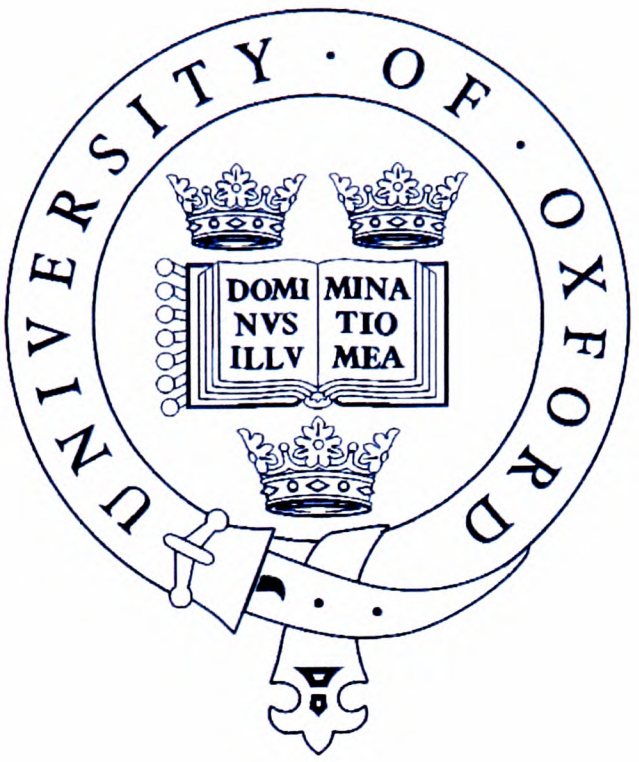

Staphylococcus aureus Toxins: Expression and Control.

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Abstract

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Staphylococcus aureus Toxins: Expression and Control.

Staphylococcus aureus was discovered in 1880 by the Scottish surgeon Sir Alexander Ogston. Its ability to utilise a panoply of secreted and cell associated virulence factors, combined with an ability to rapidly adapt to antibiotic selective pressure, has led it to become one of the most important Gram positive pathogens of our time. It plays a significant role in chronic diseases such as eczema, as well as the acute and potentially life threatening conditions toxic shock syndrome (TSS), meningitis. pneumonia and staphylococcal scalded skin syndrome (SSSS) to name just a few. Its presence in hospitals is endemic in many countries, where methicillin resistant *S. aureus* (MRSA) strains have earned the 'superbug' moniker.

Using a large, well-defined, clinical strain collection in combination with isogenic lab strains, this study endeavored to characterise the genetic factors associated with *S. aureus* toxicity, and identify novel means of controlling toxin expression.

We found that:

- A broad range of toxicities exists among *S. aureus* strains, that the beta- and delta-haemolysins are involved, and that hospital acquired-MRSA (HA-MRSA) strains are less toxic than MSSA or community acquired-MRSA (CA-MRSA).
- NaCl and the emollient Oilatum downregulate toxin expression.
- The *mecA* gene, conferring methicillin resistance, mediates mutation rates.
- The large type II SCC*mec* element of HA-MRSA confers a significant metabolic burden which is mitigated by the down regulation of toxins.
- The smaller type IV SCC*mec* element of CA-MRSA does not confer a significant metabolic burden, enabling CA-MRSA to utilse their full arsenal of virulence factors.

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"I soon found that there were two forms of micrococcus: one in the form of chains or necklaces to which the name 'streptococcus' had been given — I do not know by whom, which produced the more violent inflammation, and the other growing in masses or clusters, to which I gave the name 'staphylococcus' which cause a less violent inflammatory disease."

Sir Alexander Ogston (1844–1929).

1 Introduction.

This thesis utilises a diverse collection of clinical isolates and isogenic laboratory strains to determine patterns and associations between genotypes and phenotypes of *Staphylococcus aureus*. The use of a large number of clinical isolates enables the identification of factors that are likely to be clinically relevant from the noisy background found in natural samples (reducing the number of findings that may be highly significant *in vitro*, but play a reduced role *in natura*); isogenic laboratory strains are then used to confirm these findings.

Staphylococcus aureus is a well studied organism, this introduction provides an up to date review of its biology and interaction with human hosts, with a focus on those areas relevant to subsequent chapters.

1.1 *Staphylococcus aureus.*

Staphylococcus aureus (*S. aureus*) is a facultatively anaerobic, non-motile, Gram positive cocci and a common human commensal. It often has a golden

colour (*aureus* translates as golden) caused by the carotenoid pigment staphyloxanthin which is thought to function as an antioxidant (Clauditz *et al.*, 2006). When viewed under a microscope *S. aureus* appears to form clusters, much like bunches of grapes, due to the cells dividing in two planes (Prescott *et al.*, 1999). *S. aureus* is able to grow in a wide variety of environments, with a temperature range of 6.5–46 °C, pH 4.2–9.3 and a sodium chloride solution of up to 3 molar (Prescott *et al.*, 1999).

S.aureus predominantly lives without causing any damage as a commensal in the nares (nasal cavity) and on the skin. Up to 20% of the adult population can be described as persistent carriers with a further 60% being intermittent carriers (Peacock *et al.*, 2001). As well as humans, *S. aureus* colonises livestock such as cows, pigs, sheep and chickens (De Oliveira *et al.*, 2000; De Neeling *et al.*, 2007; Goni *et al.*, 2004; Witte *et al.*, 1977); and companion animals, particularly cats and dogs, but also horses and rabbits (Bender *et al.*, 2005; Pak *et al.*, 1999; Cuny *et al.*, 2006; Goni *et al.*, 2004). These animals provide a potential reservoir for human infection, indeed where isolates have been taken from companion animals and their owners they have been shown to be genetically indistinguishable (Baptiste *et al.*, 2005).

1.2 Disease.

One of the main reasons for the high interest in studying *S. aureus* is its role in disease. *S. aureus* carriage is non-problematic in the majority of hosts, where it lives as a commensal on the skin and nares. It is however, an opportunistic pathogen capable of causing infections at many different sites (Figure 1.1 on page 4) and frequently causing infections in patients with underlying disease, surgical patients, those with prosthetic devices, individuals undergoing dialysis,

and patients with diabetes (Petti *et al.*, 2001; Diekema *et al.*, 2001). *S. aureus* is responsible for approximately 32–47% of all skin/soft tissue infections and is the leading cause of infection of the bloodstream and lower respiratory tract (as identified by the worldwide SENTRY Surveillance Program, Jan 1997–Dec 1999) (Diekema *et al.*, 2001).

S. aureus produces a broad arsenal of cell associated and secreted virulence factors (Table 1.1 on page 6) capable of causing disease and exacerbating the symptoms of non-staphylococcal disease. These factors are not all present in all strains and as such different strains show varying levels of virulence. For the majority of Staphylococcal diseases including: Bacteremia (blood), pneumonia (lungs), rhinosinusitis (Paranasal sinuses), mastitis (mammary gland), phlebitis (veins), meningitis (brain & spinal cord), urinary tract infections, osteomyelitis (bone) and endocarditis (heart valves), a combination of virulence factors are thought to be involved (Lowy, 1998a). There are also a number of diseases where individual factors can be singled out as the predominant cause.

1.2.1 Toxic Shock Syndrome.

Toxic Shock Syndrome (TSS) is an acute and potentially fatal illness caused by the super antigen 'Toxic Shock Syndrome Toxin-1' (TSST-1). TSST-1 is a 22 kDa single chain protein with the unique ability amongst staphylococcal enterotoxins to cross the mucosa (Clements *et al.*, 2000). Once in the body TSST-1 is capable of forming a bridge between macrophages and T-cells. This is achieved by binding the major histocompatibility complex class 2 molecules (MHC-II) of macrophages with the T-cell receptors (TCR) on $CD4^+$ T-cells. Unlike the specific binding that normally occurs, TSST-1 binds outside of the peptide binding groove (Figure 1.2 on page 5) (Kim *et al.*, 1994), inducing T-cell

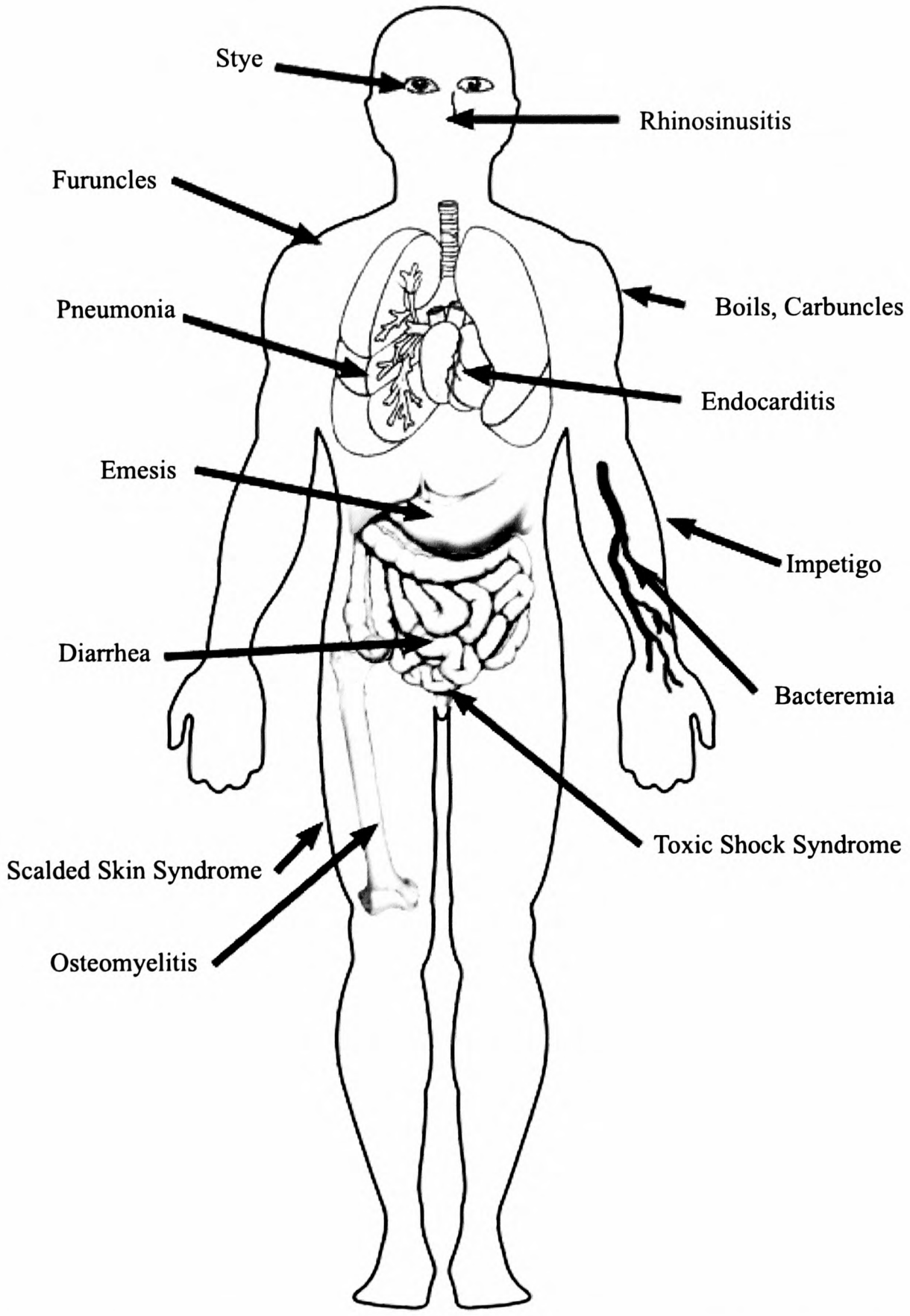

Figure 1.1: **Sites of *S. aureus* Infection**. *S. aureus* is an opportunistic pathogen that can cause infection in multiple sites.

proliferation and a corresponding release of large amounts of host cytokines. The cytokine release includes, though is not limited to, interferon-γ, IL-2, and TNF-β from T-cells, and IL-1 and TNF-α from macrophages (McCormick *et al.*, 2001). An inflammatory cascade is induced resulting in capillary leak syndrome, hypotension (low blood pressure), hypoalbuminemia (low levels of blood albumin), generalised nonpitting edema (excessive accumulation of fluid in tissue), vasodilation, disseminated intravascular coagulation (blood clotting throughout body), myocardial suppression, renal failure, acute respiratory distress syndrome and multiple organ failure (McCormick *et al.*, 2001; Dinarello, 2000).

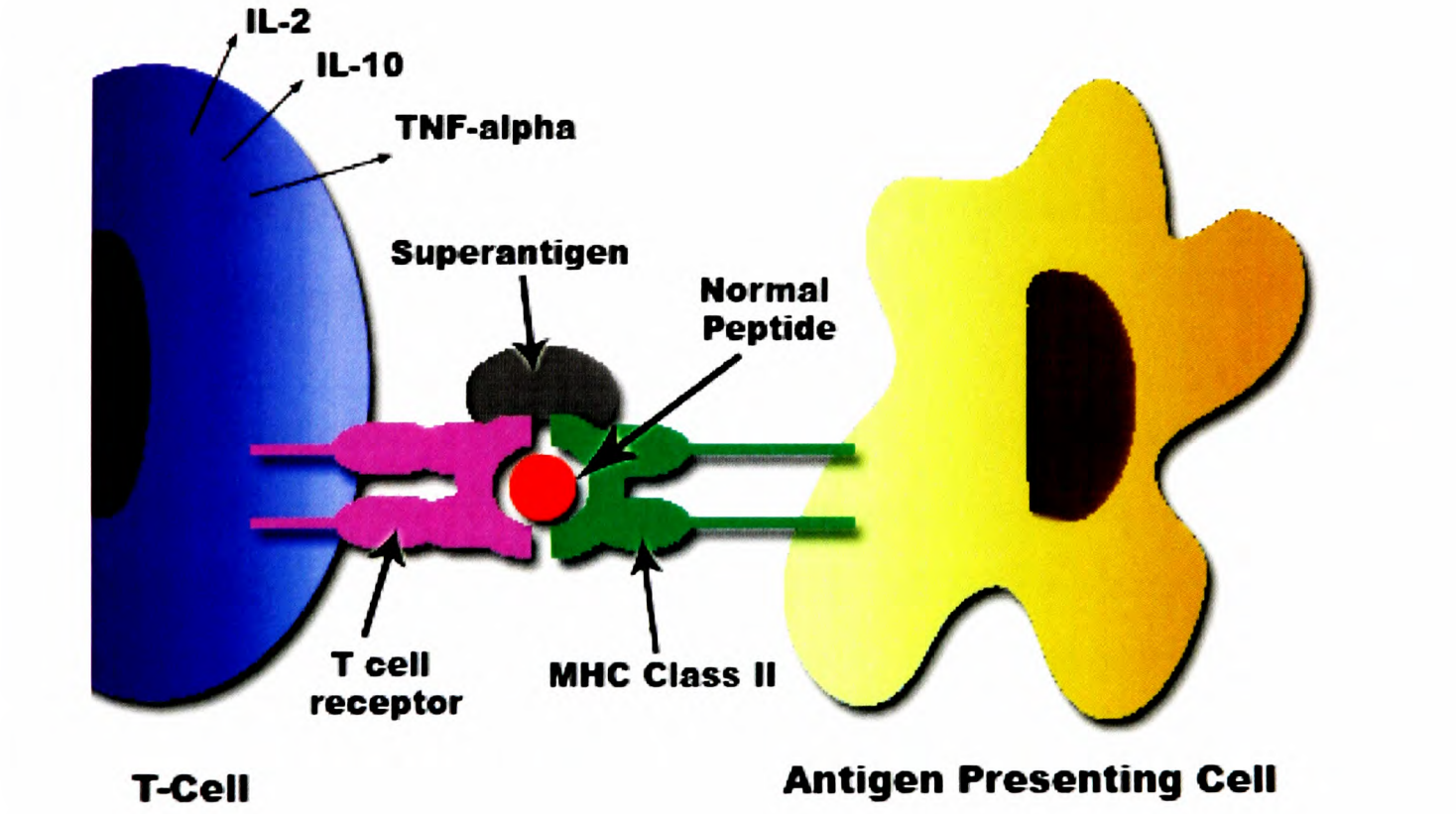

Figure 1.2: **Superantigen activation of T cells.** Superantigens such as the Toxic Shock Syndrome Toxin-1 (TSST-1) bind the major histocompatibility complex class 2 molecules (MHC-II) of macrophages on the outside of the peptide binding groove and T-cell receptors (TCR) on $CD4^+$ T-cells. This binding induces T-cell proliferation and a massive pro-inflammatory cytokine release.

Secreted factors	Cell-associated factors
Superantigens:	Protein A
Toxic shock syndrome toxin-1	Capsular Polysaccharide
Enterotoxins (A to E, G to Q)	Polysaccharide intercellular adhesin
Exfoliative toxins (A–D)	Peptidoglycan
Cytotoxins:	**Adhesins for:**
α-toxin	Fibronectin
β-toxin	Fibrinogen
δ-toxin	Collagen
γ-toxin	Elastin
P-V Leukocidin	Vitronectin
Staphylococcal exotoxin-like proteins	von Willebrand factor
Enzymes:	Bone Sialoprotein
Coagulase	Thrombospondin
Ironate lyase	Lactoferrin
Lipases	Plasmin
Proteases	
Staphylokinase	
Epidermal cell differentiation inhibitors (A–C)	

Table 1.1: **Virulence factors associated with *S. aureus***

1.2.2 Staphylococcal Scalded Skin Syndrome & Bullous Impetigo.

Staphylococcal Scalded Skin Syndrome (SSSS) and bullous impetigo are characterised by red and blistering skin. The diseases mainly occur in young children although adults can also be affected (Elias *et al.*, 1977). The toxins involved are the epidermolytic toxins (ET's) of which *S. aureus* has three serological forms: ETA, ETB and ETD. ETA and ETB are both linked to SSSS in humans with ETB being more frequently isolated in children (Ladhani, 2003). The ET's are serine proteases that cleave desmoglein (part of the cadherin family that join cells together) between the third and fourth extracellular domains (Iwatsuki *et al.*, 2006). This results in a separation of the layers of the epidermis and widespread blistering. In patients with bullous impetigo the toxin produces blisters locally at the site of infection whereas in cases of the scalded-skin

syndrome it circulates throughout the body, causing blisters at sites distant from the infection (Ladhani, 2001). When contracted by children the risk of fatality is relatively low (<5%), among adults the syndrome often occurs in those who have renal disease or who are immunosuppressed, and the subsequent risk of death can be as high as 60% (Cribier *et al.*, 1994).

1.2.3 Furuncles.

Furuncles or boils are caused when hair follicles become infected. They may be caused by fungi or a number of bacterial species, though *S. aureus* is the main cause of infection. The toxin associated with furuncles is the two component cytotoxin Panton-Valentine leukocidin (PVL). PVL is made up of lukS-PV and lukF-PV and shows specific lytic activity against polymorphonuclear cells, monocytes and macrophages. Amongst clinical isolates as a whole PVL is uncommon, occurring <5% of the time (Yamasaki *et al.*, 2005); in studies examining furuncles however, PVL-positive strains account for between 40 & 93% of strains isolated (Durupt *et al.*, 2007; Lina *et al.*, 1999; Couppie *et al.*, 1994). PVL-positive strains have also been associated with cases of severe necrotising pneumonia in otherwise healthy children and young adults (Gillet *et al.*, 2002).

1.2.4 Atopic Dermatitis (Eczema).

Atopic dermatitis (AD) or eczema is a common, chronic, inflammatory skin disease, often linked with one or both parents having a history of AD, asthma or allergic rhinitis (Allen *et al.*, 2001). The International Study of Asthma and Allergies in Childhood (ISAAC) epidemiological research programme (established in 1991) examined data from over 700,000 children from 156

centers in 56 countries and concluded that cases of eczema are on the rise, especially among young children (Asher *et al.*, 2006). In the United States the prevalence approaches 17% in school-aged children (Boguniewicz *et al.*, 2006) and health insurance companies are thought to spend in the region of $0.9 to $3.8 billion per year, a figure similar to the expenditure on diseases such as emphysema, psoriasis, and epilepsy (Ellis *et al.*, 2002). A recent study reported that in England almost 1 in 9 people will be diagnosed with eczema at some point in their lives, making it one of the most common chronic conditions to effect the English population (Simpson *et al.*, 2009).

Patients with AD are highly vulnerable to infection with cutaneous viral, fungal, and bacterial species (Lübbe, 2003). Indeed any form of dermatitis leads to an increased susceptibility to infection. The most common infective agent of people with AD is *S. aureus*. Studies have found >90% of patients with AD are colonised or infected with *S. aureus* (Baker, 2006). This compares with just 5–30% of normal individuals (Breuer *et al.*, 2001).

The AD skin provides good conditions for *S. aureus* with colonisation densities reaching 10^7 colony-forming units cm^2 (Gong *et al.*, 2006). The bacterial density, as well as toxin production, is directly related to lesion severity (Tomi *et al.*, 2005; Guzik *et al.*, 2005). Once colonised with *S. aureus* the superantigens SEA, SEB and TSST-1 can cause an exacerbation of the inflammatory immune response via the polyclonal activation of superantigen-specific TCR Vβ families of T-cells (Figure 1.2 on page 5) (Baker, 2006). Besides the superantigens, α-haemolysin in AD skin has been shown to activate T-cells in the absence of antigen-presenting cells (Breuer *et al.*, 2005).

Oral treatment of AD with antibiotics has not been shown to be effective (Ewing *et al.*, 1998; Boguniewicz *et al.*, 2001). Topical treatment with antimicrobials and corticosteroids leads to an initial reduction in bacterial density

and improvements in skin lesions (Breuer *et al.*, 2002), though once treatment has stopped *S. aureus* is able to recolonise the skin. The use of antibiotics for long term treatment has obvious downfalls, prolonging selection pressure for antibiotic resistance. Fusidic acid is commonly used in topical preparations to treat AD. In a study by Shah & Mohanraj (2003) fusidic acid resistant strains were found on 78% of patients with AD compared to just 9.6% of samples cultured from non-dermatology patients.

The increasing prevalence of antibiotic resistance, contamination of topical treatments and the observation that nasal carriage of *S. aureus* is higher amongst patients with AD (39-82%) may account for the persistence of *S. aureus* colonization (Gilani *et al.*, 2005; Roth, 1987).

1.3 Evasion of Host Defences.

S. aureus has co-evolved with humans over a long period of time. It is capable of living asymptomatically within the moist squamous epithelium of the anterior nares and to a lesser extent the skin. Infections most commonly result from breaches in the hosts innate immunity, such as damage to mucosal and cutaneous membranes (Lowy, 1998b). If this opportunity arises, *S. aureus* is equipped with a panoply of tools for the evasion of the host immune system and the establishment of infection.

Upon breaching the first line of defence provided by the skin and mucosal membranes, a microbial invader is met with the innate immune system, comprising: inflammation, the complement system, and activated immune cells. To counter this, *S. aureus* has evolved ways of resisting phagocytosis, inhibiting neutrophil chemotaxis, resisting antimicrobial peptides, and killing host immune cells.

1.3.1 Protein A.

Protein A is a cell wall-bound 42 kDa protein containing four or five homologous immunoglobulin G (IgG) binding domains (Uhlen *et al.*, 1984). The binding domains bind to the Fc region of IgG molecules, coating the bacterium with IgG in the incorrect orientation for neutrophil recognition (Figure 1.4 on page 15). As well as binding IgG, Protein A has been shown to bind Von Willebrand factor and tumour-necrosis factor receptor 1 (Hartleib *et al.*, 2000; Gómez *et al.*, 2004). It is thought that this protects the bacterium from phagocytosis and therefore increases its virulence (Palmqvist *et al.*, 2002). Protein A is also capable of promoting biofilm development in the absence of exopolysaccharides, leading to the development of biofilm-associated infections (Merino *et al.*, 2008).

1.3.2 Clumping Factors A & B.

Clumping factors A & B (ClfA & ClfB) are two structurally related fibrinogen-binding MSCRAMMs — Microbial surface components recognizing adhesive matrix molecules (OBrien *et al.*, 2002). During exponential phase (*in vitro*) ClfB is predominantly expressed, giving way to ClfA during the stationary phase (Foster, 2005). ClfB plays an important role in normal colonisation of the nares, with strains deficient in ClfB being rapidly eliminated (Wertheim *et al.*, 2008). During infection bacterial cells become coated in fibrinogen (Figure 1.4 on page 15), protecting them from phagocytosis by macrophages & neutrophils and allowing them to remain virulent (Josefsson *et al.*, 2001; Palmqvist *et al.*, 2004).

1.3.3 Inactivation of Complement.

The complement system is a biochemical cascade comprising of more than thirty proteins. It helps to clear pathogens by directly killing foreign cells and by regulating effectors of the immune response. This is achieved via three pathways: Classical, Alternative and Lectin. The three pathways converge at one step: cleavage of the central complement protein, C3 (Figure 1.3 on the following page). C3 is composed of an α and β chain (110 and 75 kDa respectively) and is abundant in serum (Janssen *et al.*, 2005). Cleavage of C3 yields C3a, a 9 kDa chemo-attractant molecule, and C3b, a 176 kDa microbial surface attachment molecule that marks microbes for uptake by phagocytes (Rooijakkers & van Strijp, 2007).

In steady-state physiological conditions, activation of the complement system is strictly regulated by a series of 'regulator of complement activation' (RCA) proteins (Hammel *et al.*, 2007). Various pathogens use these RCA proteins by recruiting them to the bacterial cell surface and attenuating the complement cascade (Lindahl *et al.*, 2000). *S. aureus* is no exception and has evolved a number of strategies whereby it modulates this complement response.

***Staphylococcus* Complement Inhibitor (SCIN).**

SCIN, *Staphylococcus* complement inhibitor, is a 10 kDa excreted protein that blocks all complement pathways (Rooijakkers & van Strijp, 2007). This is achieved by binding to and stabilising C4bC2a and C3bBb which are present in the classical and lectin, and alternative pathways respectively. The function of C4bC2a and C3bBb is to cleave C3, forming C3a and C3b which covalently attach to the bacterium, promoting opsonization (the process of making cells more susceptible to phagocytosis). The presence of SCIN therefore inhibits

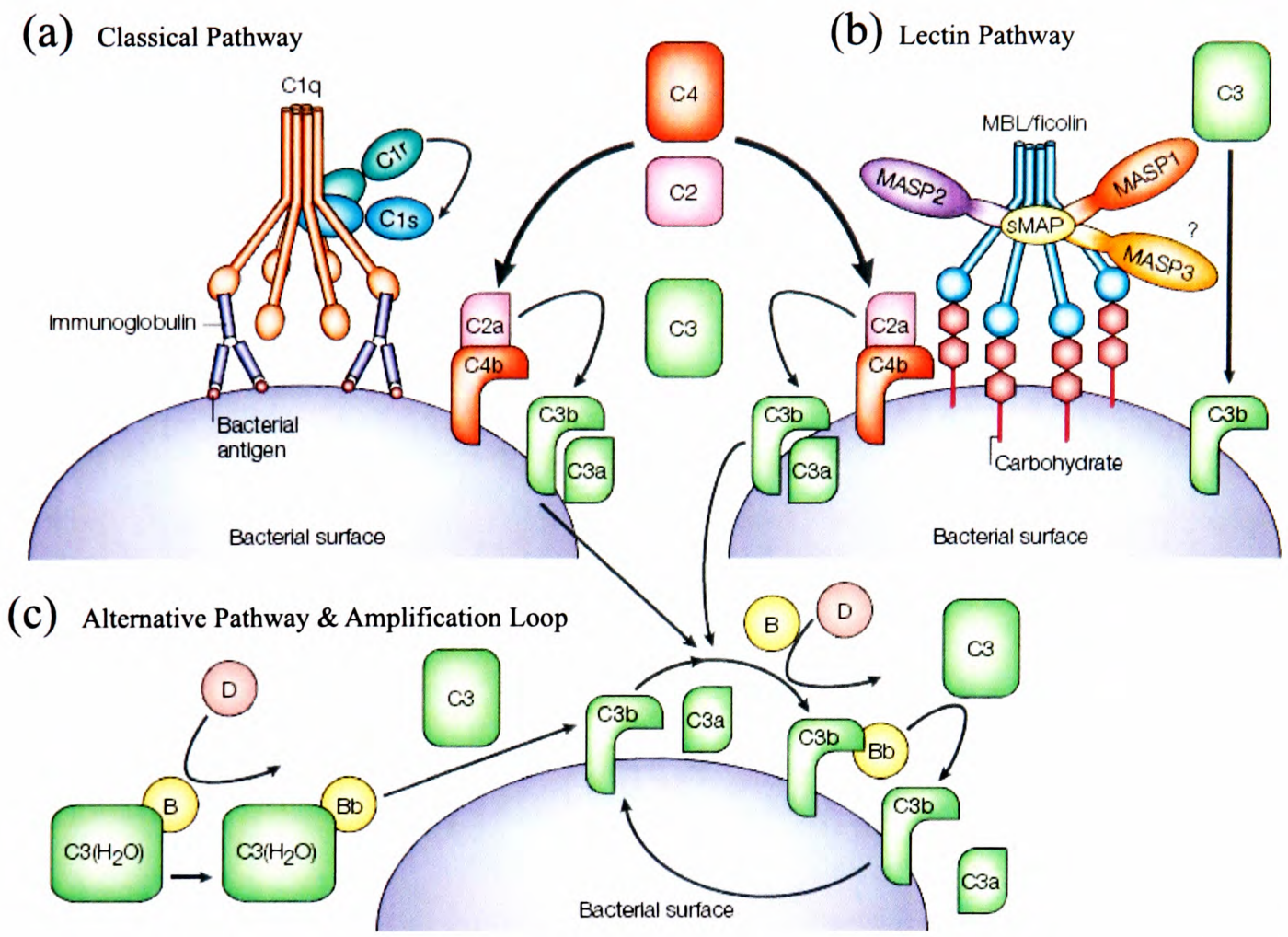

Figure 1.3: **Pathways for complement activation**. (**a**)The classical pathway is initiated by the binding of the C1 complex to antibodies that are bound to antigens on the surface of bacteria. The C1 complex consists of C1q and two molecules each of C1r and C1s. The binding of the recognition subcomponent C1q to the Fc portion of immunoglobulins results in autoactivation of the serine protease C1r. C1r then cleaves and activates C1s, which translates the activation of the C1 complex into complement activation through the cleavage of C4 and C2 to form a C4bC2a enzyme complex. C4bC2a acts as a C3 convertase and cleaves C3, which results in products that bind to and cause the destruction of invading bacteria. (**b**) The lectin pathway is initiated by the binding of either mannose-binding lectin (MBL) or ficolin associated with MBL-associated serine protease 1 (MASP1), MASP2, MASP3 and small MBL-associated protein (sMAP) to an array of carbohydrate groups on the surface of a bacterial cell. Similar to C1s, MASP2 is responsible for the activation of C4 and C2, which leads to the generation of the same C3 convertase (C4bC2a) as in the classical pathway. MASP1 is able to cleave C3 directly. (**c**) The alternative pathway is initiated by the low-grade activation of C3 by hydrolysed C3 ($C3(H_2O)$) and activated factor B (Bb). The activated C3b binds factor B (B), which is then cleaved into Bb by factor D (D) to form the alternative pathway C3 convertase, C3bBb. Once C3b is attached to the cell surface, the amplification loop consisting of the alternative-pathway components is activated, and the C3-convertase enzymes cleave many molecules of C3 to C3b, which bind covalently around the site of complement activation. Taken from Fujita (2002).

further C3b formation (Rooijakkers *et al.*, 2005a).

