PAO1	Nottingham collection wild type *P. aeruginosa* strain	(654)
PAOlΔ*rhlR*	*rlhlR* mutant of PAO1	(655)
PAOlΔ*rhlI*	PAO1 derivative with a Tc cassette insertion into the *rhlI* gene, Tc[r]	(656)
PAOlΔ*lasR*	PAO1 mutant with a Gm cassette insertion into the *lasR* gene, Gm[r]	(657)
PAOlΔ*lasI*	PAO1 mutant with a Gm cassette insertion into the *lasI* gene, Gm[r]	(656)
PAOlΔ*plcH*	*plcH* mutant of PAO1	(658)
PAOlΔ*pqsA*	*pqsA* mutant of PAO1	(659)
PAO1Δ*pqsE*	*pqsE* mutant of PAO1	(79)
PAO1Δ*pqsH*	*pqsH* mutant of PAO1	(79)
PAO1Δ*rhlA*	*rhlA* mutant of PAO1	(660)
Chronic isolates	n=48 isolated from patients with cystic fibrosis	Gift from M.C. Enright
Acute isolates	n=30 isolated from patients with acute injury	Gift from Southmead Hospital, Bristol

Chapter 5:

Strain	Description	Reference
RN6390B	NCTC8325 cured of three phages	(331)
RN6911	RN6390BΔ*agr::tetM*	(335)
RN4282	Wild type TSST-1 producing strain	(462)
RN6938	RN4282Δ*tst::tetM*	(661)
MSSA 101	Invasive CA-MRSA strain *tst+*	(427)
MSSA 253	Invasive CA-MRSA strain *tst+*	(427)
MSSA 279	Invasive CA-MRSA strain *tst+*	(427)

Chapter 7:

Strain/primer/plasmid	Description	Reference
Strain		
RN4220	Restriction negative derivative of 8325-4	(462)
RN6390B	NCTC8325 cured of three prophages	(331)
ALC1927	RN6390Δ*sarS*::*ermC*	(372)
ALCML1	ALC1927 with pRMC2ML1	This work
ALCML2	ALC1927 with pRMC2ML2	This work
BH1CC	Clinical isolate, type II MRSA CC8	(414)
BH1CCΔScc*mec*	SCC*mec* excised using plasmid pSR$_2$	(581)
ML121	BH1ccΔScc*mec* complemented with pRMC2	This work
ML122	BH1ccΔScc*mec* complemented with p*mec*A1	This work
ML123	BH1ccΔScc*mec* complemented with p*mec*A2	This work
ML124	BH1ccΔScc*mec* complemented with p*mec*A3	This work
ML125	BH1ccΔScc*mec* complemented with p*mec*A4	This work
FPR3757	Wild type USA300 isolate	(640)
AgrB-	FPR3757 Tn::*agr*B	(640)
95A07	FPR3757 Tn::298351	(640)
93B09	FPR3757 Tn::690868	(640)
82B04	FPR3757 Tn::717257	(640)
180A03	FPR3757 Tn::728946	(640)
207A03	FPR3757 Tn::1050930	(640)
90D01	FPR3757 Tn::1429479	(640)
137C12	FPR3757 Tn::1907464	(640)
45D06	FPR3757 Tn::2003286	(640)
179A03	FPR3757 Tn::2087951	(640)
108B09	FPR3757 Tn::2380097	(640)
184F11	FPR3757 Tn::2379899	(640)
86C03	FPR3757 Tn::2487759	(640)
168A05	FPR3757 Tn::2504661	(640)
72A04	FPR3757 Tn::2601159	(640)
Plasmid		
pRMC2	Plasmid containing a tetracycline-inducible promoter	(461)
pRMC2ML1	pRMC2 containing *sarS* amplified from strain N315	This work
pRMC2ML2	pRMC2 containing *sarS* amplified from strain 252	This work
p*mec*A1	pRMC2 containing *mecA* amplified from strain TW20	This work
p*mec*A2	pRMC2 containing *mecA*1 harbouring SNPs 78097 and 78222	This work
p*mec*A3	pRMC2 containing *mecA*2 harbouring SNP78396	This work

pmecA4	pRMC2 containing *mecA*3 harbouring SNP78119		This work
Primer			
sarS Fw	GGC<u>GGTACC</u>GGAGATAGAACAATG		This work
sarS Rv	CCG<u>AGATCT</u>GAATACAGCACACTTGCG		This work
mecA Fw	TTT<u>GGTACC</u>AGTCTTATATAAGGAGGA TATTGATG		(238)
mecA Rv	AAA<u>GAGCTC</u>CGTTATCGCTGAAATTAC		(238)