Extracellular Fibrinogen Binding Protein (Efb).

Extracellular fibrinogen-binding protein (Efb) is a 15.6 kDa excreted molecule that acts as an immunosuppressive, anti-inflammatory protein and potent inhibitor of complement-mediated innate immunity (Chen *et al.*, 2008). Efb functions by binding to, and disrupting, the function of complement component 3 (C3). The binding of C3 prevents complement activation and opsonization. Efb is capable of binding both C3b and fibrinogen simultaneously, indicating two separate binding sites (Lee *et al.*, 2004a,b).

Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS).

The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a small (14.1 kDa) protein encoded on a bacteriophage and found in >60% of clinical isolates (de Haas *et al.*, 2004). CHIPS works by directly binding the the C5a receptor (C5a, like C3a, is a chemo-attractant molecule) and the formylated peptide receptor (Postma *et al.*, 2005), preventing the cognate agonists from binding and eliciting a chemotactic response.

Staphylokinase (Sak).

Staphylokinase (Sak) is a 15.5 kDa protein whose expression is under the control of the *agr* locus (See § 1.6 on page 31 for further information on the *agr* locus). As such, Sak is predominantly expressed during late exponential phase (Novick & Fischetti, 2000). Staphylokinase binds to the bactericidal peptides α-defensins, neutralising their bactericidal properties (Bokarewa *et al.*,

2006). Sak has no enzymatic activity of its own but is able to bind plasminogen to form a 1:1 complex activating host plasminogen into plasmin (Bokarewa *et al.*, 2006). Plasmin has serine protease activity creating a bacterium-bound protease and cleaving surface bound C3b and IgG (Figure 1.4 on the facing page) (Rooijakkers *et al.*, 2005b). Plasmin is also able to digest fibrin clots, potentially enabling *S. aureus* to spread from a site of local infection to new areas (Mölkänen *et al.*, 2002). This ability has not gone unnoticed by the medical profession, staphylokinase has received much attention as a possible treatment of thrombosis—the blockage of blood vessels with clots (Collen, 1998).

Sak, along with complement activation inhibitors CHIPS and SCIN, is located on the conserved 3′ end of a β-hemolysin-converting bacteriophage, forming an immune evasion cluster (IEC). The IEC has been shown to be easily transferred among bacterial strains and found in 90% of isolates (van Wamel *et al.*, 2006).

1.4 Genomics & Evolution of *S. aureus*.

With the discovery of DNA in 1953 (Watson & Crick, 1953) a new way of looking at the biological world was born. By examining the genomes of different species (and in the case of bacteria, strains within a species) new methods of classification and inference were possible. By examining how these genomes change with regard to differing selective pressures, and by comparing closely and more distantly related species the study of evolution evolved.

Evolution is the change in genetic material in the population of a species over time. Evolution occurs when selective forces act upon point mutations, recombination events, gene silencing, and the horizontal transfer of genetic material between different bacterial species and even genera (Hacker & Carniel,

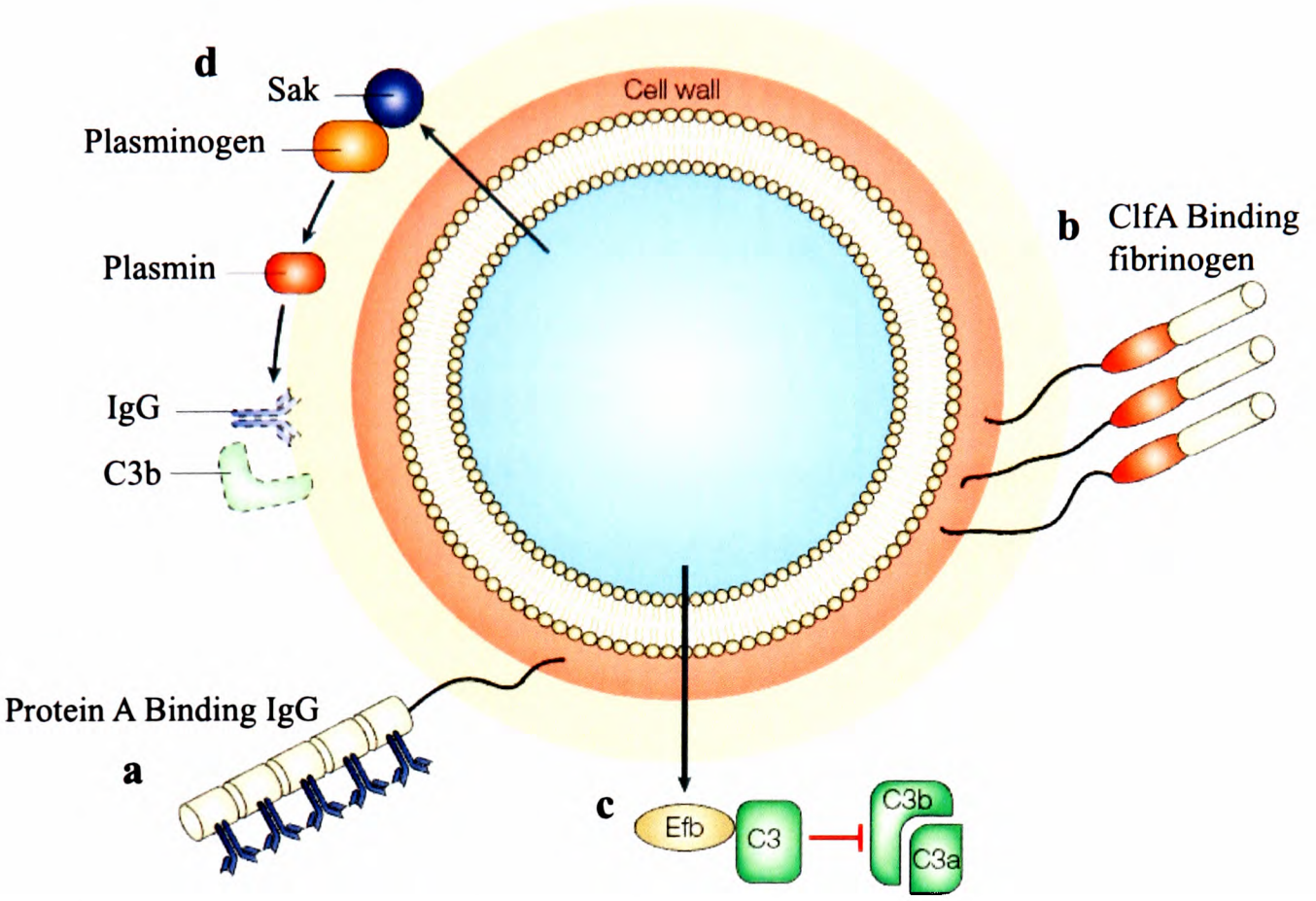

Figure 1.4: **Mechanisms by which *S. aureus* avoids opsonophagocytosis.** (**a**) Protein A with 5 IgG Fc-binding domains. (**b**) Clumping factor A (ClfA) binding the γ-chain of fibrinogen. (**c**) Fibrinogen-binding protein (Efb), binding complement factor C3 preventing its deposition on the bacterial cell surface and inhibiting opsonization. (**d**) Extracellular staphylokinase (Sak) activates cell-bound plasminogen and cleaves IgG & C3b. Modified from Foster (2005).

2001). DNA transferred horizontally between bacteria often contains antibiotic resistance determinants and virulence factors passed on via mobile genetic elements, plasmids and bacteriophage. An example is the Staphylococcal Chromosome Cassette (SCC) containing *mecA* (termed SCC*mec*). SCC*mec* is the defining feature of MRSA, though neither the SCC element nor *mecA* evolved in *S. aureus*. It is likely that *mecA* evolved in the β-lactam susceptible *Staphylococcus sciuri* (Couto *et al.*, 1996) before being grafted into SCC and jumping into *S. aureus* (de Lencastre *et al.*, 2007; Katayama *et al.*, 2003).

The *S. aureus* genome consists of between 2.8–2.9 Mb of a single circular

chromosome containing 2,592-2,748 genes (Lindsay & Holden, 2006). Approximately 78% of the genome is composed of essential genes present in all strains, the 'core' genome. The remainder is composed of non-essential genes, often resistance determinants and virulence factors, termed the 'accessory genome' (Lindsay & Holden, 2006) (See § 1.6 on page 31). The core genome shows remarkable similarity between sequenced strains, the differences arising primarily from single nucleotide polymorphisms (SNPs)* (Lindsay & Holden, 2006). SNPs may be synonymous whereby the nucleotide change does not affect the amino acid encoded or non-synonymous resulting in an amino acid change. The accessory genome is also affected by SNPs, however, a larger factor for change is the acquisition of foreign DNA via horizontally acquired elements. This can result in rapid evolution, often enabling adaptation to a new niche (Holden *et al.*, 2004). The accessory genome often carries disease specific genes: for example, Enterotoxin A — a common cause of food poisoning, Exfoliative toxin A — the cause of scalded skin syndrome, and Panton-Valentine leukocidin — a contributing factor in *S. aureus* induced necrotising pneumonia, are all carried on phages (See § 1.2 on page 2) (Betley & Mekalanos, 1985; Yamaguchi *et al.*, 2000; Narita *et al.*, 2001; Labandeira-Rey *et al.*, 2007a).

1.4.1 Relationship Between *S. aureus* Strains.

To monitor the spread and relationship of different strains of *S. aureus* and particularly MRSA a number of molecular typing methods have been developed:

- **Pulsed-field gel electrophoresis** (PFGE). Chromosomal DNA is digested with the restriction enzyme *SmaI*. The resulting DNA fragments

*Large chromosomal replacements may also occur without obvious involvement of mobile genetic elements, however this is likely a rare event *in natura* (Robinson & Enright, 2004a).

are separated by agarose gel electrophoresis in an electric field with an alternating voltage gradient. Analysis of the band patterns is undertaken by specialised software, the results of which are considered the gold standard for typing due to the ability to discriminate closely related strains (Deurenberg & Stobberingh, 2008).

- **Multilocus sequence typing** (MLST). Originally developed to characterise isolates of *Neisseria meningitidis* (Maiden *et al.*, 1998) MLST uses DNA sequences of multiple (usually seven) housekeeping genes to compare strains. For *S. aureus* 450 bp internal fragments of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) are sequenced. For each gene fragment different sequences are assigned as distinct alleles and each isolate is defined by the alleles at each of the seven housekeeping loci giving the sequence type (ST) (Enright *et al.*, 2000). For example the EMRSA-16 clone has MLST profile 2-2-2-2-3-3-2 which is defined as ST36 (`www.mlst.net`). If five or more loci are identical between strains then they are considered to be part of a clonal cluster (CC), if all seven are identical then they are considered the same clone. The putative ancestor of a CC is the ST with the largest number of single locus variants (SLVs) (Deurenberg & Stobberingh, 2008).

- ***spa* typing**. Developed by Frénay *et al.* (1996) *spa* typing utilises the polymorphic X-region of the protein A gene (*spa*) to differentiate between strains. Differences in the X-region occur through point mutations, deletions and duplications of repeats allowing discrimination between strains. The process is less expensive, laborious and time consuming compared to MLST, as only a single locus is sequenced (Deurenberg & Stobberingh, 2008). Conversely, sequencing of one region of the chromosome which is subject to recombination between unrelated clones could result in isolates

exhibiting the same *spa* type when they are shown to be unrelated by other methods (Cookson *et al.*, 2007).

- **SCC*mec* typing**. A number of protocols exist to detect sections of the Staphylococcal chromosome cassette *mec* (SCC*mec*), the defining feature of MRSA (See § 1.5.2 on page 23). A single or several multiplex PCR assays are used to classify the element by the presence of different recombinase (*ccr*) genes and J regions (Junkyard regions) (Boye *et al.*, 2007; Zhang *et al.*, 2005; Okuma *et al.*, 2002; Oliveira & Lencastre, 2002; Ito *et al.*, 2001). An obvious disadvantage of this method is that only MRSA strains can be tested and as the SCC*mec* is a mobile genetic element, it does not give any information regarding the 'host' genome.

S. aureus strains can be split into four Agr groups (I–IV) based on the ability of one group to cross inhibit other Agr groups in a heterologous manner (expanded in detail in § 1.6.1 on page 32). The Agr groups have been shown to correlate with specific genotypes using multiple methods including PFGE (pulsed field gel electrophoresis), MLST (multi-locus sequence typing) and *spa* (gene encoding Protein A) typing (Wright *et al.*, 2005b).

An early evolutionary event that may have facilitated the divergence of strains is the novel interstrain and interspecies inhibition of heterologous Agr groups (Wright *et al.*, 2005b). An example of this is the cross-inhibition by quorum-sensing pheromones between *S. aureus* and *Staphylococcus epidermidis* (Kaufmann *et al.*, 2001a). The Agr subgroup IV is closely related to subgroup I, suggesting that subgroup IV might have evolved from subgroup I by mutation under the selective pressure of competition with *S. epidermidis* (Kaufmann *et al.*, 2001a).

1.4.2 Mutation rates.

In their famous paper on mutations, Luria & Delbrück (1943) introduced the work with the observation that 'When a pure bacterial culture is attacked by a bacterial virus, the culture will clear after a few hours due to the destruction of the sensitive cells by the virus. However, after further incubation for a few hours, or sometimes days, the culture will often become turbid again, due to the growth of a bacterial variant.' Following from this initial observation they determined that bacterial resistance to infection by phage arises spontaneously in bacteria, implying the existence of genes that can be mutated, rather than being induced by contact between virus and bacterium. Furthermore, by careful analysis of the distribution of mutations they were able to provide the first appropriate mathematics for determining mutation rates. This work was the beginning of the end for the then widely held belief that bacteria adapted to environmental stress via Lamarckian inheritance, that is, by a process of directed change, rather than as the result of random mutations and natural selection (i.e. neo-Darwinian theory).

Bacteria are haploid for the vast majority of their genes, which, coupled with typically short generation times allows mutations to emerge and accumulate. This rapidly creates significant phenotypic changes in what is perceived to be real-time (Woodford & Ellington, 2007). The rapid emergence of mutations allows resistance to antibiotics such as rifampicin and fusidic acid to readily evolve, via point mutations in *rpoB* and *fusA* genes respectively (Wichelhaus *et al.*, 1999; Besier *et al.*, 2003). This limits the use of these antibiotics in monotherapy (Mason *et al.*, 2003), but enables researchers to make estimations of the mutation frequency or mutation rate within a strain (See method § 2.11 on page 65). The mutation frequency is simply the average fraction of mutant

bacteria in replicate cultures. The mutation rate is the probability of a cell sustaining a mutation during its lifetime and if worked out correctly is more accurate and reproducible than the mutant frequency (Rosche & Foster, 2000).

Mutation rates can vary significantly between species and even within strains of a species. Within a genome the mutation rate may also vary. Housekeeping genes, relevant for basic metabolism and structure are found to mutate at a low frequency (Moxon *et al.*, 1994a). A comparison between the yeast *Saccharomyces cerevisiae* and its close relative *Candida albicans* revealed that highly expressed genes evolved at a reduced rate (Pal *et al.*, 2001). The accessory genome on the other hand, important for bacterial adaptability, is often highly mutable (Martinez & Baquero, 2000; Moxon *et al.*, 1994b).

The environment and growth conditions also affect mutation rate. Stressful conditions such as starvation, oxidation, UV irradiation or 'arms-races' (for example the co-evolution of bacteria with parasitic bacteriophage) can also cause an increase in mutation rate (Tenaillon *et al.*, 2004; Pal *et al.*, 2007). The elevated rates of mutation are often brought about by mutations arising in genes responsible for DNA damage repair, especially the mismatch repair systems (MRS) (Prunier & Leclercq, 2005; O'Neill & Chopra, 2002). Damage to the MRS can lead to hypermutator phenotypes with mutation rates 100–>1000 fold higher than the base rate (Horst *et al.*, 1999). Hypermutators have been readily isolated from populations of a variety of human pathogens at frequencies ranging from 2% to as high as 57% (Sundin & Weigand, 2007). *S. aureus* mutators were found to make up almost 15% of populations isolated from the lungs of cystic fibrosis patients (Prunier *et al.*, 2003). These hypermutators may increase adaptability to novel environmental conditions, though they are also prone to the accumulation of deleterious mutations and may impede cooperation in pathogenic bacteria (Harrison & Buckling, 2005). The mutation rate within

a population is therefore not static, but an evolving feature dependent upon a multitude of parameters including population size and environment (Denamur & Matic, 2006).

1.5 Antibiotic Resistance.

Antibiotic resistance is a major problem, costing billions of dollars each year in the US alone (Palumbi, 2001) and more importantly leading to increased mortality rates (Niederman, 2001). It is therefore important to identify the major antibiotics used against *S. aureus* and the ways in which these antibiotics have been rendered ineffective.

In 1939 the first clinically tested antibiotic, gramicidin, was successfully used in the treatment of human disease (Dubos, 1939a,b). Gramicidin was too toxic for general use and it was not until three years later that the true advent of the antibiotic age arrived. In March 1942 Anne Sheafe Miller made medical history as the first patient saved by penicillin (Salyers & Whitt, 2005). Although many lives have subsequently been saved, bacterial resistance was quick to appear and spread through bacterial populations.

Bacteria can acquire resistance to antibiotics mutation or horizontal transmission. Horizontal transmission occurs through the physical introduction of genetic material from one cell to another via transduction, transformation or conjugation (Thomas & Nielsen, 2005).

The introduction of new antibiotics produces a strong selection for evolution of resistance, enabling any resistant bacteria to be rapidly selected and often detected shortly after the antibiotic's introduction. If the antibiotic is held back or used limitedly (due to more efficient antibiotics becoming available or

adverse side effects) then the emergence of resistant strains may be delayed (Figure 1.5).

The mode of antibiotic resistance can be separated into four broad categories: (i) chemical inactivation of the antibiotic, (ii) modification of the drug target, (iii) use of an alternative pathway or increased production of target metabolite and (iv) the prevention of the antibiotic from reaching the target—either through the use of membrane pumps or a physical barrier such as a biofilm or resistant membrane (Benveniste & Davies, 1973).

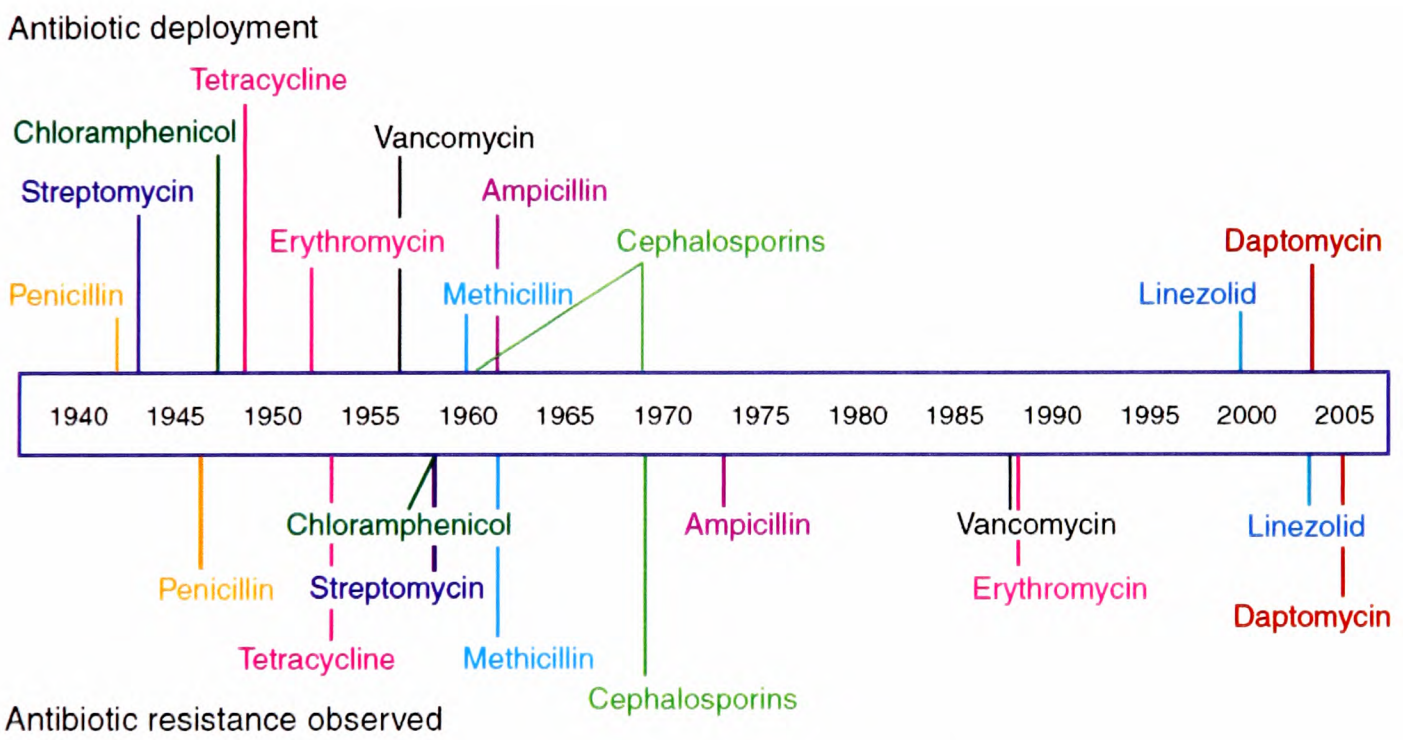

Figure 1.5: **Evolution of Resistance to Antibiotics**. Dates for the deployment of antibiotics are shown on the top of the timeline with the dates of the first observed evolved resistance shown below.

1.5.1 Penicillin.

During the Second World War penicillin (Figure 1.6 on page 26) became the first antibiotic to be introduced on a global scale. Initially its use was highly effective with >94% of *S. aureus* isolates being susceptible (Livermore, 2000).

By the early 1950s plasmids encoding penicillinase, an enzyme that degrades penicillin and other β-lactam antibiotics, were spreading rapidly through the *S. aureus* population rendering them resistant. By the late 1960s approximately 80% of *S. aureus* isolates were resistant to penicillin (Gootz, 1990). Today that number is >90% (Lowy, 2003).

Penicillin is a member of the β-lactam group of antibiotics that work by inhibiting the synthesis of peptidoglycan. This is achieved by forming a stable bond with the active site of one of the essential cell wall synthesis enzymes, the penicillin binding protein (PBP) (Park & Strominger, 1957).

S. aureus is able to overcome the peptidoglycan inhibition through chemical inactivation of penicillin. β-lactamase is secreted, causing hydrolysis of the β-lactam ring, rendering the antibiotic ineffective (Figure 1.6 on page 26). β-lactamase is encoded by the *blaZ* gene and is under control of two regulators: BlaR1 an antirepressor and BlaI the repressor (Lowy, 2003). The regulators ensure that β-lactamase is only secreted following exposure to β-lactams.

1.5.2 Methicillin.

During the 1950s work was undertaken to combat the emergence of penicillin resistance. Efforts were focused on producing a semi-synthetic antibiotic resistant to β-lactamases. The first of its kind, Methicillin (Figure 1.6 on page 26) was introduced in 1959 and was initially highly effective. Just two years after its introduction the first case of methicillin resistance was reported in a British hospital (Jevons, 1961).

Methicillin acts by inhibiting the synthesis of bacterial cell walls. This is achieved by binding to, and competitively inhibiting, the (confusingly named) penicillin-binding proteins (PBPs). The PBPs are involved in the process of

synthesising cross-linked peptidoglycan from lipid intermediates and mediating the removal of D-alanine from the precursor of peptidoglycan (Spratt, 1977). They are termed 'penicillin-binding proteins' due to their affinity for and binding of penicillin.

The ability of *S. aureus* to resist methicillin is due to the the acquisition of a foreign penicillin-binding protein with a greatly reduced affinity for methicillin, penicillin binding protein 2′ (PBP2′ or PBP2a) (Lim & Strynadka, 2002a). PBP2′ confers resistance not only to methicillin but to a broad spectrum of β-lactam antibiotics due to a significantly reduced affinity to those antibiotics (Hartman & Tomasz, 1984; Reynolds & Brown, 1985).

PBP2′ is a product of the *mecA* gene, part of the mobile genetic element 'Staphylococcal Chromosome Cassette *mec*' (SCC*mec*). Transcription of *mecA* is under the control of the regulatory element *mecR1-mecI*. MecI acts as the repressor and its corresponding sensor-transducer molecule is MecR1 (Berger-Bächi & Rohrer, 2002). The analogous set of regulatory genes for penicillinase *blaR1-blaI* also effect *mecA* expression with BlaI acting as a repressor of *mecA* (Lewis & Dyke, 2000).

A distinctive feature of most MRSA is the heterogeneous expression of methicillin resistance. This is characterized by a majority of cells with low-level resistance from which a small proportion of highly resistant cells segregate (Berger-Bächi & Rohrer, 2002). Within a culture of a clinical isolate with an intact SCC*mec* element a subpopulation of between 10^{-8} and 10^{-4} cells may express high methicillin resistance whereas the remaining cells are relatively susceptible (Wax *et al.*, 2007). Variation in resistance is due to mutational events in the staphylococcal genome, though not the SCC*mec* (Ryffel *et al.*, 1994). Heterogenous phenotypes can be converted to a homogenous phenotype by the persistent presence of β-lactam antibiotics (Finan *et al.*, 2002; Hartman

& Tomasz, 1986). Once high level resistant clones have been selected they maintain high resistance even in the absence of selective pressure, indicating a role for mutation rather than increased expression of *mecA* (Berger-Bächi & Rohrer, 2002; de Lencastre *et al.*, 1993; Tomasz *et al.*, 1991).

Mutations in a number of genes outside of SCC*mec* can lead to loss of methicillin resistance. These genes, termed *fem* (factors essential for methicillin resistance) or *aux* (auxiliary), were discovered through screening for loss of methicillin resistance by insertional inactivation (Berger-Bächi & Rohrer, 2002). So far $\geq$20 *fem* genes have been identified. Many have a role in peptidoglycan biosynthesis and turnover, or regulatory function, but so far none of them have been shown to affect PBP2′ expression (De Lencastre *et al.*, 1999; Berger-Bachi *et al.*, 1992).

Methicillin resistant *S. aureus* can be divided into at least seven groups (I–VII) based upon the characterization of the SCC*mec* element they contain (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004; Oliveira *et al.*, 2006; Takano *et al.*, 2008). All SCC*mec*s contain the methicillin resistance gene *mecA* as well as cassette chromosome recombinase genes (*ccrA* & *ccrB*, Katayama *et al.*, 2000) thought to be responsible for the excision and insertion of the cassette at a specific site, the 'open reading frame of unknown function' (*orfX*), located near the origin of replication. The variation in the SCC*mec* groups is due to additional resistance genes and 'junkyard' regions. Types IV (20.9–24.3 kb), V (28 kb), VI (20.9 kb), VII (35.9 kb) and I (34.3 kb) posses only the *mecA* gene whereas types II (53 kb) and III (66.9 kb) contain multiple determinants for resistance to non-β-lactam antibiotics (Figure 1.7) (Deurenberg & Stobberingh, 2008). Types I, II & III are considered hospital acquired MRSAs (Rychlik *et al.*, 1990). Types IV–VII SCC*mec* are found in the community with type IV predominantly found in emerging community acquired MRSA strains.

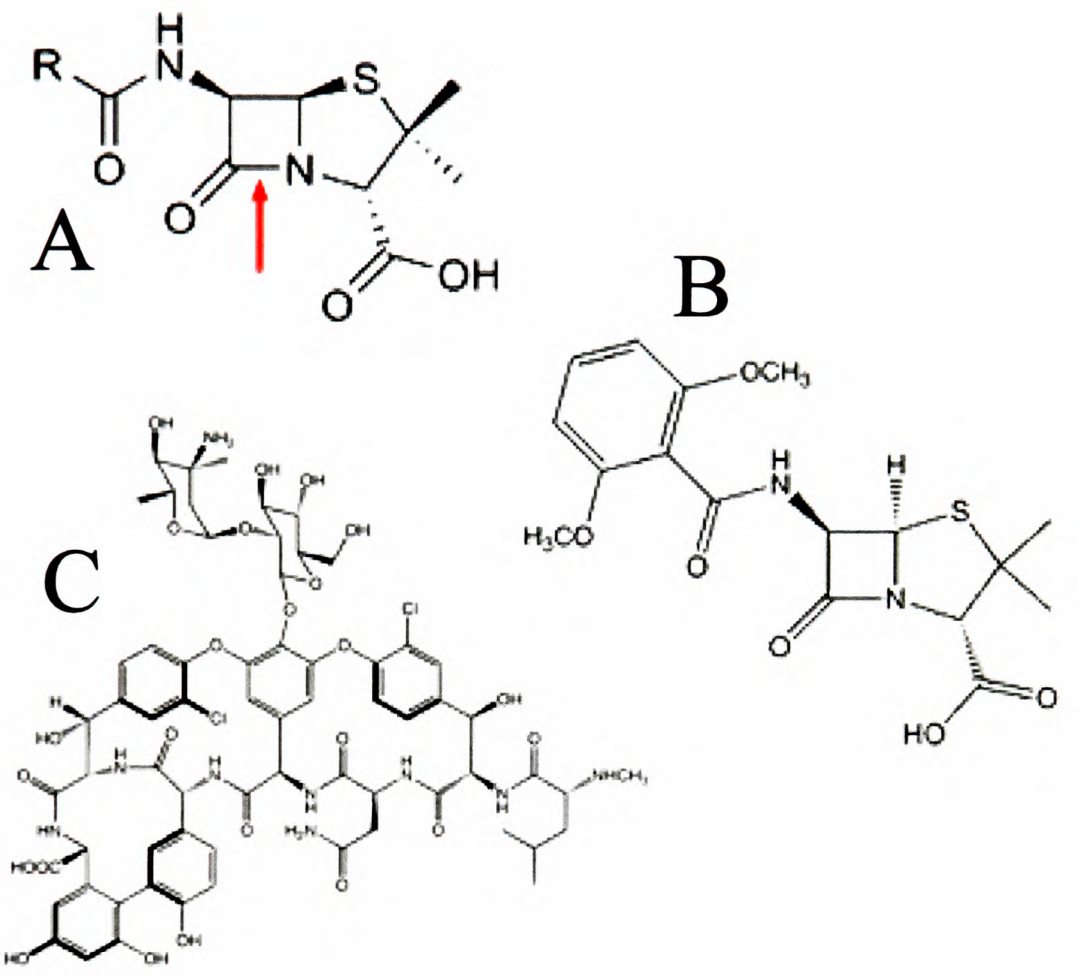

Figure 1.6: **Structure of Antibiotics**. (A) Core Structure of Penicillin. Other semi-synthetic antibiotics such as penicillin G, V, ampicillin and methicillin are based around this structure. The red arrow indicates the bond in the β-lactam ring that is hydrolysed by penicillinase. (B) Methicillin. Unlike penicillin methicillin is impervious to the actions of secreted β-lactamases. *S. aureus* overcomes this by utilising penicillin binding protein 2′ (PBP2′) encoded by the methicillin resistance gene *mecA*. PBP2′ has a much lower affinity for methicillin than the intrinsic penicillin binding proteins (PBP) (Hartman & Tomasz, 1984; Reynolds & Brown, 1985). (C) Vancomycin. Until recently vancomycin has been used as the last line of defence however recently vancomycin intermediate-resistant (VISA) and vancomycin resistant (VRSA) strains have been isolated.

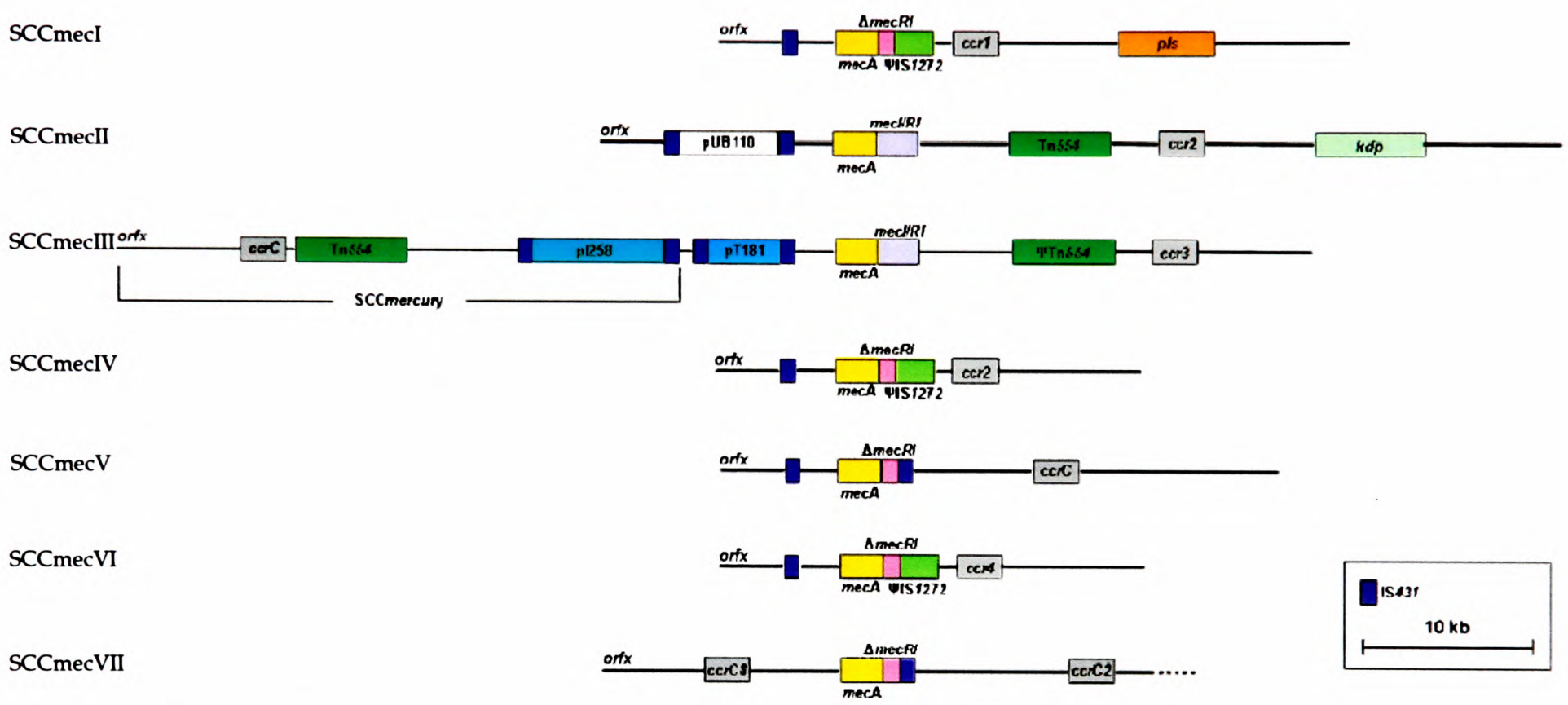

Figure 1.7: **Schematic of SCC*mec* types I-VII**. The seven SCC*mec* types I-VII showing the major elements: *ccr* genes responsible for insertion of the *mec* element, *mecA* encoding PBP2′, *mecI / RI* regulators of *mecA*, *orfX* the open reading frame of unknown function, pI258 a linearised plasmid containing erythromycin and the heavy metals arsenate & arsenite resistance determinants, pT181 a linearised plasmid containing a tetracycline resistance determinant, pUB110 a linearised plasmid containing kanamycin, tobramycin & bleomycin resistance determinants, Tn554 a transposon containing erythromycin and spectinomycin resistance determinants (ΨTn554 also contains a cadmium resistance determinant) and the insertion sequences IS431 & IS1272. The *pls* gene encoding a plasmin-sensitive surface protein (Hilden *et al.*, 1996) and the *Kdp* operon encoding an ATP-dependent transport system probably responsible for osmolarity resistance (Kuroda *et al.*, 2001) are also present within the junkyard regions of SCC*mec* I & II respectively. Modified from Deurenberg & Stobberingh (2008).

Hospital Acquired MRSA (HA-MRSA).

MRSA cases first appeared in British hospitals in 1961 before emerging throughout Europe in the 1960's and the world during the 1970s.

Voluntary reporting by hospitals in England and Wales to the Health Protection Agency (HPA) showed that the proportion of MRSA isolates from blood culture rose from around 2% in 1990 to a peak of 43% in 2002 before a slight decline thereafter (Johnson *et al.*, 2005). This situation is not repeated worldwide. Data collected from January 1999 to December 2002, involving 50,759 isolates from 495 hospitals in 26 countries showed that MRSA prevalence varied almost 100-fold, from <1% in northern Europe (Sweden, Denmark and The Netherlands) to >40% in Greece, Italy and the UK (Figure 1.8 on the facing page) (Schito, 2006; Tiemersma *et al.*, 2004). Results from the Department of Health's mandatory MRSA surveillance system in acute trusts in England (2001-2005) shows an average of 9270 cases of bacteraemia in any 6 month period with 40% being MRSA cases.

Community Acquired MRSA (CA-MRSA).

Although hospital acquired MRSA has been recorded since the early 1960s, it was not until 1993 that the first cases of community acquired MRSA (CA-MRSA) were reported (Udo *et al.*, 1993). There have been numerous definitions for CA-MRSA, though a general international consensus defines them as: 'strains isolated in an outpatient setting, or from patients within 48 h of hospital admission and whereby patients have had no history of MRSA infection and have not been hospitalised, in a nursing home or had dialysis in the prior year' (Deurenberg *et al.*, 2007).

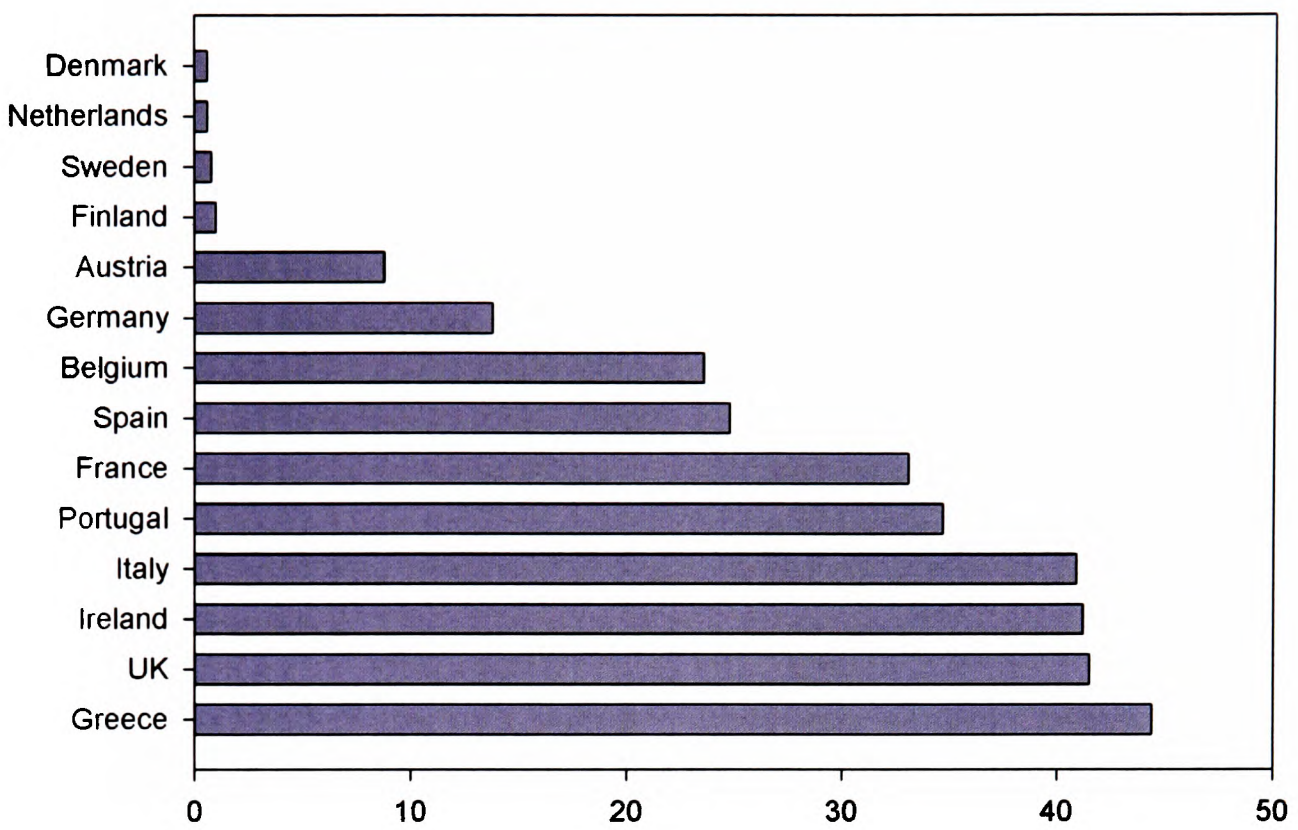

Figure 1.8: Percentage (%) methicillin-resistant *Staphylococcus aureus* (MRSA) blood isolates from hospitals (1999-2002). Data from Tiemersma *et al.* (2004).

CA-MRSA strains are phenotypically and genotypically distinct from HA-MRSA (Deurenberg *et al.*, 2007). CA-MRSA strains tend to harbor the SCC*mec* types IV & V and as such are not resistant to the non-β-lactam antibiotics. PFGE (Pulse Field Gel Electrophoresis) analysis shows CA-MRSA strains belong to clonal types unrelated to those found in hospitals (Naimi *et al.*, 2003, 2001; Groom *et al.*, 2001), and MLST (Multi Locus Sequence Typing) has shown a greater clonal diversity of CA-MRSA when compared to HA-MRSA (Okuma *et al.*, 2002; Enright *et al.*, 2002). CA-MRSA strains have also been shown to be more virulent than HA-MRSA (Chambers, 2001; Etienne, 2005).

1.5.3 Vancomycin.

Vancomycin (Figure 1.6) is a glycopeptide antibiotic that binds to D-alanyl-D-alanine residues, preventing the penicillin binding proteins from incorporating them into the cell wall (Barna & Williams, 1984). Discovered in 1956 it was soon superseded by other beta-lactam antibiotics and employed only as a drug of last resort (McCormick *et al.*, 1956). Vancomycin needs to be administered intravenously due to an inability to cross the intestinal membrane, has a number of adverse side effects and for a long time was the only drug capable of combating multi-drug resistant MRSA (Leeb, 2004). Despite this, in 1996, *S. aureus* strains showing intermediate resistance to vancomycin (VISA) with MICs of 8–16 μg ml^{-1} were isolated (Hiramatsu *et al.*, 1997). In 2002 strains exhibiting high resistance (VRSA) of $\geq$128 μg ml^{-1} were isolated (González-Zorn & Courvalin, 2003).

Low and high level resistance to vancomycin is brought about by two distinct modes of resistance. Low level resistance is due to an increase in peptidoglycan synthesis resulting in a thicker cell wall. Vancomycin binds to free D-alanyl-D-alanine residues in the wall and is prevented from entering the cell. The binding is thought to clog up the cell wall, further trapping vancomycin molecules and increasing resistance (Hiramatsu, 2001). High level vancomycin resistance found in VRSA strains is due to the horizontal transfer of the *vanA* operon, most likely from *Enterococcus faecalis* (Weigel *et al.*, 2003). The *vanA* operon enables cell wall synthesis via a precursor ending in D-alanyl-D-lactate rather than D-alanyl-D-alanine (Murray, 2000). Expression of the operon is regulated (much like *blaZ* and *mecA* for penicillin & β-lactam resistance) by two genes, *vanR* and *vanS*, forming a two-component regulatory system (Arthur & Quintiliani, 2001). Although there is great concern over the spread of the *van* operon

among *S. aureus*; there has (as of July 2008) only been nine isolates detected worldwide in six years, seven from the same region in Michigan (Taubes, 2008). This suggests that vancomycin resistance also confers heavy costs in *S. aureus*, making resistant strains uncompetitive in the absence of vancomycin.

1.6 The Staphylococcal Virulon.

A bacterial virulon consists of gene products that are either secreted or bound to the bacterial outer cell surface and provide the primary interface between the bacterium and host. The products are generally encoded by accessory genes and enable the organism to evade host defenses, to adhere to cells and the tissue matrix, to spread within the host and to degrade cells and tissues, for both nutrition and protection against host defenses (Novick & Geisinger, 2008a).

The *S. aureus* virulon consists of virulence genes primarily located in the accessory genome but also the cell associated and expressed factors encoded within the core genome (Table 1.1 on page 6). Transcriptional profiling by Dunman *et al.* (2001) revealed a total of 104 up-regulated and 34 down regulated accessory genes, using *agr* & *sarA* mutants (See §1.6.1 & 1.6.3 on page 38). Amongst the down regulated genes are a number coding for metabolic enzymes, suggesting that up regulating toxins occurs at the cost of reduced metabolic activity (Queck *et al.*, 2008). The metabolic burden requires careful regulation, being highly controlled by a number of distinct, two component regulatory systems, (TCS) and transcription factors—themselves dependent on a multitude of external and internal factors (Novick, 2003a).

1.6.1 Accessory Gene Regulator (Agr).

The main controller of the accessory genes is the 'accessory gene regulator' (Agr) (Figure 1.9 on the next page). The Agr is a two component quorum sensing (QS) system** discovered by Recsei *et al.* (1986) that up-regulates secretory proteins and down regulates surface proteins during late exponential phase *in vitro* (Novick *et al.*, 1993). The QS system acts predominately as a sensor of population density *in vitro* though *in vivo* it may also act as a sensor of limited diffusion (Redfield, 2002). If *S. aureus* is internalised by host cells, such as epithelial cells, the Agr can be induced due to the confined space, and may be essential for the bacteria to escape (Tuomanen *et al.*, 2001c; Shompole *et al.*. 2003).

The *agr* locus (≈3 kb) consists of two divergent operons controlled by the promoters P2 and P3. The P2 operon encodes four genes *agrA*, *B*, *C*, and *D* which form the two-component system and its autoinducing ligand (Novick *et al.*, 1995). A schematic of the AGR system is shown on Figure 1.9 on the facing page. AgrC is a histidine protein kinase sensor that acts as the Agr signal receptor. AgrA is the response regulator that is thought to bind to the *agr* promoters P2 and P3 completing the autoinduction circuit, though actual binding has not been demonstrable (Morfeldt *et al.*, 1996). AgrB is a polytopic transmembrane associated peptide responsible for processing and secreting the autoinducing peptide (AIP) (Zhang *et al.*, 2002), though another protein SvrA may also be involved (Garvis *et al.*, 2002). AgrD is the signalling peptide, AIP

**Quorum sensing (QS) is the method used by many bacteria to co-ordinate gene expression in relation to the local cell density of the population. The first QS system was discovered by Nealson *et al.* (1970) whilst studying the induction of luciferase synthesis in *Vibrio fischeri*, responsible for the luminosity of certain marine fish. When the concentration of an autoinducer reaches a critical level transcription of luciferase is induced, leading to bioluminescence (Nealson *et al.*, 1970; Nealson & Markovitz, 1970).

(Figure 1.10 on page 35). The *agrD* gene encodes the autoinducer propeptide which is then modified by AgrB through N and C terminal cleavage during transport to producing the autoinducing peptide (AIP). The AIPs all consist of a 5 amino acid thiolactone ring with a linear, 2 to 5 amino acid, tail and conserved cysteine residue five amino acids prior to the C terminus (Wright *et al.*, 2004; Lyon *et al.*, 2002).

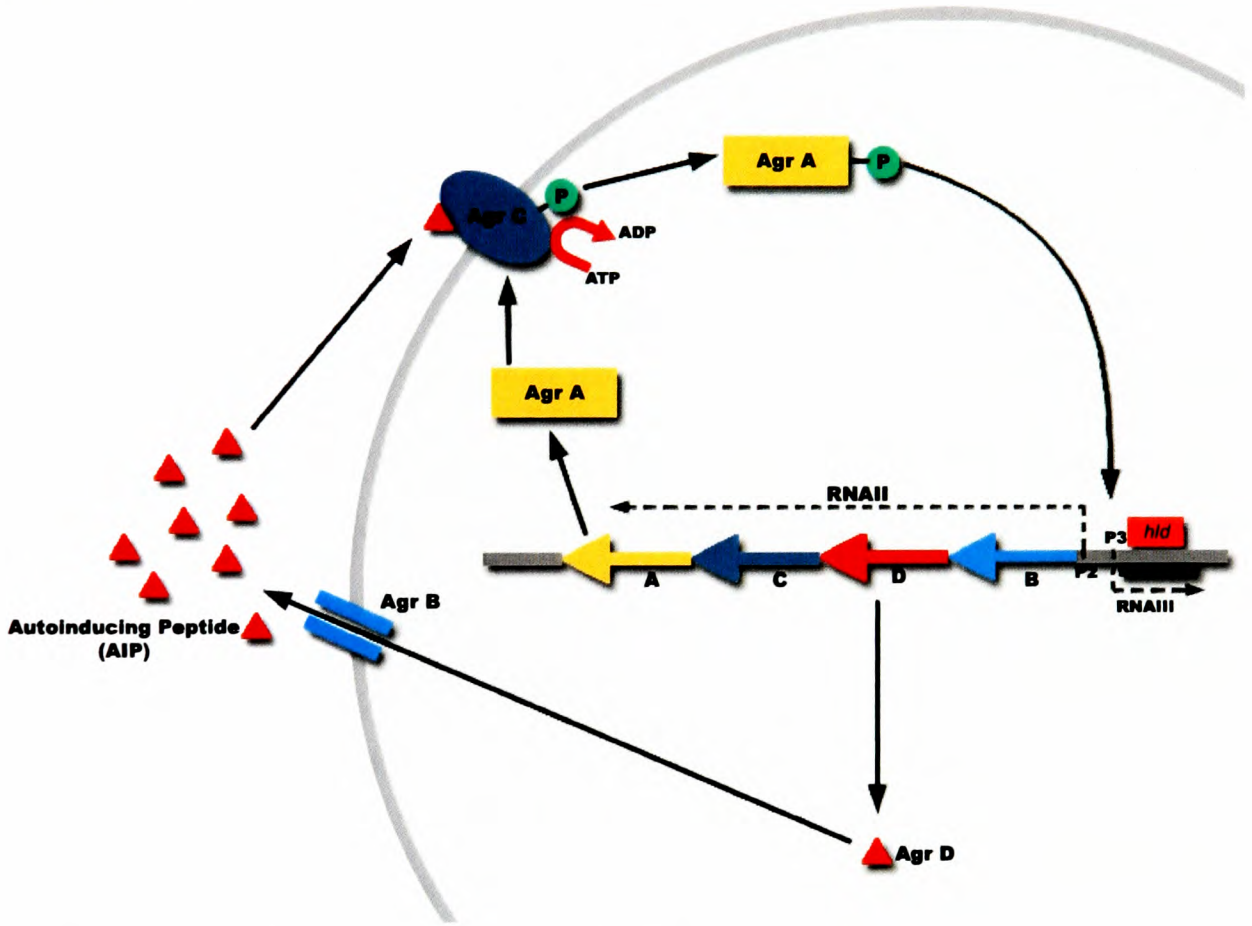

Figure 1.9: Schematic representation of the Agr system. AgrD is post-translationaly modified by AgrB to form the autoinducing peptide (AIP) and exported from the cell. AIP is detected by AgrC which activates AgrA, the response regulator. AgrA activates the two *agr* promoters P2 & P3 leading to the production of RNAIII, which controls transcription of the target genes.

Once the AIP reaches a certain threshold (around the transition to the stationary growth phase *in vitro*), AgrC activates the regulator AgrA through phosphorylation. The auto activated AgrA initiates an exponential increase of transcription rates in both the P2 & P3 promoters. The P3 operon encodes

the effector of the *agr* regulon RNAIII, a 510-nucleotide RNA molecule with a complex secondary structure (including 14 different hairpins) (Benito *et al.*, 2000). RNAIII up regulates transcription of a number of secreted proteins including α-haemolysin (α-toxin), enterotoxin B and toxic shock syndrome toxin (TSST-1) whilst down regulating production of surface proteins such as protein A and fibronectin binding proteins (Tegmark *et al.*, 1998). The specific mechanism by which RNAIII does this is not known but it is thought that it may act as an anti-repressor by binding transcriptional regulators (Arvidson & Tegmark, 2001). RNAIII is also capable of acting at the level of translation for at least two proteins, α-haemolysin and protein A (Morfeldt *et al.*, 1995; Huntzinger *et al.*, 2005).

The *S. aureus* QS system therefore switches the bacteria from a sessile phenotype to one which can move freely by repressing adhesins and producing toxins and lytic enzymes. Moreover if the bacteria are in a biofilm state when the AIP reaches quorum, the biofilm is dispersed through an Agr dependent mechanism (Boles & Horswill, 2008), potentially aided by δ-haemolysin, encoded by RNAIII, acting as a detergent due to its amphipathic nature.

Although Agr activation is a self contained system, it can also be up($\uparrow$) or down($\downarrow$) regulated by a number of other genes: *sarA*$\uparrow$, *sarU*$\uparrow$, *mgrA*$\uparrow$, *sarX*$\uparrow$, *clpP*$\uparrow$, *cvfA*$\uparrow$, *srrAB*$\downarrow$, $\sigma^B\downarrow$, *msa*$\uparrow$, *ccpA*$\uparrow$, and *arlRS*$\downarrow$ (Novick & Geisinger, 2008b) forming an extremely complicated regulatory network (Figure 1.12 on page 43).

Experimental animal infection models and *in vitro* experiments have shown that if the Agr is not functioning, due to mutation or interference, *S. aureus* exhibits significantly reduced virulence (Abdelnour *et al.*, 1993; Cheung *et al.*, 1994; Gillaspy *et al.*, 1995; Novick, 2003a; Collins *et al.*, 2008). Despite this, Agr^- strains are still capable of colonising and being transmitted among healthy

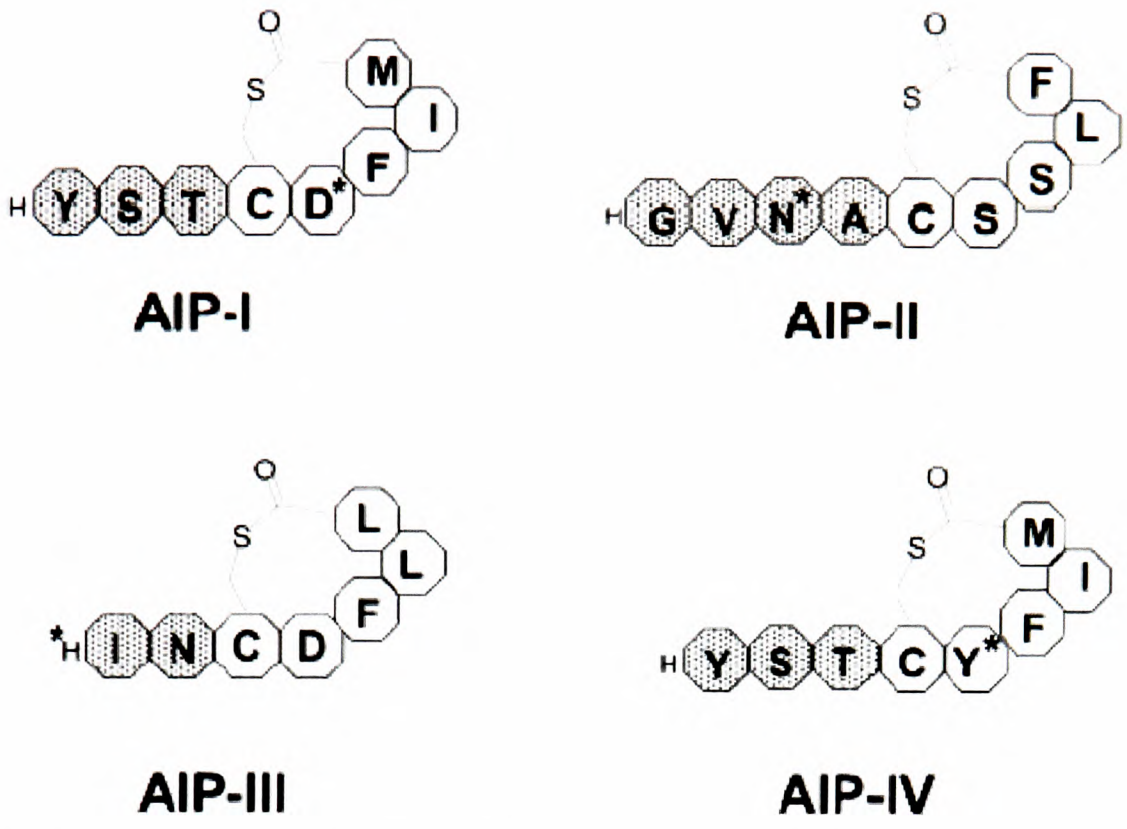

Figure 1.10: Structure of the four AIP groups modified from Lyon *et al.* (2002). The gray shaded area indicates the exocyclic tail residues whereas the clear blocks indicate the endocyclic residues. The asterisks represent residues that are critical for receptor activation.

individuals (Shopsin *et al.*, 2008b) and of causing disease (Traber *et al.*, 2008).