ST239 Strains

Isolate	Hospital	Infectious source	Reference
DEU1	Dokuz Eylul University, Turkey.	Abscess	(180)
DEU2	Dokuz Eylul University, Turkey.	Aspiration	(180)
DEU3	Dokuz Eylul University, Turkey.	Blood	(180)
DEU4	Dokuz Eylul University, Turkey.	Blood	(180)
DEU5	Dokuz Eylul University, Turkey.	Wound	(180)
DEU6	Dokuz Eylul University, Turkey.	Wound	(180)
DEU7	Dokuz Eylul University, Turkey.	Blood	(180)
DEU8	Dokuz Eylul University, Turkey.	Wound	(180)
DEU9	Dokuz Eylul University, Turkey.	Aspiration	(180)
DEU10	Dokuz Eylul University, Turkey.	Catheter	(180)
DEU11	Dokuz Eylul University, Turkey.	Blood	(180)
DEU12	Dokuz Eylul University, Turkey.	Sputum	(180)
DEU14	Dokuz Eylul University, Turkey.	Bronchoalveoler lavage	(180)
DEU15	Dokuz Eylul University, Turkey.	Aspiration	(180)
DEU16	Dokuz Eylul University, Turkey.	Blood	(180)
DEU17	Dokuz Eylul University, Turkey.	Blood	(180)
DEU19	Dokuz Eylul University, Turkey.	Peritoneum fluid	(180)
DEU20	Dokuz Eylul University, Turkey.	Blood	(180)
DEU22	Dokuz Eylul University, Turkey.	Wound	(180)
DEU23	Dokuz Eylul University, Turkey.	Urine	(180)
DEU25	Dokuz Eylul University, Turkey.	Blood	(180)
DEU27	Dokuz Eylul University, Turkey.	Catheter	(180)
DEU28	Dokuz Eylul University, Turkey.	Blood	(180)
DEU29	Dokuz Eylul University, Turkey.	Blood	(180)
DEU30	Dokuz Eylul University, Turkey.	Wound	(180)
DEU35	Dokuz Eylul University, Turkey.	Tracheal secretion	(180)
DEU36	Dokuz Eylul University, Turkey.	Plevral fluid	(180)
DEU37	Dokuz Eylul University, Turkey.	Blood	(180)
DEU38	Dokuz Eylul University, Turkey.	Aspiration	(180)
DEU39	Dokuz Eylul University, Turkey.	Catheter	(180)
DEU40	Dokuz Eylul University, Turkey.	Wound	(180)
DEU41	Dokuz Eylul University, Turkey.	Blood	(180)

DEU42	Dokuz Eylul University, Turkey.	Blood	(180)
DEU43	Dokuz Eylul University, Turkey.	Blood	(180)
DEU46	Dokuz Eylul University, Turkey.	Blood	(180)
DEU47	Dokuz Eylul University, Turkey.	Wound	(180)
DEU49	Dokuz Eylul University, Turkey.	Blood	(180)
DEU50	Dokuz Eylul University, Turkey.	Tracheal secretion	(180)
HU2	Hacettepe University, Turkey.	Blood	(180)
HU4	Hacettepe University, Turkey.	Tracheal secretion	(180)
HU5	Hacettepe University, Turkey.	Catheter	(180)
HU6	Hacettepe University, Turkey.	Sputum	(180)
HU7	Hacettepe University, Turkey.	Abscess	(180)
HU8	Hacettepe University, Turkey.	Abscess	(180)
HU9	Hacettepe University, Turkey.	Liver cyst	(180)
HU10	Hacettepe University, Turkey.	Catheter	(180)
HU11	Hacettepe University, Turkey.	Blood	(180)
HU12	Hacettepe University, Turkey.	Catheter	(180)
HU13	Hacettepe University, Turkey.	Brain abscess	(180)
HU14	Hacettepe University, Turkey.	Catheter	(180)
HU15	Hacettepe University, Turkey.	Catheter	(180)
HU16	Hacettepe University, Turkey.	Spinal fluid	(180)
HU17	Hacettepe University, Turkey.	Brain abscess	(180)
HU18	Hacettepe University, Turkey.	Catheter	(180)
HU19	Hacettepe University, Turkey.	Tracheal secretion	(180)
HU21	Hacettepe University, Turkey.	Spinal fluid	(180)
HU23	Hacettepe University, Turkey.	Spinal fluid	(180)
HU24	Hacettepe University, Turkey.	Blood	(180)
HU25	Hacettepe University, Turkey.	Abscess	(180)
HU26	Hacettepe University, Turkey.	Blood	(180)
IU1	Istanbul University, Turkey.	Blood	(180)
IU2	Istanbul University, Turkey.	Blood	(180)
IU3	Istanbul University, Turkey.	Blood	(180)
IU4	Istanbul University, Turkey.	Sputum	(180)
IU6	Istanbul University, Turkey.	Blood	(180)
IU7	Istanbul University, Turkey.	Catheter	(180)
IU8	Istanbul University, Turkey.	Blood	(180)
IU9	Istanbul University, Turkey.	Nasal swab	(180)
IU10	Istanbul University, Turkey.	Blood	(180)
IU11	Istanbul University, Turkey.	Blood	(180)
IU12	Istanbul University, Turkey.	Abscess	(180)
IU13	Istanbul University, Turkey.	Abscess	(180)
IU14	Istanbul University, Turkey.	Sputum	(180)

IU15	Istanbul University, Turkey.	Transtracheal asp. fluid	(180)
IU16	Istanbul University, Turkey.	No info.	(180)
IU17	Istanbul University, Turkey.	No info.	(180)
IU18	Istanbul University, Turkey.	No info.	(180)
IU19	Istanbul University, Turkey.	No info.	(180)
IU20	Istanbul University, Turkey.	No info.	(180)
MU1	Marmara University, Turkey.	Blood	(180)
MU2	Marmara University, Turkey.	Blood	(180)
MU3	Marmara University, Turkey.	Wound	(180)
MU4	Marmara University, Turkey.	Abscess	(180)
MU5	Marmara University, Turkey.	Wound	(180)
MU6	Marmara University, Turkey.	Blood	(180)
MU7	Marmara University, Turkey.	Blood	(180)
MU9	Marmara University, Turkey.	Wound	(180)
MU11	Marmara University, Turkey.	Blood	(180)
MU19	Marmara University, Turkey.	Blood	(180)
MU20	Marmara University, Turkey.	Blood	(180)

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