Agr Group.

The Agr is a variable system with four main allelic groups (I–IV) found amongst *S. aureus* strains. The split is based on the ability of AIPs (Figure 1.10) from one group to cross inhibit other Agr groups in a heterologous manner, resulting in bacterial interference of Agr rather than growth (Figure 1.11 on the following page) (Ji *et al.*, 1997). It has been shown *in vitro* by Boles & Horswill (2008) that *S. aureus* in a biofilm exposed to AIP of the same group will activate Agr and biofilm dispersal will ensue. Exposure to AIP from a interfering Agr type will result in non activation of Agr and the biofilm remains.

A link has been suggested between the Agr groups and disease, particularly for

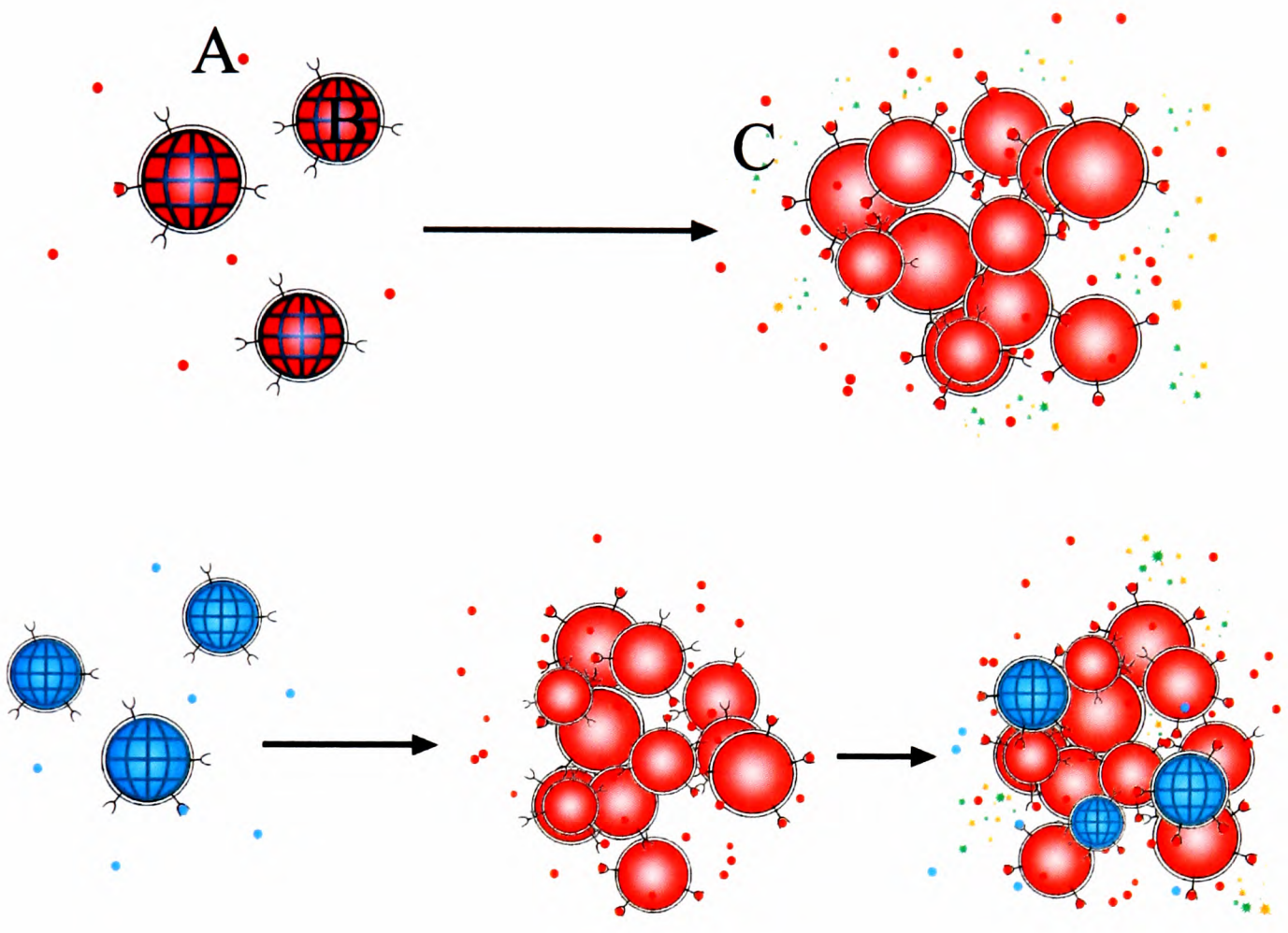

Figure 1.11: **Agr interference in *Staphylococcus aureus*.** There are currently four known Agr types of *S. aureus*. The AIP (auto-inducing peptide) is the effector molecule secreted by the bacteria. 1: The top panel shows the activity of the AIP when a single strain of *S. aureus* is growing in isolation. At low bacterial cell densities the Agr signal molecule is present at low concentrations, resulting in low-level binding of the AIP (A) to the Agr receptor on the bacterial cell surface and continued expression of cell surface proteins (B) such as protein A and fibronectin binding protein. Under these conditions the regulatory cascade that is necessary for the full expression of virulence factors is not triggered. At higher cell densities, the AIP is at a higher concentration and subsequently binds to the Agr receptor on the cell surface, which triggers the regulatory cascade necessary for the full expression of virulence determinants (C) and down-regulation of surface proteins. 2: The bottom panel shows how the AIP of one *S. aureus* strain can interfere with the colonization of an invading strain. As the strain of one Agr type (blue) attempts to invade an established colony of a strain of a different Agr type (red), the relative density of the AIP of the established colony will saturate the Agr receptors on the cell surface of the invading species. This prevents the AIP of the invader species from binding to its receptor and inducing the regulatory cascade necessary for the full expression of its virulence determinants. Adapted from Massey *et al.* (2006)

toxin mediated disease. Work on a collection of 198 *S. aureus* isolates (isolated from 14 asymptomatic carriers, 66 patients with suppurative infection, and 114 patients with acute toxemia) showed a correlation between *agr* groups I & II and Enterotoxin-mediated disease, Group III were involved in TSST-1 mediated disease—specifically menstrual toxic shock syndrome and *agr* group IV strains were involved in generalized exfoliative syndrome and bullous impetigo (Jarraud *et al.*, 2002). Group III strains have also been implicated in leukocidin induced necrotizing pneumonia in young immunocompetent patients (Gillet *et al.*, 2002) and a study comparing variability in *agrD* from globally collected strains found that of 137 MRSA isolates 130 belonged to Agr group I (van Leeuwen *et al.*, 2000).

1.6.2 *S. aureus* Exoprotein Expression locus (*sae*).

The *sae* locus is composed of 4 genes. Two genes make up a two-component system (TCS) encoding SaeR (26.9 kDa) a response regulator and SaeS (39.5 kDa) a sensor kinase (Giraudo *et al.*, 1999; Bronner *et al.*, 2004) with the two remaining genes *saeP* and *Q* found upstream. The function of SeaP & SeaQ has only recently been investigated, where it is thought they play a role in the regulation of the TCS (Adhikari & Novick, 2008).

The *sae* locus is thought to act downstream of *agr* and is essential for the transcription of *hla*, *hlb* and *coa*, though it does not affect expression of *agr* or *sarA* (Giraudo *et al.*, 2003, 1997). This makes Sae an important regulatory system in its own right. As well as regulating many virulence factors *in vitro*, Sae has been shown to be an important element for the expression of virulence genes *in vivo* (Rampone *et al.*, 1996; Goerke *et al.*, 2001, 2005). The *sae* locus responds to several environmental stimuli including high salt concentration,

low pH, glucose and sub-inhibitory antibiotic concentrations (Novick, 2003a).

1.6.3 Staphylococcal Accessory Regulator (*sarA*) and its Homologues.

The *sarA* operon was initially described by Cheung *et al.* (1992) whilst examining a clinical isolate exhibiting reduced fibrinogen binding. In this strain *sarA* was inactivated which resulted in the up regulation of α-toxin, lipase and serine protease and the down regulation of fibrinogen and fibronectin binding proteins and coagulase—the opposite effect to an *agr* mutation[†]. The *sarA* locus consists of three overlapping transcripts, each with a distinct promoter (P1–P3) (Bayer *et al.*, 1996). The three transcripts encode the 14.7 kDa DNA binding protein SarA and are expressed during early exponential phase through to the stationary phase (Manna *et al.*, 1998). Promoters P1 & P2 are recognised by σ^A (a general housekeeping sigma factor) and are expressed constitutively. P3 is recognised by σ^B (the stress or stationary-phase sigma factor, homologous to *Bacillus subtilis* σ^B) and is activated during postexponential phase or during periods of metabolic stress (Manna *et al.*, 1998).

SarA appears to bind as a dimer to regions of DNA rich in adenine and thymine (AT). This includes a region between the P2 & P3 promoters of the *agr* operon where it up-regulates the levels of RNAII & RNAIII (Heinrichs *et al.*, 1996). There appears to be no consensus as to what constitutes a SarA binding site (Rechtin *et al.*, 1999; Chien & Cheung, 1998; Novick, 2003a). Indeed, it has recently been shown that SarA can bind to its own promoter

[†]When the *sarA* mutation was transferred to lab strains derived from 8325–4 an opposite phenotype was observed with increased production of protein A and a decrease in haemolysins. This can (at least) be partially explained by the discovery of a mutation in *rsbU*, which encodes a phosphatase that activates σ^B in 8325–4 and its derivatives. (Blevins *et al.*, 2002; Cheung & Projan, 1994; Kullik *et al.*, 1998).

	Putative Function
SarA	Activates genes via *agr* and *agr* independent pathways
SarR	Negative regulator of *sarA* and positive activator of *agr*
SarS	Activator of protein A synthesis
SarT	Activator of *sarS* and repressor of α-haemolysin synthesis
SarU	Positive regulator of *agr*
SarV	Regulator of autolysis, repressed by SarA and MgrA
SarX	Negative regulator of *agr*, activated by MgrA
SarY	Unknown function
SarZ	Positive regulator of *hla*
MgrA	Regulator of autolysis and *agr*
Rot	Repressor of toxin synthesis, opposite to *agr*

Table 1.2: **SarA protein family.** Modified from Cheung *et al.* (2007).

and down-regulate *sarA* expression (Cheung *et al.*, 2008). The production of exoproteins (α-toxin, δ-hemolysin, γ-hemolysin) and cell wall associated proteins (fibronectin-binding proteins, collagen adhesins) are up-regulated by SarA binding directly to conserved regions within promoters (Sar boxes) in an Agr independent manner (Dunman *et al.*, 2001). SarA can also directly repress *aur* (metalloproteinase; aureolysin) and *sspA* (V8 protease; serine protease) (Oscarsson *et al.*, 2006).

Since the discovery of *SarA* a number of homologues have been discovered making a large and complex regulatory family of Sar proteins (Table 1.2). The complexity of the regulatory family is illustrated in Figure 1.12 on page 43.

SarS (SarH1).

SarS (also known as SarH1 for Sar homologue 1) consists of two homologous halves, each with a degree of identity to SarA (32% & 34% respectively) and at 29.9 kDa is approximately twice its size (Tegmark *et al.*, 2000; Arvidson

& Tegmark, 2001). Expression of *sarS* appears to be repressed by *sarA* and *agr*. SarS itself represses *hla* (α-haemolysin) whilst up-regulating *spa* (protein A) transcription (Tegmark *et al.*, 2000). It has been hypothesised that the increased transcription of *spa* in *agr* and *sarA* mutants is predominantly the result of increased SarS production rather than repression of *spa* transcription by *agr* and SarA as previously thought (Arvidson & Tegmark, 2001).

SarR (Sar Repressor, SarH5).

SarR (13.7 kDa) is a repressor of *sarA* that likely effects the transcription of genes via *sarS* (Arvidson & Tegmark, 2001). Recent experimentation has shown SarR affects the transcription of *aur* and *sspa* by directly up-regulating them and not just via the down regulation of *sarA* (Gustafsson & Oscarsson, 2008).

SarT (SarH2).

SarT (14.0 kDa) is a repressor of α-haemolysin (Schmidt *et al.*, 2001) that is thought to act upstream of *sae* or *rot* and not downstream of *sae* in *hla* repression (Li & Cheung, 2008). SarT also represses *sarU* (Manna & Cheung, 2003) though it can bind to and activate *sarS* (Schmidt *et al.*, 2003).

SarU (SarH3).

SarU (29.7 kDa) is composed of two homologous halves, each with a high degree of identity to SarA (33% & 30% respectively) (Arvidson & Tegmark, 2001) and is a positive activator of *agr* expression. *sarU* is repressed by SarT which has a knock on effect, repressing the expression of RNAII and RNAIII

of *agr* (Manna & Cheung, 2003).

MgrA (Rat/NorR.)

MgrA is a positive regulator of *agr* and also modulates α-haemolysin and protein A expression, in an *agr* independent manner, by binding directly to the *hla* (up-regulates) and *sarS* (down-regulates) promoters (Ingavale *et al.*, 2005). MgrA is also responsible for activation of *sarX* as well as the down regulation of *sarV* (Manna & Cheung, 2006; Manna *et al.*, 2004).

SarX.

sarX is activated by MgrA and is expressed maximally during stationary phase growth (Manna & Cheung, 2006). SarX binds to the *agr* intergenic promoter region with high affinity, and acts as a negative regulator of *agr* (Manna & Cheung, 2006).

SarZ.

SarZ positively regulates *hla* by binding to its promoter (Kaito *et al.*, 2006).

SarV (SarH4).

SarV (14 kDa) is a regulator of autolysis though its expression is low, or even undetectable, under *in vitro* growth conditions. The low expression levels can be accounted for by the fact that *sarV* is repressed by SarA and MgrA (Manna *et al.*, 2004).

Rot (Repressor of Toxins).

Rot (15.6 kDa) is a SarA homologue identified by McNamara *et al.* (2000). It was termed the 'repressor of toxins' as a mutation in *rot*, in an *agr*-null background, increased the production of α-haemolysin and other proteases. The downregulation of *hla* by Rot is mediated via the repression of *sae*, especially at the *sae* P3 promoter (Li & Cheung, 2008). Rot was later shown to be a global regulator of gene expression by Saïd-Salim *et al.* (2003) down regulating 60 genes and up-regulating 86 genes. Amongst the up regulated genes is *spa* which encodes protein A. Previous studies showed that *spa* is positively regulated by *SarS* (*SarH1*) (Tuomanen *et al.*, 2001a; Tegmark *et al.*, 2000). Using isogenic mutant strains lacking *rot* and *agr*, and *sarS* and *agr*, it was elucidated that Rot is required for *sarS* (*sarH1*) expression (Li & Cheung, 2008). Sigma factor B (σ^B) has a repressive effect on *rot* during the post exponential growth phase and the function of Rot (but not the transcription of *rot*) is repressed by Agr (Hsieh *et al.*, 2008).

1.7 Attenuating Toxin Expression.

S.aureus has the ability to rapidly adapt to new antibiotics (Figure 1.5 on page 22) and to re-infect patients with chronic disease (Hall, 2004). An alternative approach to the treatment of *S. aureus* infection is to identify the proteins that cause tissue damage and the manifestation of disease. For example in eczema particularly it has been shown that toxin production is directly related to lesion severity (Tomi *et al.*, 2005; Guzik *et al.*, 2005). These proteins can then be targeted either directly or at the genetic level, by reducing the transcription of their genes. It has been hypothisised that inhibiting

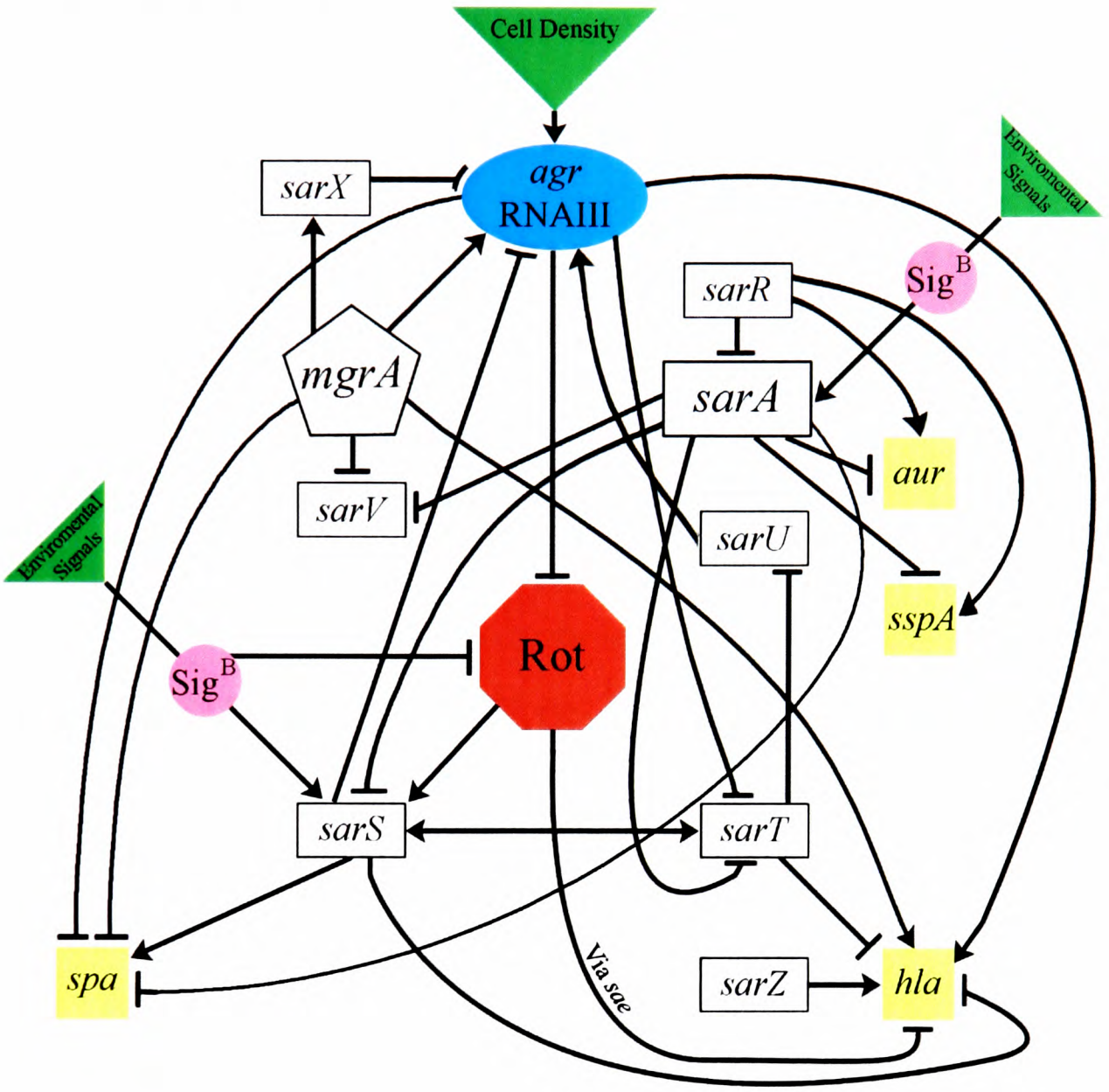

Figure 1.12: **Regulatory interactions involving SarA and its homologues**. SarA and its homologue's form a complex network of virulence regulators. The arrows indicate up-regulation and the bars down-regulation. Further information on the interactions can be found in § 1.6.3 on page 38. Figure is based upon Novick (2003a) with additional information from Cheung *et al.* (2008); Oscarsson *et al.* (2006); Cheung *et al.* (2007); Gustafsson & Oscarsson (2008); Li & Cheung (2008); Hsieh *et al.* (2008); Manna *et al.* (2004).

virulence, rather than growth, may impose weaker selective pressures than those imposed by current antibiotics (Clatworthy *et al.*, 2007). A treatment that lowers toxin expression may therefore have longer lasting benefits than one that kills bacteria as patients rapidly become recolonised when treatment ceases.

A number of methods have been proposed in the literature for the control of toxin expression in *S. aureus*.

1.7.1 The Agr & Autoinducing Peptides (AIPs).

The Agr groups (I–IV) are defined by the mutual ability of one group to cross inhibit the others (See § 1.6.1 on page 32 & Figure 1.11 on page 36). Interference from different groups has been shown to repress *agr in vitro* (Ji *et al.*, 1997). A reduction in virulence mediated via Agr group interference has been demonstrated *in vivo* using the tobacco hornworm (*Manduca sexta*) and clinical *S. aureus* strains (Fleming *et al.*, 2006).

As well as inhibition from groups of *S. aureus* (intraspecies), the *agr* response can be inhibited by pheromone's from closely related species (interspecies) such as *Staphylococcus epidermidis* (Kaufmann *et al.*, 2001b) and by more distantly related species such as *Pseudomonas aeruginosa* (Qazi *et al.*, 2006) (See § 1.7.4 on page 47). Synthetic peptides based upon the AIPs have also been tested (Novick, 2003a; Chan *et al.*, 2004). The use of synthetic peptides to control toxin expression is not without drawbacks. AIPs tend to be unstable, particularly at a physiological pH, with AIP II having an effective biological lifetime *in vivo* of 3 hours (Wright *et al.*, 2005a). The relatively short lifetime of the inhibitory AIPs may result in delayed *agr* activation, rather than complete blocking of function. Also co-colonisation of mutually inhibiting groups is often observed in disease states (Goerke *et al.*, 2003), so one group may be inhibited whilst another is at a sufficient density to become activated.

Inactivation or suppression of the *agr* results in an increased expression of adhesion factors (Shenkman *et al.*, 2002; Vuong *et al.*, 2000). This may result in an acute infection becoming a chronic one (Figure 1.11 on page 36).

1.7.2 Inhibition of RNAIII.

RNAIII is the effector of the *agr* response, activating transcription of secretory protein genes and repressing transcription of surface protein genes. RNAIII itself also contains a small open reading frame coding δ-haemolysin (*hld*).

Balaban *et al.* (2001) have suggested that a second quorum sensing system is present in *S.aurues* comprising of RAP (RNAIII activator protein) and its target TRAP (target of RNAIII activator protein). Interference of RAP would therefore lead to attenuated virulence. One method they have used is the small peptide RIP (RNAIII inhibiting peptide) which competes with RAP to bind TRAP and thus inhibit the RNAIII response (Gov *et al.*, 2001).

This work however has been shown to be controversial. Further studies by other groups have proposed that RIP, initially thought to be isolated from an exoprotein deficient mutant of the foggi strain, RN833 (Balaban & Novick, 1995) was in fact not RN833 but likely (85%) to be *S. warnerii*; further to this the inhibitory effect was not down to a second QS system but to an AIP like molecule produced by *S. warnerii* (Novick *et al.*, 2000).

1.7.3 Addition of Simple Solutes.

Incorporation of simple substances such as glucose or sodium chloride into media or creams is a cheap and potentially easy way to attenuate virulence. Glucose added to media *in vitro* has been shown to inhibit the Agr response, though this is thought to be as a result of glucose metabolism by-products lowering pH, rather than glucose *per se* (Regassa *et al.*, 1992).

It has long been known that *S. aureus* can tolerate high concentrations of NaCl (up to 10% w/v) with no inhibition of growth (Parfentjev & Catelli, 1964). It is

only more recently that the effects of these substances on virulence have been examined. Chan & Foster (1998) found that *S. aureus* strain 8325–4 grown in Brain Heart Infusion broth (BHI) containing 1M NaCl (~6%) had significantly decreased transcriptional expression of *spa*, *hla* and *tst* (encoding Protein A, α-hemolysin and toxic shock syndrome toxin) and that this was mediated via *agr*. It has been suggested that the effect of NaCl on toxin expression was due to osmotic stress placed on the bacteria. When Koprivnjak *et al.* (2006) examined the effect of NaCl, $MgCl_2$, $CaCl_2$ and sucrose on *S. aureus* they found that the *dlt* operon (responsible for D-Alanylation of the cell envelope associated teichoic acids) was transcriptionally repressed in response to high concentrations of Na^+ and moderate concentrations of $Mg2^+$ and $Ca2^+$ but not sucrose. This suggests the cells were responding to cation concentration rather than osmotic pressure.

1.7.4 Interfering Factors and Competition from other Organisms.

As discussed previously, *S. aureus* strains can be clustered by AGR type into four cross-inhibiting groups. These groups have been shown to inhibit *agr* function *in vitro* using supernatant or purified AIPs (Ji *et al.*, 1997; Jarraud *et al.*, 2000) and have also been shown to work in a more realistic mixed infection model *in vivo* (Fleming *et al.*, 2006).

In the natural environment, bacteria live in complex communities of varied species. It should be unsurprising therefore that various forms of signaling, cues, and coercion have evolved between species and that these may have the potential for therapeutic use.

Lactobacillus reuteri.

Lactobacillus reuteri (*L. reuteri*, previously known as *Lactobacillus fermentum*) is a Gram-positive bacterium that naturally inhabits the gut of mammals and birds. A particular strain, *L. reuteri* RC-14, was found to inhibit *S. aureus* infection in a rat surgical-implant model (Gan *et al.*, 2002). This effect appears to be independent of the broad-spectrum antibiotic reuterin which is produced as a by product of glycerol fermentation in some *L. reuteri* strains, but not RC-14 (Cadieux *et al.*, 2008). Further work has shown that *L. reuteri* RC-14 is capable of producing one or more molecules that inhibit the expression of both staphylococcal superantigen-like protein 11 (SSL11), a putative staphylococcal exotoxin, and RNAIII the effector molecule of the *agr* locus (Laughton *et al.*, 2006).

Pseudomonas aeruginosa.

Pseudomonas aeruginosa (*P. aeruginosa*) is a free living, Gram-negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae*. *P. aeruginosa* is increasingly recognised as an emerging opportunistic human pathogen of clinical relevance (Lyczak *et al.*, 2000), often coming into contact with *S. aureus* when infecting the lungs of cystic fibrosis patients. It has been hypothesised that during co-culture *P. aeruginosa* lyses *S. aureus*, gaining access to sequestered iron (Mashburn *et al.*, 2005).

Virulence and secondary metabolite production in *P. aeruginosa* is mediated via an N-acylhomoserine lactone (AHL) quorum sensing system[‡] . The two

[‡]Unlike in *S. aureus* where AIP is detected by a cell wall associated receptor (AgrC), autoinduction by AHL molecules occurs when an AHL signal binds to its cognate receptor inside the cell, at a threshold concentration of the signal which correlates to the number of bacterial cells present (Popat *et al.*, 2008).

main AHLs are the short-chain molecule, N-butanoyl-L-homoserine lactone (C4-HSL), and the long-chain molecule N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Telford *et al.*, 1998). The long chain molecule 3-oxo-C12-HSL present in spent culture supernatants from *P. aeruginosa* is capable of down-regulating exotoxin production in *S. aureus* by inhibiting *agr* and *sarA* (Qazi *et al.*, 2006). This is due to an interaction between 3-oxo-C12-HSL and the *S. aureus* membrane in a saturable, specific manner. The inhibitive effect was maintained when the acyl chain lengths were varied (Qazi *et al.*, 2006).

As well as interference caused by AHLs, *P. aeruginosa* produces another compound. 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), that is known to inhibit the growth of Gram-positive bacteria (Machan *et al.*, 1992). Interestingly, longterm exposure of *S. aureus* to HQNO has been shown to select for small colony variants (SCVs) (Hoffman *et al.*, 2006). SCVs produce small, non-pigmented and non-haemolytic colonies that exhibit atypical characteristics including reduced coagulase production, failure to use mannitol, and increased resistance to aminoglycosides, trimethoprim-sulfamethoxazole, cationic peptides, and cell-wall-active antibiotics (Kipp *et al.*, 2005).

Corynebacterium species.

Corynebacterium species live in a diverse range of ecological niches as well as making up part of the normal human skin flora. In a Japanese study Uehara *et al.* (2000) found that a wildtype strain of *Corynebacterium* was capable of replacing both MSSA and MRSA strains in the nares of *S. aureus* carriers. They hypothesised that this is due to competition for a specific attachment molecule, rather than any bactericidal effect (Uehara *et al.*, 2000). Whether or

not *Corynebacterium* can outcompete *S. aureus* outside of the nares has yet to be shown. The replacement of one species of bacteria with another may not be advisable in immunocompromised patients, as even the most benign of species may cause disease (Sotorra *et al.*, 2007).

Hydrogen peroxide (H_2O_2) producing species.

A number of bacterial species produce the strong oxidizing agent hydrogen peroxide (H_2O_2), among them are *Lactobacillus* species thought to protect pregnant women against vaginosis (Hillier *et al.*, 1993) and the viridans group *streptococci* which make up part of the normal flora in the oral cavity. Uehara *et al.* (2001b) found that newborns colonised with viridans group *streptococci* were significantly less likely to be subsequently colonised with MRSA and that this was partially attributed to H_2O_2 production (Uehara *et al.*, 2001a).

Lysostaphin.

Lysostaphin is a 27 kDa zinc metalloenzyme first identified in *S. simulans* and now produced commercially as a recombinant protein in *Bacillus sphaericus* (Schindler & Schuhardt, 1964; Trayer & Buckley, 1970). Lysostaphin lyses staphylococcal cells by hydrolysing glycylglycine bonds in the polyglycine bridges which form cross links between glycopeptide chains in the cell wall peptidoglycan (Kumar, 2008). The idea of using lysostaphin as a therapeutic agent in *S. aureus* infections has been around since the 1960s (Schaffner *et al.*, 1967; Zygmunt & Tavormina, 1972), though due to the unavailability of a pure recombinant protein at the time the idea was abandoned. Recently a number of animal model studies have shown the potential worth of lysostaphin as a treatment for bacteriemia (Kokai-Kun *et al.*, 2007), and the treatment of

neonatal *S. aureus* infections (Oluola *et al.*, 2007). Although there is certainly potential in the use of lysostaphin there are also limitations. Resistance can be obtained either through mutations in *femA* (*f*actor *e*ssential for *m*ethicillin resistance) or *lyrA* (*ly*sostaphin *r*esistance A) which change the binding site for lysostaphin, or through acquisition of the lysostaphin immunity factor (lif) found on the pACK1 plasmid (Kusuma *et al.*, 2007; Gründling *et al.*, 2006; Ehlert *et al.*, 2000).

1.7.5 Use of antibiotics at sub-inhibitory concentrations.

Subinhibitory (sub MIC) levels of antibiotics have been shown to cause numerous transcriptional changes in bacteria (Yim *et al.*, 2006; Tsui *et al.*, 2004). If these changes result in a reduction in toxin production, then there is a potential for therapeutic use. Ohlsen *et al.* (1998) examined the influence of subinhibitory doses of 31 antibiotics on the expression of *hla* and discovered: β-lactam antibiotics (n=17) caused an up-regulation of between 4–8 fold compared to a control, aminoglycosides (n=5) decreased expression significantly with tobramycin inducing a 54% reduction in expression, clindamycin reduced activity by 98%, erythromycin strongly repressed *hla* activity and the remaining antibiotics had little effect. Interestingly, clindamycin has been shown to extend the expression of Panton-Valentine leukocidin (PVL) mRNA in culture, though actual production of PVL is repressed (Stevens *et al.*, 2007). Linezolid, one of the few antibiotics capable of treating MRSA infections, has also been shown to attenuate a number of virulence factors including enterotoxins A & B, bifunctional autolysin, autolysin, protein A, and α- and β-haemolysins (Bernardo *et al.*, 2004). Antibacterials are not the only potential source for toxin inhibitors, cerulenin an antibiotic that inhibits fatty acid synthesis and

traditionally used as an antifungal (it may also induce apoptosis in certain human cancer cell lines, Moche *et al.*, 1999) has been shown to reduce exoprotein production via interference at the transcription level (Adhikari & Novick, 2005).

1.8 Conclusion.

There are many avenues of *S. aureus* research currently being undertaken, and still more to be discovered. *S. aureus* possesses many virulence factors, has the ability to rapidly evolve resistance, and is capable of contributing to multiple disease states. Because of these factors it is likely that one cure all, Panacea, is unattainable. Instead a number of specific, targeted, treatments will be required. This study endeavors to gain insights into the differences between *S. aureus* strains, establish links between toxicity and the underlying causes, and attempt the attenuation of toxicity with a specific view towards eczema treatment.

2

Materials & Methods.

2.1 Materials.

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

Item	Description	Supplier
Agarose	DNA/RNA gels	Fisher Scientific, UK
Antibiotics	Rifampicin, Tetracycline, Oxacilin, Fusidic acid	Sigma, UK
APS	Ammonium persulfate	Sigma, UK
BHI	Brain heart infusion	Oxoid, UK
Bis-polyacrylamide	30% polyacrylamide solution	Sigma, UK
$CaCl_2$	Calcium chloride	Sigma, UK
Chloroform	Solvent	Fisher Scientific, UK
Dream Taq	DNA polymerase and PCR reagents	Fermentas, UK
EDTA	Ethylenediaminetetraacetic acid	Sigma, UK
Ethanol	Solvent	Fisher Scientific, UK
EZBlue	Protein gel staining solution	Sigma, UK
FastRead counting chamber	Cell counting slide	Immune Systems, UK
FBS	Foetal bovine serum	Gibco, UK
Filter, 0.22μm	Syringe driven filter	Millipore, UK
GenElute	Mamalian total RNA purification kit	Sigma, UK
GPS solution	L-Glutamine-Penicillin-Streptomycin solution	Sigma, UK
Isopropanol	Solvent	Fisher Scientific, UK
KOH	Potasium hydroxide	Sigma, UK
LB	Luria-Bertani agar/broth	Sigma, UK
Lysostaphin	Cell wall lysing enzyme	Sigma, UK
Mouse anti-Hld	Monoclonal antibody against δ-haemolysin	Abcam, UK
NaCl	Sodium chloride	Sigma, UK
NaOH	Sodium Hydroxide	Sigma, UK
Opti4CN	Colorimetric horseradish peroxidase (HRP) substrate	BioRad, UK
PBS	Tissue culture grade Phosphate-buffered saline	Gibco, UK
Proteinase K	Broad-spectrum serine protease	Sigma, UK
rabbit anti-mouse-HRP	Horseradish peroxidase conjugated Polyclonal against mouse IgG	Abcam, UK
RNA Later	RNA stabilising agent	Ambion, UK
RNase	Ribonuclease	Ambion, UK
RNA Secure	RNA stabilising agent	Ambion, UK
RPMI	Roswell Park Memorial Institute cell culture media	Gibco, UK
RT-PCR Kit	One step reverse transcriptase PCR kit	Qiagen, UK
SDS	Sodium dodecyl sulfate	Sigma, UK
TAE	Tris-acetate EDTA buffer	Sigma, UK
TBS	Tris-Buffered Saline	Sigma, UK
TMED	N, N, N/, N/-tetramethylethylenediamine	Sigma, UK
Trypan Blue	Vital cell stain	Sigma, UK
TSA	Tryptone soya agar	Oxoide, UK
Turbo DNase	Fast acting DNase	Ambion, UK

Table 2.1: Materials used throughout thesis.

2.2 Bacteria.

2.2.1 Bacterial Strains.

Frequently used strains are listed in Table 2.2 on the next page. Wildtype isolates recovered from patients with invasive *S. aureus* disease (77 hospital acquired and 48 community acquired) and 81 isolates recovered from healthy individuals within Oxfordshire, United Kingdom, between 1997 and 1998 using a prospective case control design are described previously (Peacock *et al.*, 2002; Feil *et al.*, 2003; Roche *et al.*, 2003; Enright *et al.*, 2002). Details of these strains including T-cell toxicity data can be found in Appendix A on page 185.

2.2.2 Storage and growth.

All strains were routinely stored at -80°C in 25% glycerol stocks until needed. Strains were streaked onto either tryptone soya agar (TSA) or Luria-Bertani (LB) agar plates containing appropriate antibiotics where relevant. Single colonies were transferred to 5 ml of brain heart infusion (BHI) and propagated in a shaking incubator at 37°C to an optical density (OD) at 600 nm of 2.0.

2.3 T-cells.

Immortalised human T2 cells (from here on in described as T-cells) were obtained from the ATCC and described previously (Salter & Cresswell, 1986). T-cells were propagated in 50 ml aliquots of RPMI, supplemented with 10% heat inactivated foetal bovine serum (FBS), 1 mM L-glutamine, 200 units ml^{-1}

Strain	Description	Reference
8325–4	Lab strain NCTC8325 cured of three prophages	Novick (1967)
DU1090	8325–4 Δhla::Em^r	O'Reilly *et al.* (1986)
RN6390B	NTCC8325 cured of three prophages	Peng *et al.* (1988)
RN6911	RN6390B Δagr::*tetM*	Novick *et al.* (1993)
DU5719	β-haemolysin -ve Phage 42E lysogen of 8325–4	Patel *et al.* (1987)
RN4282	Wild type TSST-1 producing strain	Lindsay *et al.* (1998)
RN6938	RN4282 Δtst::*tetM*	Lindsay *et al.* (1998)
N315	MRSA strain isolated in Japan in 1982	Hiramatsu *et al.* (1992)
RN4220	Restriction mutant of RN450 capable of accepting foreign DNA	Kreiswirth *et al.* (1983)
BB4252	RN4220 with SCC*mec* II	This work
MRSA252	Hospital-acquired MRSA	Holden *et al.* (2004)
MSSA476	Invasive community-acquired MSSA strain	Holden *et al.* (2004)
MSSA3	Invasive community-acquired MSSA strain	Peacock *et al.* (2002)
MSSA16	Invasive community-acquired MSSA strain	Peacock *et al.* (2002)
MSSA117	Invasive hospital-acquired MSSA strain	Peacock *et al.* (2002)
MSSA383	Invasive hospital-acquired MSSA strain	Peacock *et al.* (2002)
MRSA720	Invasive community-acquired MRSA strain	Peacock *et al.* (2002)
MSSA859	Invasive community-acquired MSSA strain	Peacock *et al.* (2002)
MSSA3067	Carriage community-acquired MSSA strain	Peacock *et al.* (2002)
MSSA3105	Carriage community-acquired MSSA strain	Peacock *et al.* (2002)
MSSA3106	Carriage community-acquired MSSA strain	Peacock *et al.* (2002)

Table 2.2: Frequently used Bacterial Strains.

penicillin, and 0.1 mg ml^{-1} streptomycin, at 37°C in a humidified incubator with 5% CO_2 in air. Cells were split and transferred to new media every 48 hours.

2.4 T-cell Toxicity Assay.

The T-cell toxicity assay was designed *de novo*. Conditions of use were optimised before experimental work began. The number of T-cells and bacterial cells were examined, as was the toxicity of cells at different points of the growth curve and with different incubation times. Depending upon the experiment, two different assays were employed, one utilising washed bacterial cells and the

other using bacterial culture supernate containing exogenous toxins.

2.4.1 Method A — Washed Bacterial Cells.

T-cells were propagated in T-cell media for 48 hours then harvested by centrifugation, 5 minutes at 700g, and gently washed and re-suspended in 1 ml of warm tissue culture grade phosphate buffered saline (PBS). Washing was repeated to ensure all antibiotics were removed. T-cells were diluted in PBS to a final cell concentration of 3.2 x 10^6 ml^{-1}. Fifteen microliters of cells were transferred to a sterile microfuge tube for each assay undertaken.

S. aureus strains were propagated to an OD 600 of 2.0 in 5 ml BHI, or BHI supplemented with 1 M NaCl. 750 μl cells were harvested by centrifugation, 1 min at 15,000g, and the supernatant discarded. Cells were washed and re-suspended in 750 μl tissue culture grade PBS to remove exogenous toxins, for a total of two washes. After the final wash the cells were diluted 1:2 in PBS and 15 μl added to a microfuge tube containing T-cells. Each tube was gently mixed and incubated for 30 minutes at 37°C.

In each experiment 8 *S. aureus* isolates and a control (PBS and T-cells) were used. The number of T cells lysed by the *de novo* production of *S. aureus* proteins was measured by addition of 15 μl of 0.4% trypan blue. Cells were counted as, alive (white) and dead (blue) in a Fast-Read counting chamber. Two grids per sample were counted and the percentage survival calculated.

2.4.2 Method B — Exogenous Toxins.

T-cells were propagated in T-cell media for 48 hours then harvested by centrifugation, 5 minutes at 700g, and gently washed and re-suspended in 1 ml

of warm tissue culture grade PBS. Washing was repeated for a total of two washes. T-cells were diluted in PBS to a final cell concentration of 3.2 x 10^6 ml^{-1}. 15 μl of cells were transferred to a sterile microfuge tube for each assay undertaken.

S. aureus strains were grown to an OD of 2.0 in 5 ml BHI. 750 μl of cells were harvested by centrifugation, 2 min at 15,000g. 15 μl of supernate was removed and added to the microfuge tube containing T-cells. Each tube was gently mixed and incubated for 10 minutes at 37°C. In each experiment 8 *S. aureus* isolates and a control (PBS and T-cells) were used. The number of T-cells lysed by the exogenous proteins produced by *S. aureus* whilst in the media was measured by addition of 15 μl of 0.4% trypan blue and the enumeration of alive (white) and dead (blue) T-cells in a Fast-Read counting chamber. Two grids per sample were counted and the percentage survival calculated.

2.5 Western Blot.

Protein Isolation.

Starter cultures were grown overnight in 2 ml BHI. 100 μl of starter culture was used to inoculate 100 ml BHI in 250 ml flasks. Flasks were incubated at 37°C and shaken at 200 RPM for 14 hours. 1 ml of cells were transferred to sterile microfuge tubes and harvested by centrifugation, 2 min at 15,000g. Cells were washed in PBS, re-harvested by centrifugation and re-suspended in 140 μl dH_2O. 1 μl of DNase, 1 μl of RNase, 3 μl of lysostaphin, and 10 μl of 10% SDS was added to cells, vortexed, and incubated at 37°C for 30 minutes to lyses cells and degrade DNA/RNA. Cell lysate was centrifuged at 15,000g, 4°C, for 15 minutes to pellet cellular debris. Supernate was transferred to sterile tubes

for use in SDS-PAGE.

SDS-PAGE.

A 15% resolving gel was made using: 3.75 ml 1 M Tris, pH8.8; 100 μl 10% SDS (Sodium Dodecyl Sulfate); 5 ml 30% Bis-polyacrylamide; 1.5 ml dH_2O; 100 μl APS (Ammonium persulfate); and 6 μl TMED (N, N, N′, N′-tetramethylethylenediamine). A stacking gel was made using: 375 μl 1 M Tris, pH6.8; 30 μl 10% SDS; 355 μl 30% polyacrylamide; 2.25 ml dH_2O; 30 μl APS; and 5 μl TMED.

Ten microliters of sample was combined with 5 μl loading dye and run in a gel alongside a protein ladder at 200V for 25 minutes.

Western Blotting.

SDS-PAGE gels were run in duplicate. One gel was stained using the EZBlue^{TM} gel staining reagent, as per manufacturers instructions, to visualise total protein bands. Proteins from the remaining gel were transferred to a nitrocellulose membrane using a semi-dry transfer cell. The nitrocellulose membrane with transferred protein was blocked with a 1% BSA blocking solution overnight at 4°C. The membrane was washed 3 times in TBS for 5 minutes. The membrane was incubated for 1 hour at room temperature in a 1:10,000 dilution of primary antibody, mouse anti-Hld, washed 3 times in TBS for 5 minutes, then incubated for 30 minutes at room temperature in a 1:10,000 dilution of secondary antibody, rabbit anti-mouse-HRP. The membrane was washed 3 times in TBS for 5 minutes then developed using the Opti4CN^{TM} substrate reagent kit as per manufacturer's instructions enabling visualisation of a specific Hld band.

2.6 RNA Extraction.

Samples to be used for RNA extractions were harvested by centrifugation, resuspended in RNA Later and stored at -80°C until needed. Samples stored in RNA Later were thawed and harvested by centrifugation, 15,000g for 1 minute, supernatant was discarded. Cells were washed with 500 μl of 50 mM EDTA before being harvested by centrifugation, 15,000g for 1 minute, and the supernatant discarded. Cells were resuspended in 100 μl of 50 mM EDTA containing 7 μl of lysostaphin, 10 mg ml^{-1}, briefly vortexed, and incubated at 37°C for 2 minutes to lyse cells. The remaining steps were carried out using the GenElute Mammalian Total RNA Purification Kit. 40 μl ml^{-1} of RNA secure was added to the elution solution and the kit used in accordance with the manufacturers instructions. Any residual DNA was removed using Turbo DNase in accordance with the product instructions. RNA purity was checked by running 5 μl of pure, total RNA on a 1% agarose gel.

2.7 Polymerase Chain Reaction.

Primers used for the polymerase chain reaction (PCR) and reverse transcriptase-PCR are listed in Table 2.3 on the next page. For each of the primers the optimal temperature and number of cycles was established. Thermocycler conditions are listed in Tables 2.4 on page 62, 4.6 on page 109 & 2.6 on page 62.

Gene	Forward Primer 5′–3′	Reverse Primer 5′–3′	Product Size
hla†	AGCGAAGTCTGGTGAAAACC	GGCCTTATTGGTGCAAATGT	288 bp
sec‡	TGTATGTATGGAGGTGTAAC	AATTGTGTTTCTTTTATTTTCATAA	102 bp
gyrB†	TCCACAAGTCGCACGTACAG	TCCATCCACATCGGCATCAG	415 bp
SCC*mec*‖	GGCCAACGAATACTAGCCAA	TGCACTTTTAACTGCTTGGG	764 bp

Table 2.3: **PCR Primers used in this study.** † Sequences derived from *S. aureus* N315 genomic DNA, GenBank accession BA000018. ‡ described previously by Sharma *et al.* (2000). ‖ Primers overlap the SCC*mec* II junction in MRSA252, GenBank accession BX571856.

PCR mix.

Reaction mix containing 0.5 μl genomic DNA in water, 0.25 μl Fermentas Dream Taq, 2.5 μl Fermentas Dream Taq 10X buffer, 1 μl dNTP mix (containing 10 mM each of dATP, dCTP, dGTP, and dTTP), 0.5 μl each of forward and reverse primers (25 pM μl^{-1}), and RNase/DNase free water to bring the total reaction volume to 25 μl per sample.

RT-PCR.

RNA samples were balanced by spectrophotometry. Readings at an absorbance of 260nm were taken to derive the nucleic acid content and samples were diluted accordingly

A one-step RT-PCR kit containing 5 x reaction buffer, RT-PCR enzyme mix, dNTP's and RNase free water was used. Up to 3 μl of RNA was used per reaction and added to 5 μl 5 x buffer, 1 μl mixed enzyme, 1 μl dNTP mix (containing 10 mM each of dATP, dCTP, dGTP, and dTTP), 0.5 μl each of forward and reverse primers (25 pM μl^{-1}), and RNase free water to bring the total reaction volume to 25 μl per sample.

Step	Function	Temperature	Duration
1	Reverse Transcription	50°C	30 minutes.
2	Activation of DNA Polymerase and inactivation of reverse transcriptase	95°C	15 minutes.
3	Denaturation	94°C	30 seconds.
4	Annealing	53°C	30 seconds.
5	Elongation	72°C	45 seconds.
6	Go to step 3		25 times.
7	Final Elongation step	72°C	3 minutes.
8	Storage	4°C	Forever.

Table 2.4: **Alpha haemolysin (*hla*) RT-PCR cycle.**

Step	Function	Temperature	Duration
1	Reverse Transcription	50°C	30 minutes.
2	Activation of DNA Polymerase and inactivation of reverse transcriptase	95°C	15 minutes.
3	Denaturation	94°C	30 seconds.
4	Annealing	50°C	30 seconds.
5	Elongation	72°C	45 seconds.
6	Go to step 3		25 times.
7	Final Elongation step	72°C	3 minutes.
8	Storage	4°C	Forever.

Table 2.5: **Staphylococcal enterotoxin C (*sec*) RT-PCR cycle.**

Step	Function	Temperature	Duration
1	Initial denaturation	95°C	1 minute.
2	Denaturation	94°C	30 seconds.
3	Annealing	55°C	30 seconds.
4	Elongation	72°C	45 seconds.
5	Go to step 2		25 times.
6	Final Elongation step	72°C	3 minutes.
7	Storage	4°C	Forever.

Table 2.6: **SCC*mec* junction PCR cycle.**

2.8 Gel Electrophoresis and Densitometry.

For the separation and viewing of PCR products 2% agarose gels were used. Gels were made by boiling 3 g of agarose with 150 ml Tris-acetate EDTA buffer (TAE) until the agarose was fully dissolved. 3 μl of ethidium bromide was added to the molten gel to enable visualisation. Samples, controls and an appropriate DNA ladder (Q-BIOgene) were run at 90 Volts. Gels were visualised using a standard 'Gel Doc' system. Densitometric analysis was performed using the Quantity One 1D analysis software from Bio-Rad.

2.9 Statistical Analysis.

Before analysis all data were checked to determine if parametric statistics could be used. If the data were 'normal' (i.e. following a normal distribution) then an appropriate statistical analysis was carried out i.e. general linear models (GLM) with post hoc Turkey pairwise comparisons, or two tailed T tests. If the data were not normal but could be made normal by a transformation (e.g. square-root arcsine transformation — a standard transformation for percentage data) then this was employed and parametric tests used. If the data could not be normalised then non-parametric tests were performed to determine significant ($\alpha = 0.05$) difference (e.g. Mann-Whitney test).

2.10 Relative Fitness Assay.

Two methods were employed for the relative fitness assays, modified from Lenski *et al.* (1991), one utilising diluted BHI media and the other utilising spent (pre-cultured) BHI media. Both of these methods were designed to

place *S. aureus* in a more demanding environment than standard rich media, thus making relative fitness differences more distinguishable. Spent media was employed for two reasons: firstly, to reduce nutrients available to *S. aureus* and secondly, so that the endogenous auto inducing peptides (AIPs) present in the media would activate the Agr system before a high density of cells was reached, increasing the metabolic burden (See § 1.6.1 on page 32).

In both cases the fitness of a strain was defined as a measure of the reproductive success of the population, which can be expressed as the natural logarithm of the ratio of the final and initial cell densities of the culture.

$$\text{relative (Darwinian) fitness } = \frac{ln[A(1)/A(0)]}{ln[M(1)/M(0)]}$$

where:

$A(0)$ = Estimated density of test strain at time 0

$M(0)$ = Estimated density of Marker strain at time 0

$A(1)$ = Estimated density of test strain at time 1 day

$M(1)$ = Estimated density of Marker strain at time 1 day

ln = natural logarithm (logarithm to the base e)

2.10.1 Method A, 25% BHI media.

Test and marker (MSSA466 — resistant to tetracycline) strains were propagated overnight in BHI to an OD 600 of 2.0. This insures that all cells are in a similar physiological state at the start of the experiment. Fresh BHI was diluted 1:4 with dH_2O and inoculated with 10^3 cfu ml^{-1} of the marker strain and a test strain. Initial cell numbers were confirmed by plating. Competitions were

undertaken at 37°C in a shaking incubator (160 RPM) for 24 hours. Final cell numbers were enumerated by serial dilutions on TSA plates (total cell count), and TSA plates containing 2 μg ml^{-1} tetracycline (marker strain count). Relative fitness was determined using the starting and final cell numbers for the two strains.

2.10.2 Method B, pre-cultured BHI media.

100 ml of BHI was inoculated with strain 8325–4 and propagated at 37°C in a shaking incubator (180 RPM) for 24 hours. Cells were harvested by centrifugation and supernatant filter sterilised with a 0.22 μm syringe driven filter. The sterile supernate was used instead of diluted BHI. The protocol was followed as in method A.

2.11 Mutation Rate.

Strains were propagated overnight in BHI to an OD 600 of 2.0 to ensure all cells were in an equivalent physiological state. Fresh BHI was inoculated with approximately 10^3 cfu ml^{-1} and incubated at 37°C in a shaking incubator (180 RPM) for 24 hours. Representative samples were serial diluted and plated on TSA plates to determine the total cell count. Two 1 ml samples were taken from each strain (and where isogenic strains were used, replicate) and harvested by centrifugation. Pellets were re-suspended in 200 μl sterile PBS, 100 μl aliquots were spread onto rifampicin (30 mg l^{-1}) TSA plates and/or fusidic acid (10 mg l^{-1}) TSA plates. Plates were incubated for 24 hours at 37°C and the colony forming units enumerated to determine the number of mutants per 1 ml broth.

Mutation rates were determined using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) Method (Sarkar *et al.*, 1992; Ma & Sarkar, 1992). Maximum-likelihood methods use all the data from an experiment to estimate the m (number of mutations per culture) most likely to have produced the observed distribution. Thus, they are the gold standard for estimating m (Rosche & Foster, 2000).

The MSS-MLE method uses an initial estimate of m to generate the probability of observing r mutants on selective medium, pr (Eq. (1)). The likelihood function is the product of the probabilities for each observed value of r (Eq. (2)). The value of m is then adjusted until the likelihood function reaches a maximum (Sarkar *et al.*, 1992; Ma & Sarkar, 1992). The mutation rate (μ), that is the probability of a cell sustaining a mutation during its lifetime, was assumed to be distributed evenly over a cells division cycle (Luria & Delbrück, 1943). It was therefore defined as m/N_t, where N_t represents the average of the cell counts across the cultures. Confidence intervals are calculated according to the method of Stewart (1994) who discovered that the natural log of m is normally distributed when using the MSS-MLE method. The confidence intervals are derived from an approximation of this distribution (Eq. (3)) (Stewart, 1994). The MSS-MLE and confidence intervals were calculated using the *F*luctuation *A*na*L*ysis *C*alculat*OR* — FALCOR (`http://www.keshavsingh.org/protocols/FALCOR.html`).

$$p_0 = e^{-m}; \quad p_r = \frac{m}{r}\sum_{i=0}^{r-1}\frac{p_i}{(r-i+1)} \qquad (1)$$

$$f(r|m) = \prod_{i=1}^{c} f(r_i|m) = (p_0^{c_0})(p_1^{c_1})(p_2^{c_2})\ldots(p_{rmax}^{c_{rmax}}) \qquad (2)$$

$$CL_{+95\%} = ln(m) + 1.96\sigma(e^{1.96\sigma})^{-0.315}$$
$$CL_{-95\%} = ln(m) - 1.96\sigma(e^{1.96\sigma})^{+0.315} \quad ;\text{where } \sigma_{ln(m)} \approx \frac{1.225m^{-0.315}}{\sqrt{C}} \qquad (3)$$

2.11.1 Statistical analysis.

The observations made by Stewart (1994) (that the natural log of m is normally distributed) implies that normal statistics can be used with the $ln(m)$ values determined by the MSS-MLE method. Whether two $ln(m)$ values are significantly different can therefore be determined by a standard t test (Eq. (4)) (Rosche & Foster, 2000). Differences in N_t are not considered in Equation (4) and cultures with differing cell numbers cannot be compared. To take into account different cell numbers Equation (5) was used. The value of t was then used to determine the level of significance (P-value) in a standard two-tailed t table with the degrees of freedom of $C1 + C2 - 2$.

$$t = \frac{ln(m_1) - ln(m_2)}{\sqrt{\sigma_1^2/C_1 + \sigma_2^2/C_2}} \quad t_{0.05(2),(C1+C2-2)} \qquad (4)$$

$$t = \frac{ln(\mu_1) - ln(\mu_2)}{\sqrt{\sigma_1^2/C_1 + \sigma_2^2/C_2}} \quad t_{0.05(2),(C1+C2-2)} \qquad (5)$$

Corrections for Multiple Comparisons.

As more tests are performed on a data set the chance of a type I error (i.e. rejecting the null hypothesis when it is true) increases. To correct for this the alpha level ($\alpha = 0.05$) was reduced using the Šidàk Equation (6) (Abdi, 2007) where α[PT] is the probability of making a type I error in an individual test (alpha per test or testwise alpha), α[PF] denotes the probability of making a type I error on the family of tests (alpha per family, familywise or experi-

Term	Definition
r	observed number of mutants in a culture
m	number of mutations per culture
p_0	proportion of cultures without mutants
p_r	proportion of cultures with r mutants
μ	mutation rate
N_t	final number of cells in a culture
C	number of cultures
σ	standard deviation
$\prod$	product (to be multiplied)
ln	natural logarithm (logarithm to the base e)

Table 2.7: **Definition of terms.**

mentwise alpha), and C is the number of tests. The Šidàk Equation is often simplified to the Bonferonni Equation (7) as it is easier to compute by hand (Abdi, 2007), though the Šidàk Equation shall be used in this instance.

$$\alpha[PT] = 1 - (1 - \alpha[PF])^{\frac{1}{C}} \quad (6)$$

$$\alpha[PT] \approx \frac{\alpha[PF]}{C} \quad (7)$$

The Šidàk Equation (6) was applied in a simple, sequentially rejective procedure as described in Holm (1979). For example if 6 independent tests are carried out on a data set, then the 6 P values obtained are arranged in order from most to least significant. Multiple α values are obtained using the Šidàk equation with C=6, C=5...C=2. If the lowest P value is $\leq$ to the lowest alpha value (for 6 tests this would be 0.0085) then H_0 is rejected and the second lowest P value compared with the second lowest alpha value (which would be 0.0102). This is continued until a P value is greater than its adjusted alpha value, whereby the null hypothesis is accepted for it and subsequent hypotheses, or until all null hypothesis are rejected.

2.11.2 Jackpots.

Due to their inherently stochastic nature, mutations may arise early in the growth period. This produces a large clone of identical descendants, resulting in a so called 'jackpot' culture (Saunders *et al.*, 2003). Jackpot cultures can have a large effect on methods of determining the mutation rate that utilise the mean. As the median is used in these experiments the effects of jackpot cultures are limited, however, where the number of mutants were non-determinable (due to large number) they were discarded from analysis.

2.12 DNA Purification.

Genomic DNA from bacterial cells was extracted using a modified method from Pospiech & Neumann (1995).

Cells were propagated in 30 ml BHI at 37°C in a shaking incubator (180 RPM) overnight. Cells were split into two 15 ml aliquots, harvested by centrifugation and resuspended in 5 ml SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). 30 μl lysostaphin (10 mg ml^{-1}) was added and incubated at 37°C for 1 hour. 1/10 volume of 10% SDS and 0.5 mg ml^{-1} proteinase K were added and incubated at 55°C for 2 hours with occasional inversion. 1/3 volume of 5 M NaCl and 1 volume chloroform were added and incubated at room temperature for 30 minutes with frequent inversion. Samples were centrifuged at 4500g for 15 minutes and the aqueous phase transferred to a new tube using a blunt ended pipette tip. DNA was precipitated by addition of 1 volume isopropanol and transferred to a microfuge tube. DNA was washed with 70% ethanol before being air dried and resuspended in DNase free water.

2.13 $CaCl_2$ Transformations.

Solutions used for $CaCl_2$ Transformations were:

LB, BHI, Physiological saline (9 g NaCl L^{-1}), Tris-Maleate buffer pH 7 (solution A 47.4 g L^{-1} Tris-Maleate, Solution B 0.2 N NaOH. 250 ml solution A, 240 ml solution B and 510 ml ddH_2O were combined and the pH adjusted to 7 with KOH.), Tris-Maleate Ca (as Tris-Maleate plus $CaCl_2$ to a final concentration of 0.1 M).

Strains to be transformed were grown on LB agar plates at 37°C overnight. Cells were suspended in 1 ml saline and diluted in LB broth to a final OD 540 nm of 0.2. LB broth with bacteria were diluted 1:10 in fresh LB to a final volume of 100 ml per transformation. Cells were propagated at 35°C in a shaking incubator (120 RPM) to an OD 540 of 0.2 ($\approx$ 3 hours). Cells were harvested by centrifugation at 4°C, pooled and washed with 10 ml cold Tris-Maleate buffer. Cells were again harvested by centrifugation at 4°C and resuspended in 1 ml, per 100 ml culture, Tris-Maleate-Ca buffer. 100 μl of DNA (100–500 μg ml^{-1}) previously extracted as per Method 2.12 on the preceding page was placed in 15 ml sterile tubes on ice and 1 ml cells added. Cells and DNA were incubated for 2.5 minutes on ice followed by 2.5 minutes in a 35°C water bath. Cells were harvested by centrifugation at room temperature and resuspended in 1 ml BHI before being incubated at 37°C for 1 hour. Cells were again harvested by centrifugation at room temperature and resuspended in 200 μl physiological saline. 100 μl aliquots were plated on LB plates containing 5 μg ml^{-1} oxacillin and incubated at 37°C for up to 48 hours. Any colonies that grew were picked and streaked onto fresh oxacillin 250 μg ml^{-1} LB agar plates.

3

Toxicity of Genetically Diverse *S. aureus* Strains.

3.1 Abstract.

Staphylococcus aureus is a major human pathogen capable of secreting toxins that significantly contribute to host damage (Ferry *et al.*, 2005). It is responsible for approximately 32–47% of all skin/soft tissue infections and is the leading cause of infection of the bloodstream and lower respiratory tract (Diekema *et al.*, 2001). Here we examined the ability of 207 clinical isolates of *Staphylococcus aureus* with known genetic and phenotypic characteristics to lyse T cells. We found a broad range of toxicities amongst the isolates, a significant difference between the AGR groups, that beta-haemolysin is involved and that methicillin-resistant *S. aureus* strains are less toxic than methicillin-susceptible *S. aureus* strains. A significant interaction between Toxic Shock Syndrome Toxin-1

(TSST-1) and delta-haemolysin was also observed.

3.2 Introduction.

Genomes of sequenced *S. aureus* strains reveal a range in size from 2.820–2.903 million base pairs, predicted to contain between 2592 and 2748 genes (Lindsay & Holden, 2006). Approximately 78% of the genome incorporates genes present in all strains, and forms the core genome. Genes relating to essential metabolic and housekeeping functions are primarily found in the core genome, alongside certain species specific virulence factors. The remaining genome has been termed the 'accessory genome' as it shows great plasticity and incorporates nonessential genetic information. The accessory genome principally consists of mobile (or once mobile) genetic elements that show a strong tendency to transfer horizontally between strains (Lindsay & Holden, 2004). These elements include: bacteriophages and temperate phage (prophage that integrate their DNA into that of the host through lysogeny are common in *S. aureus* with most strains carrying at least one), *S. aureus* pathogenicity islands (SaPI), staphylococcal chromosomal cassettes (SCC), genomic islands (defined as a relatively large chromosomal region acquired from another bacterium by lateral gene transfer (Ala'Aldeen & Hiramatsu, 2004)), plasmids, and transposons (Lindsay & Holden, 2006; Fitzgerald *et al.*, 2001). The transposons, plasmids and SCCs tend to carry resistance genes, whilst phages and SaPIs typically carry virulence genes.

The plasticity of the accessory genome has enabled *S. aureus* to adapt to multiple niches. Strains with specific accessory genes have been linked to different geographical areas and to different diseases (Lindsay & Holden, 2006; Kuroda *et al.*, 2001). An example is hospital acquired MRSA (HA-MRSA);

HA-MRSA strains predominantly contain the large (53–66.9 kb) type II or III SCC*mec* element conferring multi-drug resistance, and enabling MRSA to become endemic in many hospitals (Jones, 2003; Ito *et al.*, 2001). Recently emerged community acquired MRSA (CA-MRSA) strains contain a significantly smaller SCC*mec* element, typically type IV or V (20.9–29 kb) (Ma *et al.*, 2002). The smaller SCCmec elements provide resistance to β-lactam antibiotics only. The reason HA-MRSA has remained in a hospital setting and not spread widely into the community during the last half century is not fully understood, though it may be due to costs associated with the large SCC*mec* elements (See Chapter 6 on page 135 & Chapter 7 on page 153).

It has been proposed that CA-MRSA strains are more virulent than HA-MRSA (Chambers, 2001; Etienne, 2005), making them phenotypically and genotypically distinct, however, until specific characteristics are identified and isogenic mutants constructed it is difficult to definitively test this.

Studies linking disease characteristics with specific genes in *S. aureus* are often hampered by the difficulty of genetically manipulating clinical isolates (Waldron & Lindsay, 2006), the fact that laboratory strains are no longer a faithful representation of those causing disease today (Grundmeier *et al.*, 2004), and the fact that *S. aureus* often has multiple genes whose activities can be substituted for by other genes (Greene *et al.*, 1995).

In this study an alternative approach was adopted whereby a large, genetically diverse, *S. aureus* strain collection (n=207) was assayed for specific characteristics of each isolate using a T-cell toxicity assay developed specifically for the purpose. Washed bacterial cells were incubated with T-cells for 30 min (alternatively supernatant from the bacterial cells was incubated with T-cells for 10 min) and the number of T-cells lysed by the *de novo* production of *S. aureus* proteins enumerated (See Method 2.4 on page 56 for full details).

A large amount of genetic information relating to this strain collection was available, including: Agr type, clonality as determined by MLST (multilocus sequence typing—strains were considered to be of the same clonal cluster [CC] if 5 out of the 7 sequenced housekeeping genes were identical), the presence or absence of 33 adhesins and toxins, and the antibiotic resistance profile (Feil *et al.*, 2003; Peacock *et al.*, 2002; Roche *et al.*, 2003) (Table 3.1, See Appendix A on page 185 for full details). This enabled associations between the known factors and toxicity to be determined. To confirm the data from the clinical strains a collection of isogenic mutant strains were also employed.

Bacterial determinant	Putative function
Adhesins:	
FnBPA	Adhesin for fibronectin
ClfA & ClfB	Adhesins for fibrinogen
Protein A	Binds to Fc region of immunoglobulin & Von Willibrand factor
Cna	Adhesin for collagen
SdrC, SdrD & SdrE	Unknown; putative adhesins
Bbp	Adhesin for bone sialoprotein
EbpS	Adhesin for elastin
Map/Eap	Major histocompatibility complex class II analogue protein
Toxins:	
TSST-1[a]	Exotoxin with superantigen activity
Enterotoxins A, B, C, D, E, G, H, I, and J	Exotoxins with superantigen activity
Exfoliative toxins A and B	Exotoxins with superantigen activity
α-haemolysin	Cytolytic pore-forming toxin
β-haemolysin	Sphingomyelinase
δ-haemolysin	Cytolytic toxin
Panton-Valentine leukocidin	Bicomponent leukocidin
γ-haemolysin	Bicomponent leukocidin
Others:	
ica locus	Polysaccharide intercellular adhesin
Coagulase	Binds prothrombin, activating conversion of fibrinogen to fibrin
Efb	Binds to fibrinogen
V8 protease	Serine protease

Table 3.1: Bacterial determinants examined in this study. [a]Toxic Shock Syndrome Toxin. Modified from Peacock *et al.* (2002).

3.3 Results.

The toxicity of a large number of *S. aureus* strains were assayed *in vitro* using a T-cell toxicity assay. Information on the isolates including their *agr* type, virulence profile, antibiotic profile and origin was available (Table 3.1 on the previous page), enabling a detailed analysis to be performed and facilitating the identification of factors involved in toxicity. The strain associated factors and raw results can be found in Appendix A on page 185. A frequency histogram of the toxicity data can be found in Figure 3.1.

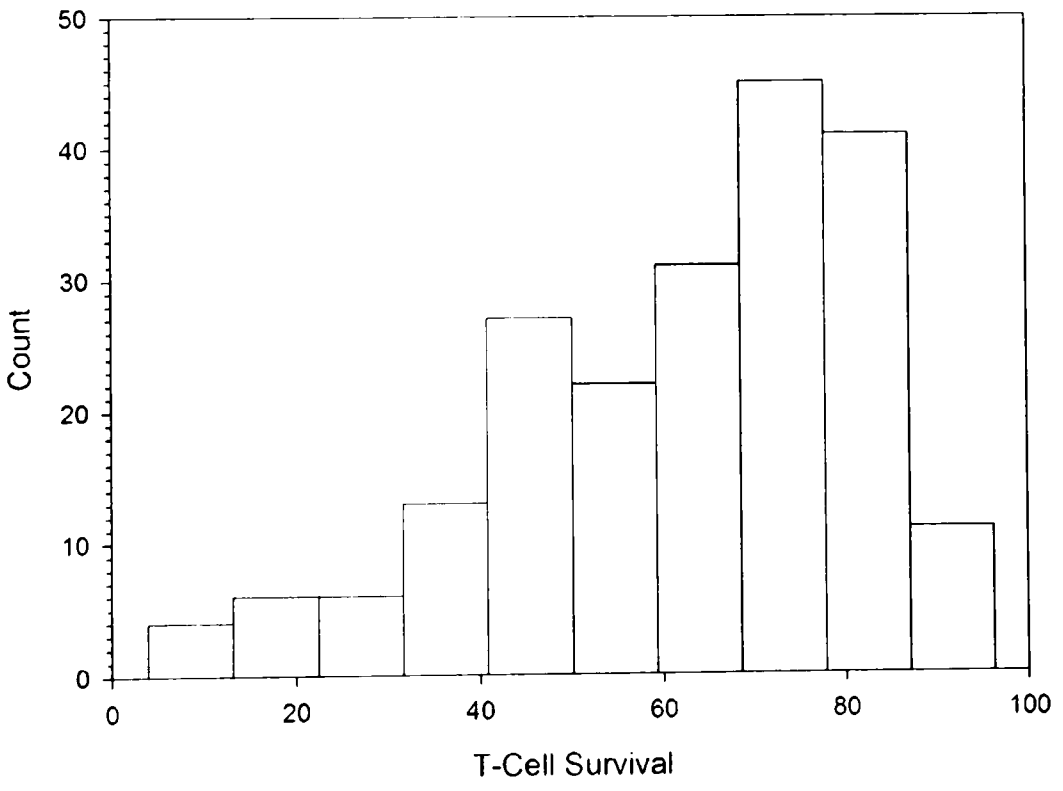

Figure 3.1: **Frequency histogram of toxicity data.**

3.3.1 Statistics.

Data were square-root arcsine transformed (a standard transformation for percentage data), and then squared because of left skew. A maximal general linear model (GLM) with interactions was made and non-significant terms removed, leaving the minimal significant model. The model found that Agr group (P<0.001) and β-haemolysin (P<0.001) were significant factors, and that a significant interaction between Toxic Shock Syndrome Toxin-1 (TSST-1) and δ-haemolysin (P=0.048) was present.

Source	DF	Seq SS	Adj SS	Seq MS	F	P
agr	3	2.8604	1.3449	0.9535	8.17	0.000
beta	1	1.5821	0.4064	1.5821	13.55	0.000
tst	1	0.0023	0.0052	0.0023	0.02	0.889
delta	1	0.1269	0.1026	0.1269	1.09	0.299
tst*delta	1	0.4628	0.4628	0.4628	3.96	0.048
Error	193	22.5339	22.5339	0.1168		
Total	200	27.5684				

3.3.2 Accessory Gene Regulator (Agr) Group.

The Agr system is a global regulator of *S. aureus* toxin expression, forming part of a complex regulatory network (See § 1.6.1 on page 32). The Agr system can be divided into at least four specificity groups (I–IV). Groups are defined by the ability of auto-inducing peptides (AIPs) (Figure 1.10 on page 35) from one group cross inhibiting heterologous Agr groups.

The Agr group is considered to have a biological significance as groups are predicted to correlate with specific biotypes and disease states. The Agr groups in this study were shown by statistical analysis to play a part in *S. aureus* toxicity (Figure 3.2). To tease apart the GLM information, Tukey post hoc tests were used. Agr group I strains were determined to be significantly more toxic than AGR group II strains (P=0.0306, T= 2.775). There was no significant difference between the other groups.

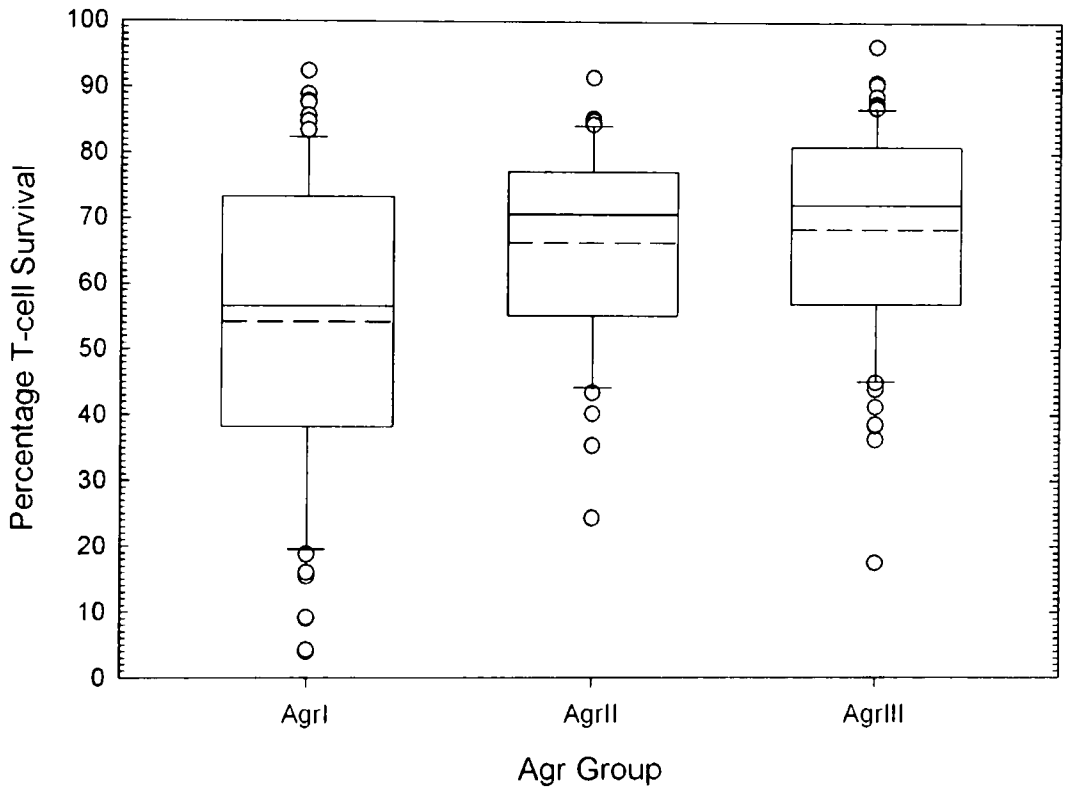

Figure 3.2: **Agr group I strains are significantly more toxic than AGR group II.** Percentage T-cell Survival by Agr group. 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (○). AgrI (n=77), AgrII (n=47), AgrIII (n=75), AgrIV (n=5) is not included due to small sample size. Agr grouping was shown to be significant factor in T-cell toxicity (P<0.001) using a general linear model. AgrI has significantly higher toxicity than AgrII (P=0.0306, T= 2.775) as shown by Tukey post hoc test.

3.3.3 Clonality of Strains.

The Agr group of a strain is strongly associated with clonality. Therefore, toxicity of the clonal clusters (CCs 1–51) was examined. T-cell survival rates varied from 45.5% +/- SE 6.79 (CC16) to 72.2% +/- SE 5.85 (CC1) amongst the clusters. Using the median value (62.3%) to dichotomize the data into high and low toxicity, it was found that CCs 1, 12, 15, 22, 30, 39, and 51 were all in the low-toxicity range, with T-cell survival rates of 72.7, 72.1, 67.8, 62.4, 68.2, 62.7, and 67.9%, respectively. CCs 5, 8, 9, 16, 25, and 45 were all in the high-toxicity range, with T-cell survival rates of 59.1, 58.9, 60.4, 45.5, 52.9, and 56.6%, respectively. Only one CC of AgrI fell into the lower toxicity range, and that was CC22, the toxicity of which fell only just above the point of dichotomization of the strains. All other AgrI CCs were in the high-toxicity range. All MRSA strains were found in the low-toxicity group of strains. δ-haemolysin was expressed by only 65.4% of strains in the low-toxicity group but was expressed by 87.7% of the strains in the high toxicity group. β-haemolysin was expressed by only 70.9% of the strains in the low-toxicity group but was expressed by 91% of the strains in the high-toxicity group, and the toxic shock syndrome gene was found in 38% of the low-toxicity strains but only 5.3% of the high-toxicity strains. No single factor was indicative of either high or low toxicity, and other factors associated with the genetic backgrounds of the strains may also contribute.

3.3.4 Beta-haemolysin.

A large number of the wildtype strains (n=163) produced the sphingomyelinase, β-haemolysin. Sphingomyelin is a cell membrane lipid that accounts for approximately 18% of the total lipid by weight of red blood cell plasma

membranes (Alberts *et al.*, 2002). Together with cholesterol, sphingomyelin forms highly organised rafts, or micro-domains, in T-cell membranes, which are thought to play an important role in T-cell receptor signalling and activation (Tani-ichi *et al.*, 2005).

Survival for T-cells incubated with β-haemolysin positive strains (n=163) was 61.9% (median), 59.4% (mean) compared to 77.1% (median), 74.5% (mean) for non-β-haemolysin producing strains (n=39) (Figure 3.3). The GLM analysis indicated that the presence of β-haemolysin was a significant factor of toxicity, P<0.001.

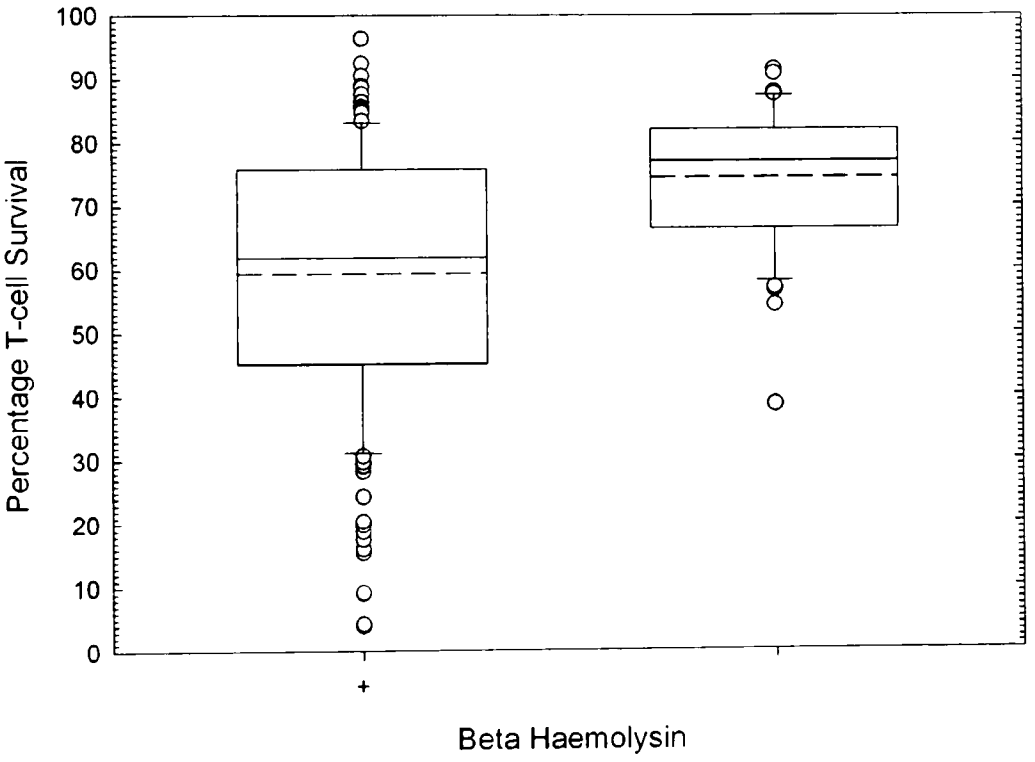

Figure 3.3: **β-haemolysin positive strains are more toxic than negative strains.** Percentage T-cell survival by β-haemolysin positive strains (n=163) and negative strains (n=39) β-haemolysin was shown to be significant factor of toxicity (P<0.001, T=-4.58, DF=9) using a general linear model.

The β-haemolysin producing lab strain 8325–4 was examined alongside an isogenic β-haemolysin mutant, DU5719, (a phage 42E lysogen of 8325–4) using the T-cell toxicity assay. The data were normal so a two sample T-test was

used and the β-haemolysin producing strain was shown to be significantly more toxic (P=0.0013, T=-4.58 DF=9) (Figure 3.4), confirming the findings of the clinical strains.

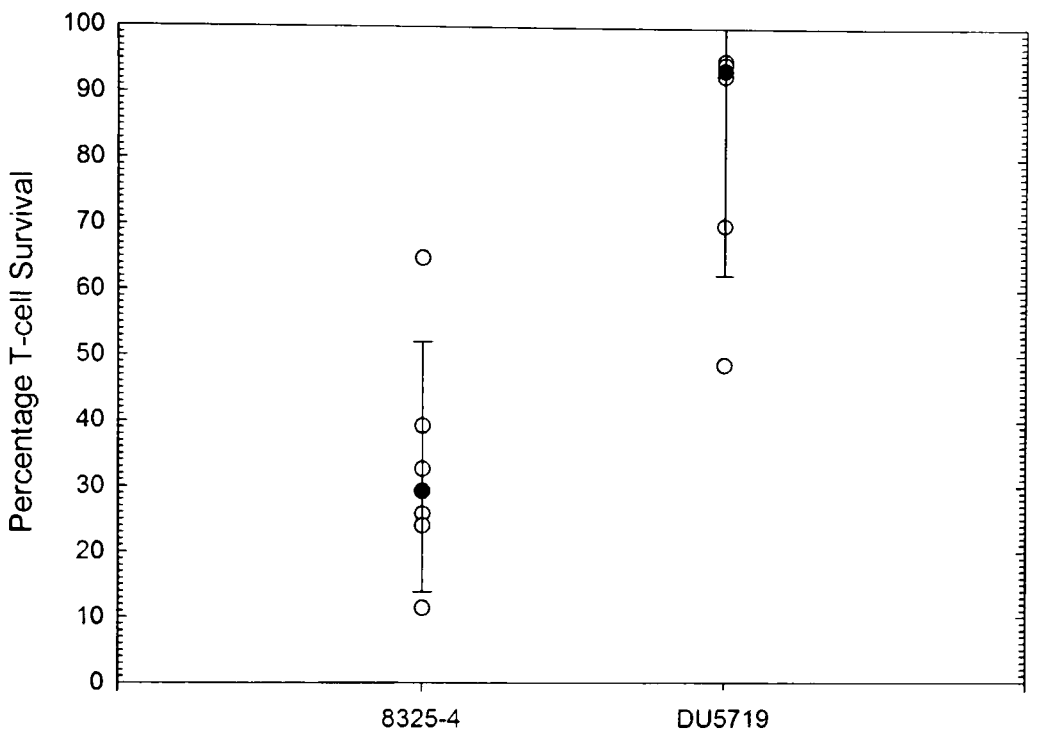

Figure 3.4: **Wild type β-haemolysin strain is more toxic than isogenic knockout.** Percentage T-cell survival when incubated with supernate from β-haemolysin producing strain 8325-4 (n=6) and isogenic *hlb*$^-$ strain DU5719 (n=6). • Median, ○ Data points, Error bars indicate 95% confidence level. β-haemolysin was shown to be significant (P<0.01) using a two sample T-test.

3.3.5 TSST-1 & Delta-haemolysin Interaction.

Delta-haemolysin is a 26 amino acid amphipathic (having one hydrophobic and one hydrophilic end) peptide, encoded by a gene residing within the intracellular effector of the *agr* regulon, RNAIII. A negative δ-haemolysin phenotype is therefore an indicator of a dysfunctional Agr system (See § 1.6 on page 31). Conversely, the presence of δ-haemolysin indicates a functioning Agr system, and an ability to express the associated extracellular toxins.

Strains used in this study were previously tested for δ-haemolysin production using a haemolysin assay (Peacock *et al.*, 2002). An *S. aureus* strain that produces a large zone of β-haemolysin, but not α or δ-haemolysin, is streaked alongside a test strain on an agar plate containing sheep erythrocytes. The β- and δ-haemolysins act synergistically to lyse the sheep red blood cells (Hebert & Hancock, 1985). Therefore, δ-haemolysin produced by a test strain results in a zone of enhanced haemolysis in areas where the lysin overlaps with the β-haemolysin zone and is scored as δ^+.

Toxic Shock Syndrome Toxin (TSST-1) is a superantigen capable of binding macrophage major histocompatibility complex class 2 molecules (MHC II), outside of the peptide binding groove, and T-cell receptors (TCR) on $CD4^+$ T-cells (Figure 1.2 on page 5). This crosslinking of macrophage and T-cells induces T-cell proliferation, and a corresponding release of large amounts of host cytokines. Unlike other *S. aureus* superantigens (SE A, B, C, D, D and G), TSST-1 does not have emetic activity. TSST-1 producing strains in this study were previously characterised using specific primers for *tst* (gene encoding TSST-1 protein) in a PCR reaction (Peacock *et al.*, 2002).

TSST-1 producing strains were less toxic than non TSST-1 producing strains (Figure 3.6 on page 85), and δ-haemolysin negative strains less toxic than those producing δ-haemolysin (Figure 3.8 on page 87). The GLM analysis showed a significant interaction between δ-haemolysin and TSST-1 (P=0.048), so the interactions were examined in more detail (Figure 3.5 on the next page). The strains with a functioning Agr system, and lacking *tst*, were significantly more toxic.

A number of strains (n=27) were δ-haemolysin$^-$, tst^+. Due to the nature of the assay used to test for δ-haemolysin it was hypothesised that the presence of *tst* could mask an intact Agr system, potentially by down regulating or interfering

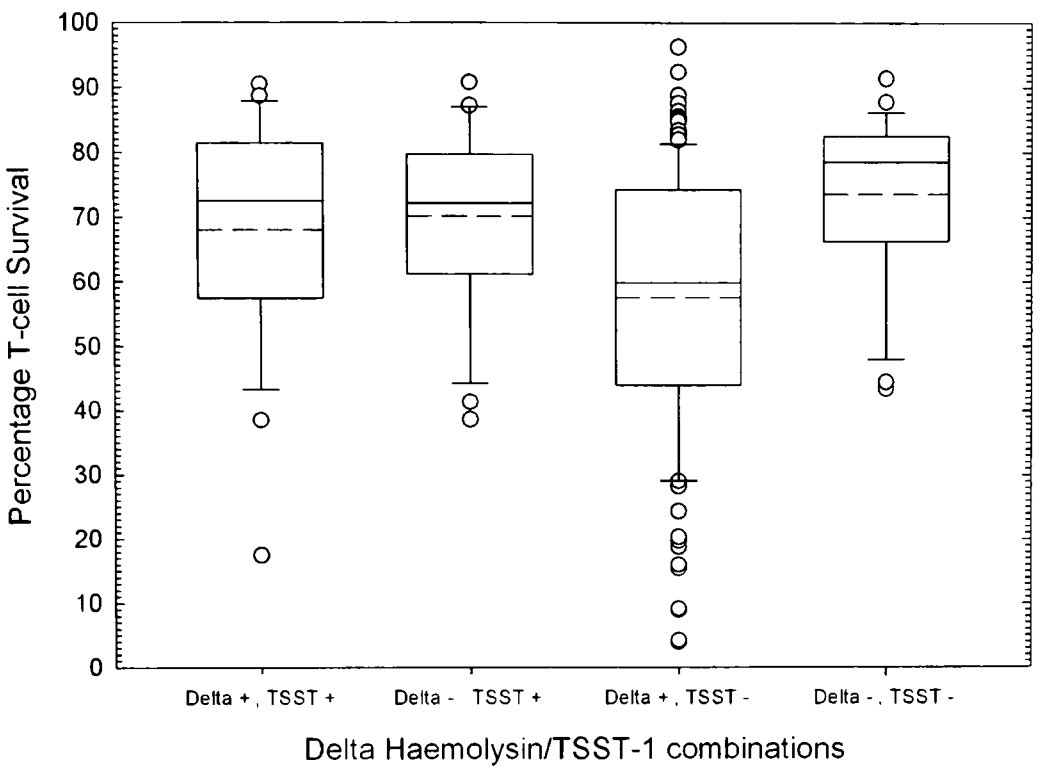

Figure 3.5: **Strains with a functioning Agr system and lacking *tst* have a higher toxicity than other combinations.** Percentage T-cell survival by δ-haemolysin/TSST-1 production combinations. δ^+ tst^+ (n=26), δ^- tst^+ (n=27), δ^+ tst^- (n=127), δ^- tst^- (n=26). A GLM with Tukey pairwise comparisons showed a significant difference between the groups, P<0.001 and that the δ^+ tst^- had a significantly higher toxicity than δ^- tst^+ (P=0.01), δ^- tst^- (P<0.001) but that there was no other significant differences.

with toxin expression. This would account for the decreased toxicity observed in tst^+ strains, and the interaction seen in the statistical analysis.

In a separate study undertaken in collaboration with two final year undergraduate students, Agr function of strains from clonal cluster 30 were examined using the SDS-PAGE and Western blot for δ-haemolysin (See Method 2.5 on page 58). A comparison of the data from this study with the data from the sheep erythrocyte lysis assay demonstrated a high number of δ-haemolysin false negatives (n=20) (Table 3.2 on the next page), strains shown to be negative for δ-haemolysin using the sheep erythrocyte lysis assay but positive by Western blot. Where strains tested negative using the lysis assay but positive using the Western blot *tst* was present on 17/20 occasions (85%), indicating a potential

cause for the masking of δ-haemolysin expression.

Strain	Lysis Result[a]	Western Result[b]	*tst*[c]
21	+	-	-
45	-		+
69	-	-	-
73	-	+	+
133	-	+	+
137	-	-	+
140	+		
144	-	+	+
160	+	+	+
209	+	+	+
215	-	+	+
252	-	-	-
253	+	+	+
307	-	+	+
325	-	+	+
354	-	-	+
370	-	-	-
378	-		+
393	-	-	-
396	-	+	+
399	-	+	+
406	-	+	+
448	-	+	+
455	-	-	+
620	-	-	-
721	-	-	
906	-	+	-
908	-	+	+
3033		+	+
3037	-	-	+
3050	-	+	-
3063	-	+	+
3066	-	+	+
3068	-	+	-
3078	-	+	+
3085	-	+	+
3097	-	+	+

Table 3.2: **The presence of *tst* leads to false negatives in the sheep erythrocyte lysis assay**. Comparison of results obtained for δ-haemolysin expression using: (a) cell lysis assay (Peacock *et al.*, 2002) and (b) Western blotting. (c) *tst*, as determined by PCR. Where strains tested negative using the lysis assay but positive using the Western blot, *tst* was present on 17/20 occasions (85%). All strains are from clonal cluster 30 (CC30).

Toxic Shock Syndrome Toxin.

TSST-1 producing strains were compared to non TSST-1 producing strains. T-cell survival was found to be greater, 72% (median) 69% (mean) compared to 64% (median) 60% (mean), in the TSST-1 producing strains (Figure 3.6). To confirm a significance in the findings a comparison of RN4282, a TSST-1 producing wildtype strain, and RN6938, an isogenic *tst* inactivated strain, (RN4282 Δ*tst*::*tetM*) was undertaken using the T-cell toxicity assay (Figure 3.7 on the next page).

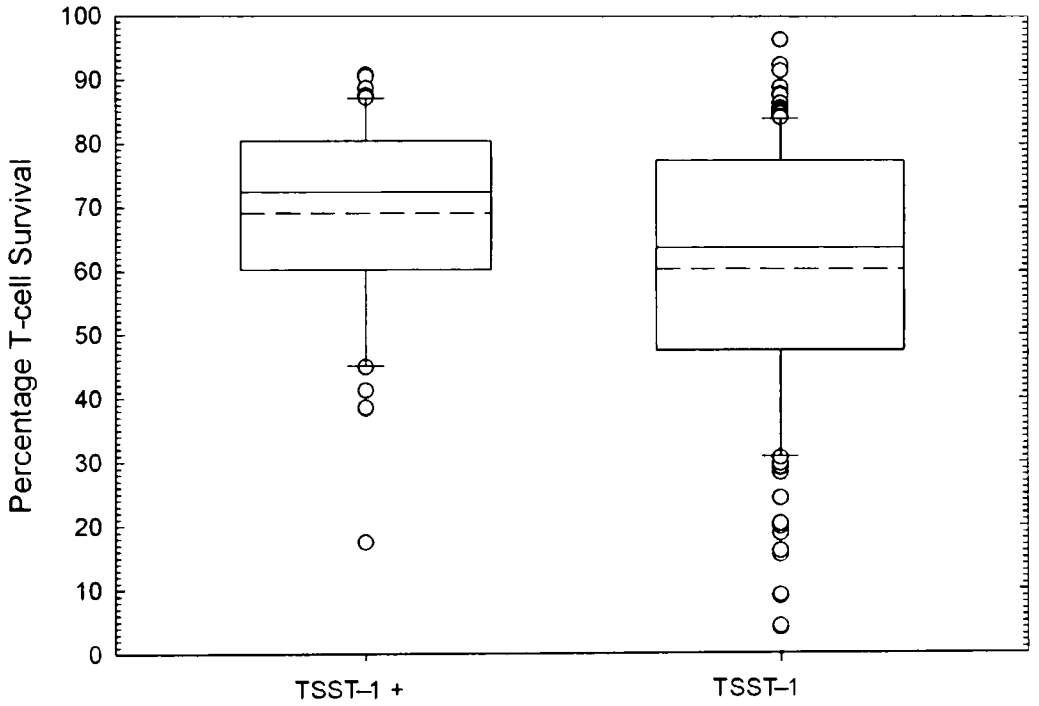

Figure 3.6: **The presence of *tst* reduces toxicity.** Percentage T-cell survival when incubated with TSST-1 positive strains (n=53) and TSST-1 negative strains (n=151).

The inactivation of the *tst* gene significantly restored toxicity, P<0.01 (Mann-Whitney test). To determine whether this effect was due to extracellular TSST-1 or to a regulatory effect RN6938 was propagated in BHI supplemented with 0.1 μg ml^{-1} TSST-1, a concentration that would likely be found in stationary phase culture (Van Langevelde *et al.*, 1997), or alternatively TSST-1 was added

to supernatant from RN6938 before incubation with T-cells. No significant effect was observed, P>0.05, suggesting the reduced toxicity of *tst* positive strains is not due to extracellular TSST-1.

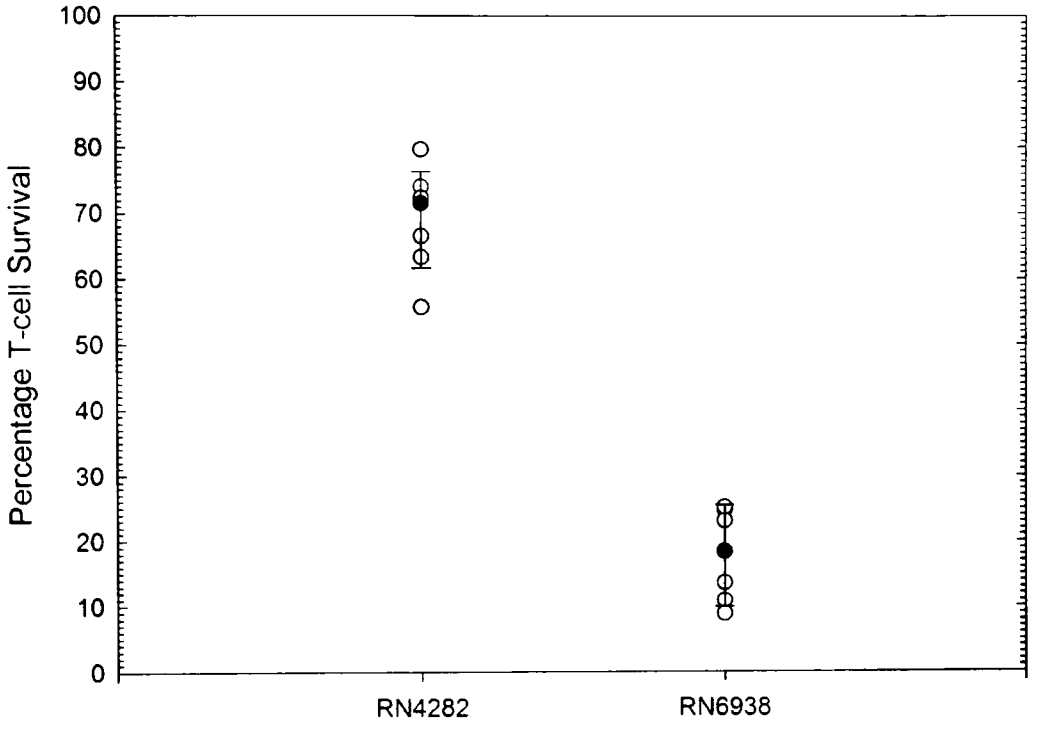

Figure 3.7: **Inactivation of *tst* restores toxicity.** Percentage T-cell survival when incubated with supernate from TSST-1 producing wild type strain RN4282 (n=6) and isogenic tst^- strain RN6938 (n=6). • Median, ○ Data points, Error bars indicate 95% confidence level. TSST-1 was shown to be significant (P<0.01) using a Mann-Whitney test.

Delta-haemolysin (Functioning Agr).

Delta-haemolysin positive strains were compared to δ-haemolysin negative strains (Figure 3.8 on the facing page), and found to have a greater toxicity, 61.7% (median) 59.2% (mean) T-cell survival compared to 77.0% (median) 72.4% (mean).

To determine the significance of the findings a comparison of RN6390B, a lab strain with a functioning Agr system, and RN6911, an isogenic *agr* inactivated

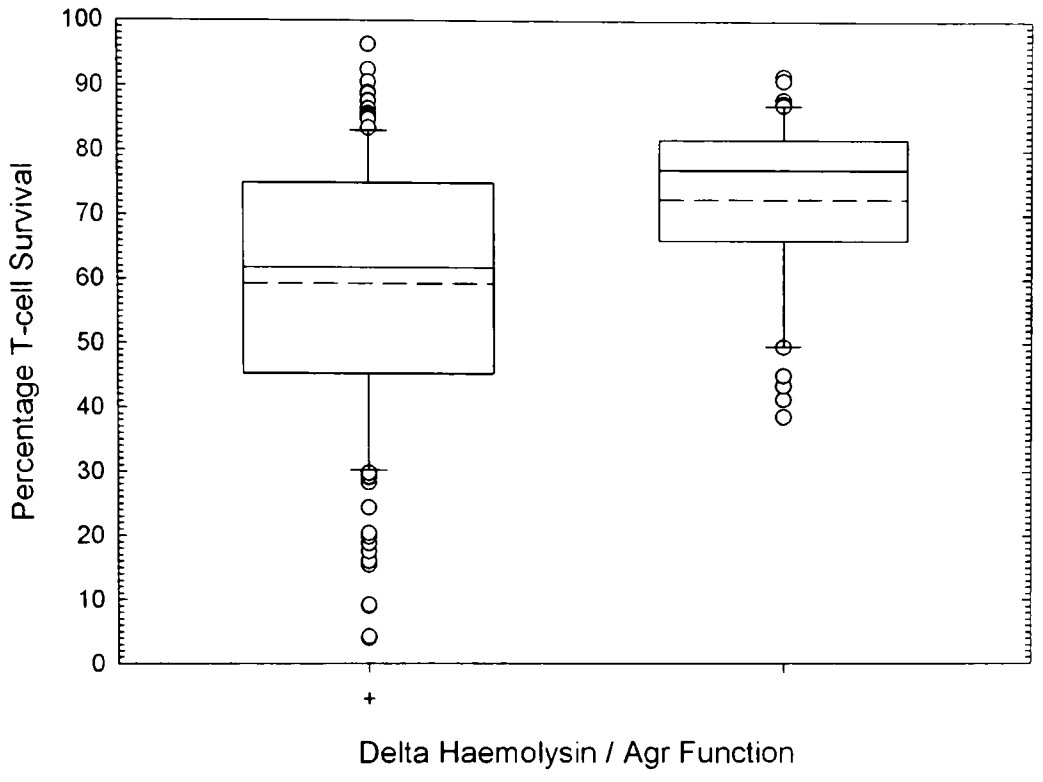

Figure 3.8: **A dysfunctional Agr system reduces toxicity**. Percentage T-cell survival when incubated with δ-haemolysin producing (functional Agr) strains (n=154) and δ-haemolysin negative (dysfunctional Agr) strains (n=51).

strain (RN6390B Δagr::*tetM*), was undertaken using the T-cell toxicity assay (Figure 3.9 on the next page). A functional Agr system was shown to be a significant contributing factor of toxicity, $P<0.01$, using a Mann-Whitney test.

3.3.6 Is There a Difference Between MRSA & MSSA Strains?

MRSA first emerged in the 1960s (Jevons, 1961) and has since become a serious problem in hospitals and more recently the community. All of the MRSA strains in the strain collection used here were hospital acquired and contained the large (53 kb) type II SCC*mec* element. The toxicity of the MRSA strains

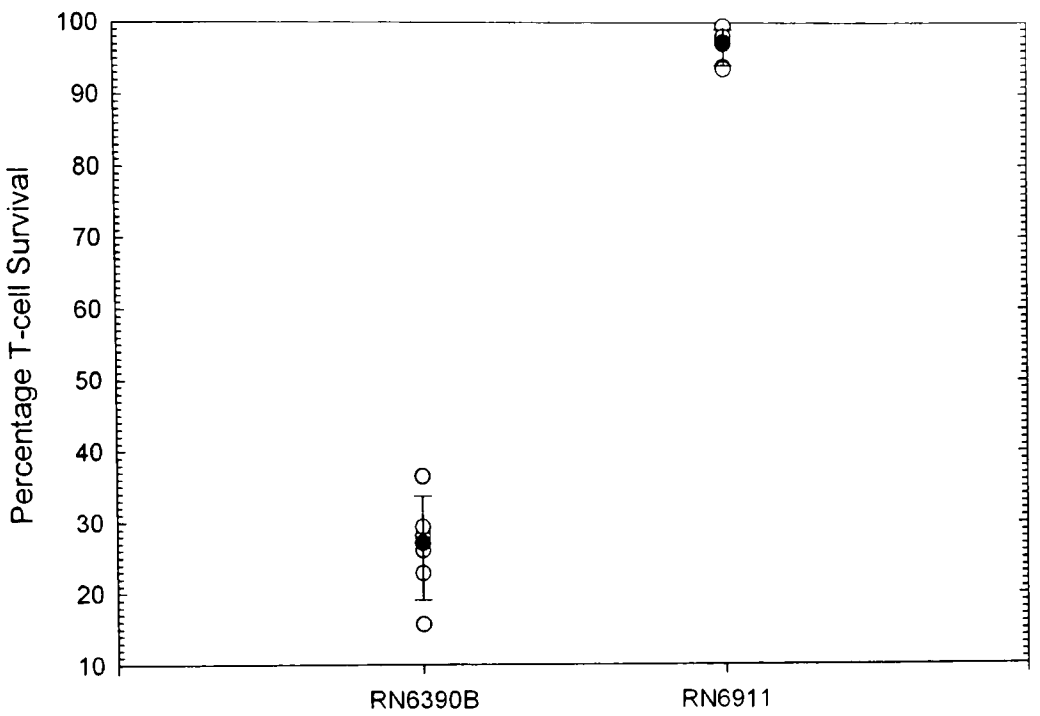

Figure 3.9: **Inactivating the *agr* locus significantly reduces toxicity**. Percentage T-cell survival when incubated with supernatant from lab strain with functioning Agr system RN6390B (n=6) and isogenic *agr*$^-$ strain RN6911 (n=6). • Median, ○ Data points, Error bars indicate 95% confidence level. A functional Agr was shown to be significant (P<0.01) using a Mann-Whitney test.

when compared to methicillin susceptible (MSSA) strains was not statistically significant in the initial analysis, though MSSA strains appeared to be more toxic (Figure 3.10 on the facing page).

Due to the clinical importance of MRSA it was decided a more detailed investigation was warranted, The results of which can be found in Chapter 6 on page 135 and Chapter 7 on page 153.

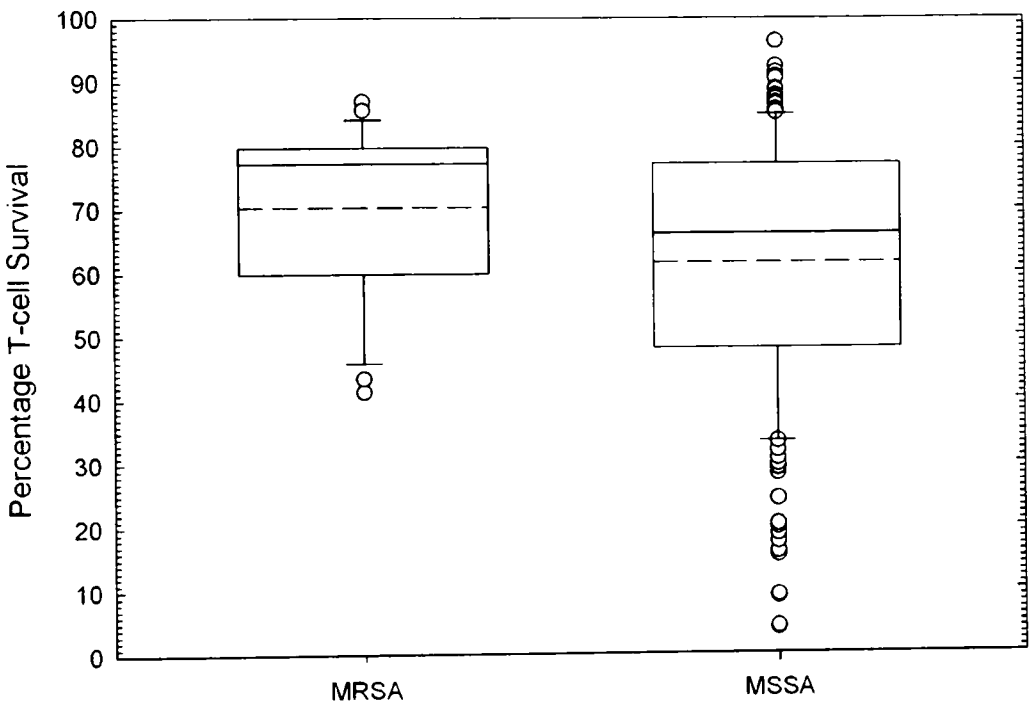

Figure 3.10: Percentage T-cell survival by MRSA strains (n=23) and MSSA strains (n=183).

3.4 Discussion.

The aim of this chapter was to examine a genetically diverse collection of well characterised strains and to see whether any novel associations between specific factors and toxicity would emerge. The results show a significant variation in the toxicity of *S. aureus* strains that can in part be accounted for by Agr group, production of β-haemolysin, a functioning/dysfunctioning Agr system, and the presence or absence of *tst*. This data has added to the data from lab strains and animal models. The T-cell toxicity assay has been shown an accurate predictor of toxicity, by confirming the affect of isogenic strains in animal models (O'Callaghan *et al.*, 1997; Dinges *et al.*, 2000; McNamara & Bayer, 2005; Vojtov *et al.*, 2002), that can be repeated cheaply and efficiently. There are also some interesting questions posed by this chapter: The HA-MRSA strains appeared to be less toxic than MSSA strains though the difference was not significant. This association will be studied in more detail in Chapter 6 on page 135 and Chapter 7 on page 153.

3.4.1 Accessory Gene Regulator (Agr) Group.

The accessory gene regulator is a two component quorum sensing (QS) system that acts as the main controller of the accessory genes, up-regulating secretory proteins and down regulating surface proteins during late exponential phase *in vitro* (Novick *et al.*, 1993). *S. aureus* strains can be split into at least four groups (I–IV) depending upon their Agr type, defined by the mutual inhibition of *agr* by heterologous auto inducing peptide (AIP) pairings and resulting in bacterial interference of Agr (Ji *et al.*, 1997).

Previous studies have shown certain disease characteristics to be more prevalent

in certain Agr groups: a study comparing variability in *agrD* from globally collected strains found that of 137 MRSA isolates 130 belonged to Agr group I, groups I and II have been linked to endocarditis and enterotoxin-mediated disease, group III were involved in TSST-1 mediated disease and leukocidin induced necrotizing pneumonia in young immunocompetent patients, and group IV strains were associated with generalized exfoliative syndromes (Jarraud *et al.*, 2002; Gillet *et al.*, 2002; van Leeuwen *et al.*, 2000). Of the tst^+ strains examined in this chapter 47/53 (88.7%) were found in AgrIII, agreeing with previous results, though of the HA-MRSA strains only 5/23 (21.7%) were found in group I, the predominant group being III with 17/23 (73.9%) strains. The grouping of the MRSA strains predominantly in Agr group III may be an artifact of the sample size, or the fact that strains were taken from one geographic location, Oxfordshire. The MRSA strains fell into two clonal clusters, CC30 (containing all of the AgrIII strains, as well as one AgrI strain) and CC22 (containing the remaining strains).

It is likely that the difference in toxicity of *S. aureus* strains is not the result of Agr group *per se*, but that Agr group is strongly linked to clonality (Jarraud *et al.*, 2002), strains with a common *agr* allele also share common virulence factors and patterns of expression.

3.4.2 Beta-haemolysin.

T-cell survival was significantly lower when T-cells were incubated with bacteria expressing β-haemolysin. The β-haemolysin is a sphingomyelinase produced in large amounts by many strains (Dinges *et al.*, 2000), the activity of which is assayed by the hot-cold lysis of sheep erythrocytes (Hebert & Hancock, 1985). β-haemolysin is regulated by Agr, though it is still detectable in Agr null

strains (Sakoulas *et al.*, 2002).

The finding that β-haemolysin production increases toxicity is one that could perhaps be anticipated from the data from animal studies, and assays using human cells — where it has been shown to lyse erythrocytes, proliferating T-lymphocytes, monocytes, resting lymphocytes, and polymorphonuclear leukocytes (O'Callaghan *et al.*, 1997; Huseby *et al.*, 2007; Marshall *et al.*, 2000; Walev *et al.*, 1996)

3.4.3 Delta-haemolysin.

Delta-haemolysin is capable of causing membrane damage in a variety of mammalian cells (Dinges *et al.*, 2000). Although its mode of action has not been conclusively determined, it is likely that its amphipathic properties enable it to act as a surfactant to disrupt cell membranes. Rogolsky (1979) found the kinetics of δ-haemolysin induced lysis to resemble those of cells exposed to detergents, a property which may also play an important role in biofilm dispersal (Novick & Geisinger, 2008b).

As well as being a haemolytically active toxin in its own right the presence of δ-haemolysin indicates a functioning Agr system. The accessory gene regulator was initially identified by Recsei *et al.* (1986) as a regulator of genes for accessory proteins, its inactivation causing a significant drop in levels of alpha-, beta-, and delta-haemolysin, toxic shock syndrome toxin (TSST-1) and staphylokinase, while protein A production was elevated. A study by Dunman *et al.* (2001) using a micro array and Agr^+ and Agr^- (RN6911) strains revealed 104 genes (including 20 putative virulence determinants, 14 being involved in extracellular factor production) were up-regulated and 34 down-regulated by Agr function. It is likely however, that many of these responses were the result of pleiotropic

effects on other parts of the exceedingly complex regulatory system (See § 1.6 on page 31 and Figure 1.12 on page 43 for a diagram indicating the complex web of regulation in *S. aureus*).

The δ-haemolysin and Agr function are intrinsically linked, it is therefore impossible to separate their effects when looking at clinical strains. Strains with dysfunctional Agr systems have been shown to have a considerably decreased virulence in numerous animal models (Fleming *et al.*, 2006; McNamara & Bayer, 2005; Abdelnour *et al.*, 1993; Booth *et al.*, 1997; Gillaspy *et al.*, 1995). A significant reduction in toxicity was also observed in the isogenic strain comparison. What is surprising in light of this was the failure of δ-haemolysin to be shown statistically significant in the initial analysis.

The *agr* locus is genetically labile *in vitro*, readily acquiring mutations resulting in Agr^- phenotypes, however, Agr negative strains have been isolated from patients and have also been found co-colonising clinical infections indicating either a role for them in certain disease states or that a certain number of 'cheaters' within the population can be tolerated (Traber *et al.*, 2008; Shopsin *et al.*, 2008a).

3.4.4 Toxic Shock Syndrome Toxin.

The presence of *tst* correlated with a decrease in toxicity. This effect of *tst* was confirmed by comparing a wildtype TSST-1 producing strain with an isogenic *tst* knockout. Levels of TSST-1 have been observed to remain constant, independent of gene dosage, suggesting that TSST-1 expression is autorepressed (Novick, 2003b). The T-cell toxicity results confirm those of Vojtov *et al.* (2002), who found that TSST-1 producing strains produce exoproteins at very low levels, $\approx$10% of what is found with an isogenic *tst* knockout. It was also

observed that the effect of the down regulation was caused by the TSST-1 protein itself, rather than mRNA or DNA (Vojtov *et al.*, 2002). The addition of TSST-1 to the T-cell toxicity assay provided no protective effect to the T-cells, neither did the growth of a *tst* knockout strain in the presence of TSST-1. This suggests that intracellular TSST-1 is the cause of the down regulation of toxins, and the observed reduction of toxicity.

Not only do TSST-1 producing strains exhibit significantly reduced exoprotein production, but wounds tend to be apurulent, as observed in cases of post surgical toxic shock (Fast, 1988; Kreiswirth *et al.*, 1986), and a murine subcutaneous abscess model (inactivation of *tst* in an isogenic strain produced the usual subcutaneous abscess) (Vojtov *et al.*, 2002). The *tst* gene itself is located on a 15.2 kb mobile genetic element, Staphylococcal pathogenicity island 1 (SaPI1), and is capable of extremely high transduction frequencies with phage 80α. SaPI1 also contains genes for a second superantigen, *sek*, the partial sequence of a third, *sel*, and the *int* gene, encoding a functional integrase necessary for the integration of the element (Ruzin *et al.*, 2001).

S. aureus is an immotile bacterium, and as such employs methods like pus formation to increase its chance of vector mediated transmission. One of the questions posed by the data and animal model experiments is why *S. aureus* keeps the *tst* gene, a mobile genetic element, if it potentially reduces its transmission rate by making wounds apurulent? Possible hypotheses are that: (i) TSST-1 production provides some other, as yet unidentified, advantageous trait, (ii) TSST-1 provides a within host advantage (Brown *et al.*, 2006; Bull, 1994) by making nutrients available without the need to employ its other virulence factors and thus significantly reducing the metabolic costs, (iii) SaPI1 may act as a piece of selfish DNA with a transduction rate high enough to ensure its survival without a net advantage to *S. aureus* (this idea is not

dissimilar, in principle, to the hypothesis put forward by Smith (2001) for the mobility of virulence factor genes. Within host selection of mutant 'cheater' strains, i.e. strains not producing a communal virulence factor, is policed by the reintroduction of virulence factor genes via horizontal transfer).

3.4.5 MRSA Vs MSSA.

The initial collection of clinical strains contained a number of hospital acquired MRSA isolates, all which contained the large (53 kb) resistance element SCC*mec* type II. Comparison of these strains with methicillin sensitive strains revealed a reduced toxicity, an observation that has also been shown in a guinea pig infective dose, and mouse lethal dose, model by Mizobuchi *et al.* (1994) — although the strains used in the experiment were not genetically characterised.

The plasticity of the *S. aureus* genome makes the singling out of individual genetic traits, from a genetically diverse collection of strains, difficult, due to the multitude of factors that could potentially be involved. When strains from the same clonal cluster (to control for genetic variation) were re-assayed for toxicity (See Chapter 6 on page 135 and Chapter 7 on page 153), a strong correlation between the toxicity and methicillin resistance was observed for SCC*mec* type II; interestingly the strains containing the smaller (20.9–24.3 kb) SCC*mec* type IV remained highly toxic, exhibiting no significant difference when compared to MSSA strains from the same clonal cluster. A recent study by Karauzum *et al.* (2008) collected clinical isolates responsible for bloodstream infections in France and compared MRSA SCC*mec* type IV isolates with MSSA strains of the same sequence type (ST) in a murine bacteremic model. The results indicated the type IV element had a variable influence on toxicity depending on ST. To confirm their findings, an MRSA SCC*mec* type IV ST30 isolate,

WSPPA (isolated in Western Samoa Adhikari *et al.*, 2002) and its isogenic mutant, ME230 (where SCC*mec* was cured) were examined and showed similar rates of mortality in a mouse model. This supports our findings that the type IV SCC*mec* element does not affect toxicity. The CA-MRSA strains have been shown to be more virulent than HA-MRSA strains (Tristan *et al.*, 2007; Chambers, 2001) though it is not known if this is due to the smaller size of the SCC*mec* insert, the difference in clonal cluster or a mixture of factors.

From the results presented in this chapter and from previous works it seems that there is a relationship between the larger SCC*mec* element and a reduction in toxicity. The reason for this however is unclear, though potential hypotheses include (i) the size of SCC*mec* type II confers a sizable metabolic burden which is overcome by compensatory mutations effecting virulence factors or virulence factor regulators. (ii) a regulator is present on the the larger SCC*mec* type II insert, (iii) clonal clusters with pre-existing mutations or a reduced production of exoproteins are able to accept the type II SCC*mec* element more readily. These hypotheses will be expanded upon and researched in later chapters.

3.5 Conclusion.

The T-cell assay was developed specifically to examine the toxicity of a large number of genetically diverse clinical strains at low cost and without the use of an animal model. This first stage analysis has enabled us to make some preliminary assumptions regarding the factors involved in *S. aureus* toxicity. This study has supported data from animal studies and has also shown that traits examined with either a small number of strains or with knockout strains, such as *tst*, are detectable when a large number of clinical strains are analysed.

4

Effect of NaCl on Toxicity and Gene Regulation.

4.1 Abstract.

Eczema is a chronic inflammatory skin disease where >90% of patients are colonised with *S. aureus*. Bacterial densities and toxin production are directly related to lesion severity. Eleven *S. aureus* strains representing the dominant clinical clonal lineages, including methicillin resistant *S. aureus* (MRSA), were assessed for toxicity before and after treatment with: 1M NaCl in nutrient broth; the emolient cream, Oilatum®, supplemented with 1M NaCl; and Oilatum® only. A significant reduction in toxicity was found in strains grown in 1M NaCl nutrient broth. The incubation of *S. aureus* in Oilatum® resulted in a reduction of toxin expression levels in relation to Oilatum® supplemented with NaCl.

4.2 Introduction.

This project is funded by Stiefel Laboratories®, a pharmaceutical company that specifically focuses on dermatology and skin care products. It is the aim of this chapter to look at the effects of NaCl and its potential inclusion in an emollient cream for the treatment of eczema.

Eczema is a common chronic inflammatory skin disease, often linked with one or both parents having a history of atopic dermatitis (AD), asthma, or allergic rhinitis. The International Study of Asthma and Allergies in Childhood (ISAAC) epidemiological research programme (established in 1991) includes data from over 700,000 children from 156 centers, in 56 countries, shows cases of eczema to be on the rise, especially among young children (Asher *et al.*, 2006). In the United States the prevalence approaches 17% in school-aged children (Boguniewicz *et al.*, 2006) and health insurance companies are thought to spend in the region of $0.9 to $3.8 billion per year, a figure similar to the expenditure on diseases such as emphysema, psoriasis, and epilepsy (Ellis *et al.*, 2002).

Patients with eczema or AD are highly vulnerable to infection with certain cutaneous viral, fungal, and bacterial species (Lübbe, 2003); indeed, any form of dermatitis leads to an increased susceptibility to infection. The most common infective agent of people with AD is that of *S. aureus*, with >90% of patients being infected (Baker, 2006) — compared to 5–30% of normal individuals (Breuer *et al.*, 2001). Colonisation densities can reach up to 10^7 colony-forming units cm^2 (Gong *et al.*, 2006). This bacterial density, as well as toxin production, is directly related to lesion severity (Tomi *et al.*, 2005; Guzik *et al.*, 2005). Once colonised with *S. aureus* the superantigens SEA, SEB and TSST-1 can cause an exacerbation of the inflammatory immune response via the polyclonal

activation of superantigen-specific TCR Vβ families of T cells (Figure 1.2 on page 5) (Baker, 2006). In addition to the superantigens, α-haemolysin in AD skin has been shown to activate T-cells in the absence of antigen-presenting cells (Breuer *et al.*, 2005).

Oral treatment with antibiotics has not been shown to be especially effective (Ewing *et al.*, 1998; Boguniewicz *et al.*, 2001), though topical treatment with antimicrobials and corticosteroids leads to a reduction in bacterial density and improvements in skin lesions (Breuer *et al.*, 2002). Once treatment has stopped however, *S. aureus* can readily recolonise the skin, exacerbating the condition. Use of antibiotics for long term treatment has obvious downfalls, prolonging selection pressure for antibiotic resistance. Fusidic acid is commonly used in topical preparations to treat AD. In a study by Shah & Mohanraj (2003), fusidic acid resistant strains were found on 78% of patients with AD, compared to just 9.6% of samples cultured from non-dermatology patients.

The increasing prevalence of antibiotic resistance, contamination of topical treatments, and the observation that nasal carriage of *S. aureus* is higher amongst patients with AD (39-82%) may account for the persistence of *S. aureus* colonization in AD (Gilani *et al.*, 2005; Roth, 1987).

Antibiotics have been used unknowingly for thousands of years; however, it was not until the discovery of penicillin by Ernest Duchesne in 1897, who noticed that certain molds killed bacteria (Duchesne, 1897), and its rediscovery by Alexander Fleming in 1928 that their study and eventual use in the early 1940s really took off (Fleming, 1944). At this time little if anything was known about the likelihood of resistance evolving and a widespread misuse of antibiotics ensued. Penicillin's initial use was highly effective, with >94% of *S. aureus* isolates susceptible (Livermore, 2000). By the early 1950s, plasmids encoding penicillinase were spreading rapidly through the *S. aureus* population rendering

them resistant. By the late 1960s approximately 80% of *S. aureus* isolates were resistant to penicillin (Gootz, 1990). Today that number is >90% (Lowy, 2003). According to the Infectious Diseases Society of America (2004) >70% of the bacteria causing hospital infections will be resistant to at least one of the antibiotics commonly used to fight them.

There is clearly a need for new antibiotics to be produced. The research and development of novel antibiotics is however, an unattractive prospect for the pharmaceutical industry (Norrby *et al.*, 2005). Antibiotics offer a low return of investment compared with non-antibiotic drugs (Katz *et al.*, 2006a; Trouiller *et al.*, 2002). The majority of antibiotics are administered for approximately a week, whereas drugs for chronic conditions such as arthritis may be administered over years (Norrby *et al.*, 2005). The ability of microbes to rapidly overcome antibiotics also makes the development of new antimicrobial an unattractive prospect for big pharmaceutical companies. Coupled to this is the rising costs of drug development, in 1987 it cost $231 million to bring a drug to market, by 2001 this had risen to $802 million (Katz *et al.*, 2006b).

The combination of a need for new treatments and reluctance from the pharmaceutical industry to spend large sums of money with limited return has led to a more economical approach becoming a necessity. In this chapter I have looked at one such approach, particularly suited to a topical treatment of diseases such as atopic dermatitis or eczema. Toxin production has been directly related to lesion severity in eczema (Tomi *et al.*, 2005; Guzik *et al.*, 2005). A method to reduce the toxicity of *S. aureus* would have a twofold advantage (i) reducing lesion severity and (ii) potentially providing a reduced selection pressure when compared to traditional antibiotic methods (Clatworthy *et al.*, 2007). Previous work on a lab strain has shown that NaCl and glucose can cause a down regulation in toxin production (Chan & Foster, 1998). This

chapter examines whether NaCl has an effect on the regulation of toxins in a representative collection of clinically significant wild type strains, representing 9 dominant clonal clusters (Moore & Lindsay, 2001) .

Toxin production has been directly related to lesion severity in eczema. A method to reduce the toxicity of *S. aureus* would have a twofold advantage, reducing lesion severity, and potentially providing a reduced selection pressure when compared to traditional antibiotic methods. A large number of the virulence factors involved in exacerbating eczema are exoproteins (e.g. α-haemolysin, superantigens), and as such can be viewed as public goods or group-beneficial traits. That is, beneficial products produced by an individual organism that can then be used by the producer as well as its neighbors (West *et al.*, 2007). By targeting the public goods, in this case the exoproteins or quorum sensing system that controls exoprotein expression, rather than killing the bacteria, it is expected that a weaker selective pressure will be evoked (Andre & Godelle, 2005). An explanation as to why the targeting of public goods may be an effective measure is given by Pepper (2008) using angiogenesis blockers for the treatment of cancer as an example: 'All genetic variation within a cancer originally arises in single cells. Imagine a cluster of cells within a tumor in which most cells produce an angiogenesis factor that can be blocked by a certain drug. Any mutation conferring resistance to this angiogenesis blocker must originate in one cell, allowing its angiogenesis factor to remain effective. A single cell cannot produce enough effective angiogenesis factor to attract the blood vessels it needs to survive, but a larger clone of descendants could. If a cytotoxin were applied, a single resistant cell would survive and recolonize the entire region. However, when an angiogenesis blocker is applied, most angiogenesis fails and all cancer cells in the region die, including the resistant cell producing effective angiogenesis factor in an insufficient amount.

Although a larger clone of resistant cells could survive the drug and proliferate, it is unlikely that the original mutant cell could proliferate to create such a clone before the drug is applied, thus before resistance is advantageous. Instead, the lone resistant cells die as their neighbours die and take with them the public goods they had provided'. Although we are not stopping the production of public goods, if they are downregulated sufficiently a similar effect may occur.

The T-cell toxicity assay has been shown to be an efficient indicator of toxicity in Chapter 3 on page 71. In this chapter I will again use the T-cell toxicity assay in combination with reverse transcription-PCR (RT-PCR) to examine mRNA expression levels of *hla* & *sec*, and by inference, expression levels, of α-haemolysin and enterotoxin C (Methods 2.4 on page 56 & 2.7 on page 60). There are a number of reasons for choosing α-haemolysin. i) α-haemolysin is the most common of the three haemolysins and is expressed by the majority of *S. aureus* strains. ii) α-haemolysin is under tight regulation from at least 3 global regulatory loci, the accessory gene regulator (*agr*), the staphylococcal accessory gene regulator (*sarA*), and the staphylococcal accessory protein effector (*sae*)(Xiong *et al.*, 2006); these regulators also control the expression of a large number of expressed virulence factors, so a decrease in *hla* may potentially signify a down regulation of a large number of factors. iii) α-haemolysin has been shown to activate T-cells in the absence of antigen-presenting cells at sublytic concentrations and may well facilitate the development of Th1 cell dominated chronic eczema (Breuer *et al.*, 2005). Enterotoxin C was chosen as staphylococcal superantigens have been frequently implicated as aggravating factors in eczema and psoriasis (Wehner & Neuber, 2001; Yarwood *et al.*, 2000; Bunikowski *et al.*, 2000).

4.3 Results.

4.3.1 T-cell toxicity.

Eleven *S. aureus* strains were picked to encompassed a broad genetic background and include lab strains, MRSA/MSSA strains, carriage strains and invasive strains from both the hospital and community (Table 4.1).

Strain	Description	CC*	Reference
8325–4	MSSA Lab strain		Novick (1967)
MSSA3	Community acquired, disease causing	CC51	Peacock *et al.* (2002)
MSSA476	Community acquired, disease causing	CC45	Peacock *et al.* (2002)
N315	MRSA lab strain		Hiramatsu *et al.* (1992)
MSSA850	Community acquired, disease causing	CC8	Peacock *et al.* (2002)
MRSA720	Community acquired, disease causing	CC22	Peacock *et al.* (2002)
MSSA16	Community acquired, disease causing	CC25	Peacock *et al.* (2002)
MSSA3105	Carriage strain	CC15	Peacock *et al.* (2002)
MSSA117	Hospital acquired, disease causing	CC12	Peacock *et al.* (2002)
MSSA3067	Carriage strain	CC45	Peacock *et al.* (2002)
MRSA252	Hospital acquired, disease causing	CC30	Peacock *et al.* (2002)

Table 4.1: **Strains used in study.** * Clonal Complex. Strains encompass a broad genetic background and include lab strains, MRSA/MSSA strains, carriage strains and invasive strains from both the hospital and community. A full list of strain characteristics can be found in Appendix A on page 185

Strains were grown in brain heart infusion (BHI, low salt) and also BHI supplemented with 1M NaCl (high salt) before assessment of *in vitro* toxicity using the T-cell toxicity assay (Method 2.4). The assay was repeated on separate days and the results visualised graphically (Figures 4.1 & 4.2). A Mann-Whitney test was performed to evaluate the difference between treatments.

Cells grown in BHI supplemented with 1M NaCl showed a significantly decreased toxicity, $P<0.01$ (Figure 4.1). The total bacterial cell numbers used in the

assay were the same, indicating a decrease in toxin production. There was no significant difference found between repeats of samples grown in the same media, P=0.89 & P=0.79 respectively for high salt and low salt samples, indicating that the experimental parameters remained consistent across differing days. This verified that NaCl reduced toxin expression in genetically diverse *S. aureus* isolates.

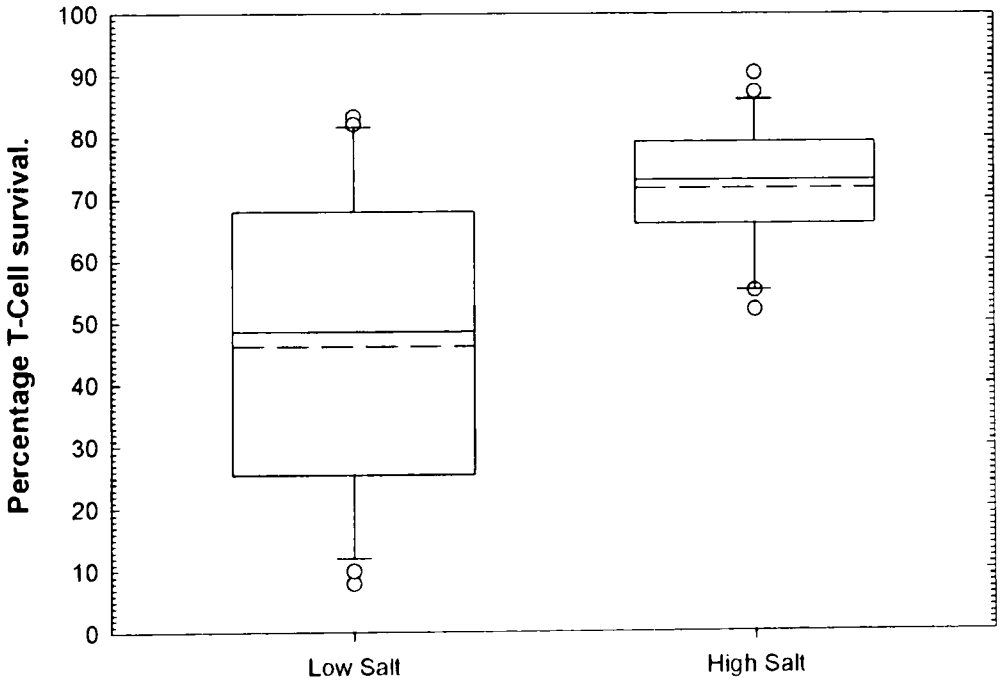

Figure 4.1: **The presence of NaCl significantly reduces the toxicity of *S. aureus*.** Percentage T-cell Survival when incubated with untreated strains (n=11) (grown in BHI) and treated strains (n=11) (grown in BHI supplemented with 1M NaCl). The 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (circles) are shown.

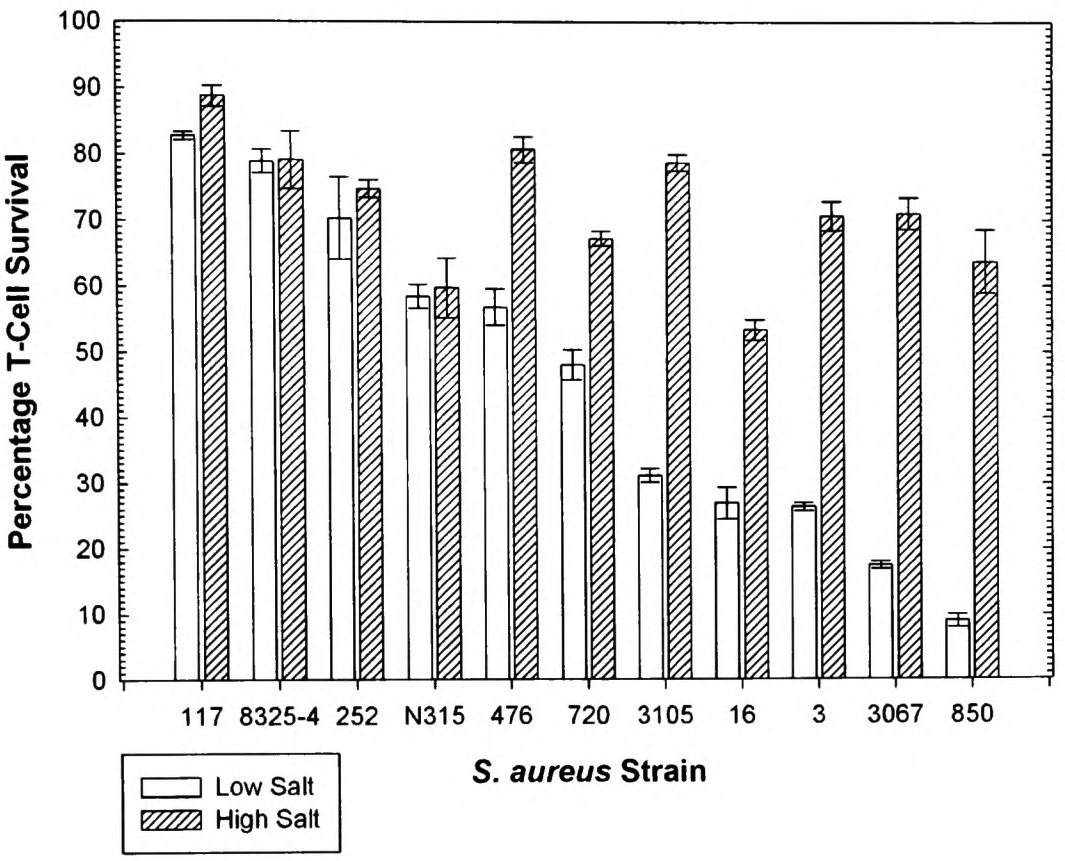

Figure 4.2: **The presence of NaCl reduces the toxicity of the majority of *S. aureus* strains.** Comparison of toxicity of treated and untreated cells, averaged from two replicates. Whiskers indicate Standard Error.

4.3.2 *hla* Expression.

The inclusion of 1M NaCl in the growth media resulted in an overall reduction in toxicity of *S. aureus* when incubated with T-cells. To confirm that the reduction in toxicity was due to a decrease in expressed toxins, a number of reverse transcription polymerase chain reaction experiments (RT-PCR) were carried out.

α-haemolysin, a 33.2 kDa polypeptide encoded by *hla*, is a pore-forming cytotoxin produced by the majority of *S. aureus* strains. α-haemolysin has been shown to be a factor in tissue damage and virulence in various animal models (Bramley *et al.*, 1989; Tuomanen *et al.*, 2001b). Its expression is

under tight regulation from at least 3 global regulatory loci, the accessory gene regulator (*agr*), the staphylococcal accessory gene regulator (*sarA*), and the staphylococcal accessory protein effector (*sae*)(Xiong *et al.*, 2006). For these reasons *hla* mRNA expression levels were determined to see if they corresponded with the reduced toxicity. mRNA from the 11 strains grown in BHI and BHI supplemented with 1M NaCl was purified (Method 2.6 on page 60) and visualised on a 1% agarose gel for signs of degradation (Figure 4.3).

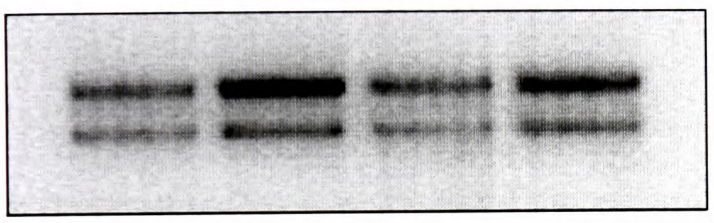

Figure 4.3: **Typical purified RNA ran on 1% agarose gel.** Ribosomal RNA is shown as clear, distinct, bands with no degradation.

To quantify the results from RT-PCR experiments it is important to ensure that RNA levels are balanced, that is the starting quantity of RNA going into an RT-PCR reaction is the same for all treatments (although the relative proportions of differing mRNAs may contrast). RNA was balanced initially by comparing the band intensities of the ribosomal RNA and running an RT-PCR using primers for the commonly used housekeeping gene *gyrB* (encoding the B unit of DNA gyrase). This method was not continued however, as it was discovered that expression levels of *gyrB* were not consistent within the two treatments. This was likely down to the osmotic effect of NaCl, which has been shown to influence the degree of DNA supercoiling and hence the expression of DNA gyrase in some *S. aureus* strains, though not all (Sheehan *et al.*, 1992). RNA was therefore balanced by measuring the absorbance of RNA at 260nm and adding appropriate amounts to the RT-PCR reaction (Method 2.7 on page 60).

RT-PCR products were run on 2% gels and stained with ethidium bromide to enable visualisation under ultra violet light. The bands clearly showed a down regulation of *hla* mRNA levels (and by inference α-haemolysin) in the majority of treated (high salt) samples compared to the untreated (low salt) samples (Figure 4.4). This down regulation was examined using the semi quantifiable method of densitometry comparison, via the analysis program 'Quantity One' (Figure 4.5 on the following page). The average intensity of each band was taken and the average background intensity subtracted. The values were expressed as density intensity/mm^2 (INT/mm^2), a significant difference was determined using a Mann-Whitney test, P<0.001.

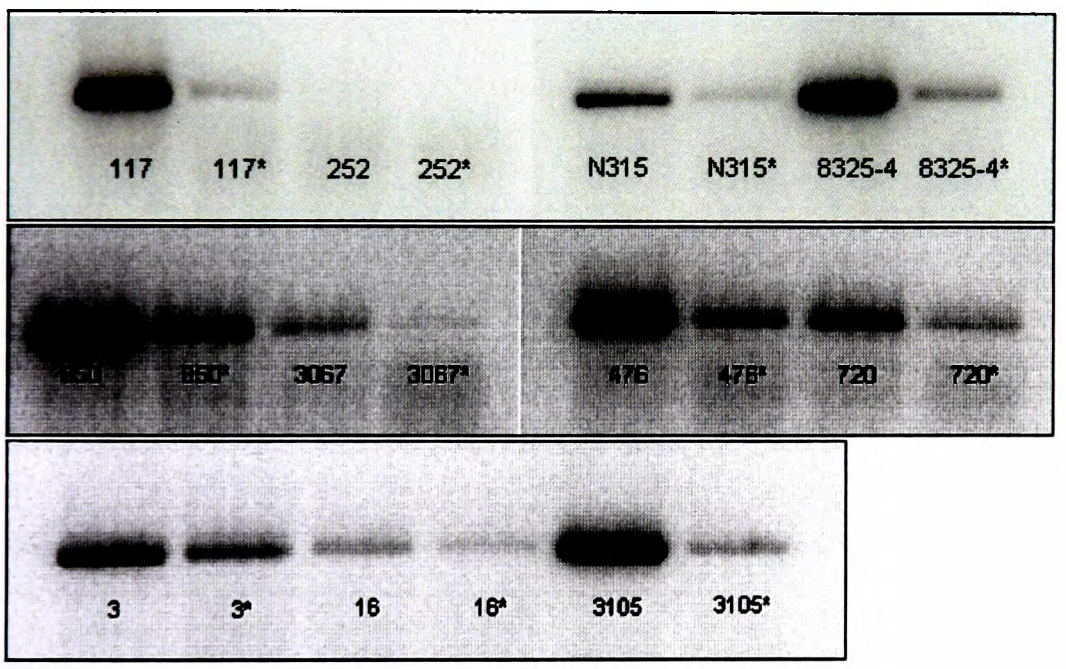

Figure 4.4: **A clear down regulation of *hla* is seen in the majority of high salt samples.** *hla* mRNA RT-PCR product run on a 2% agarose gel and stained with ethidium bromide. * indicates the sample grown in BHI supplemented with 1M NaCl, the unmarked bands indicate growth in BHI.

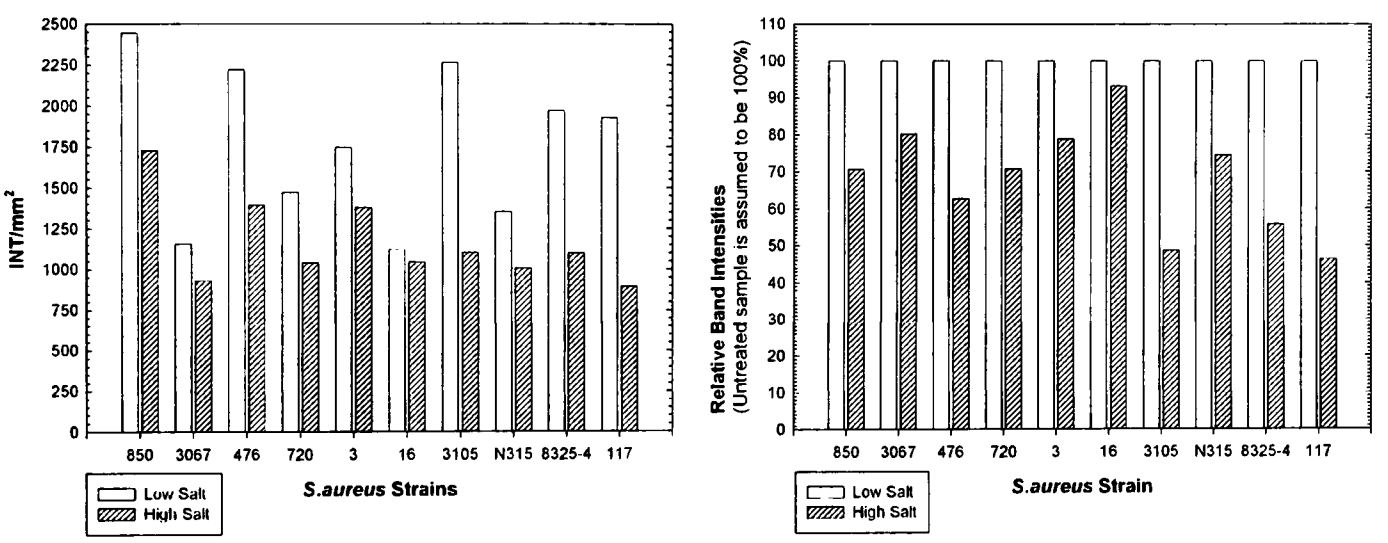

Figure 4.5: **NaCl significantly reduces *hla* expression.** Average and relative *hla* band intensities, indicating different expression levels of *hla* for strains grown in BHI and BHI supplemented with 1M NaCl. A Mann-Whitney test on the density intensity values shows a significant difference $P<0.001$.

4.3.3 *sec* Expression.

Superantigens have been frequently implicated as aggravating factors in eczema and psoriasis, it was therefore important to ascertain whether their down regulation also occurred. Of the eleven strains used for the T-cell toxicity assay and *hla* expression level analysis two were known to produce the superantigen, enterotoxin C (117 & 3067); a further two strains (3106 & 383) were used to assess the expression of *sec* mRNA levels using RT-PCR (Figure 4.6). A comparison of the band intensities undertaken using Quantity One (Figure 4.7 on the following page) and analysis of the values using the Mann-Whitney test showed that the down-regulation of *sec* mRNA levels was significant (P=0.03).

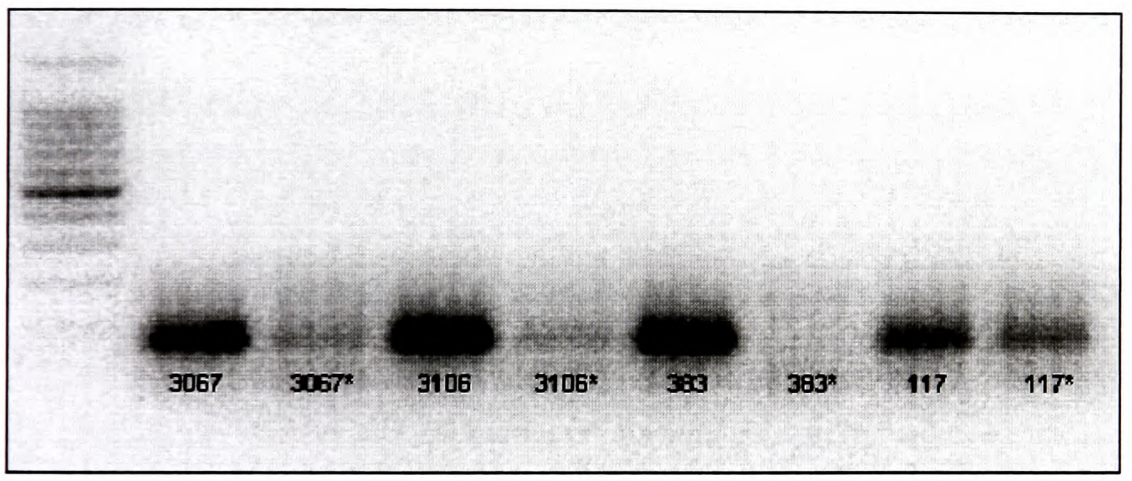

Figure 4.6: **NaCl significantly reduces *sec* expression.** *sec* mRNA RT-PCR product run on a 2% agarose gel and stained with ethidium bromide. * indicates the treated sample grown in BHI supplemented with 1M NaCl and the unmarked bands indicate growth in BHI.

4.3.4 Incorporation of NaCl in an Emollient.

The experiments using BHI had shown that the incorporation of NaCl into the growth media resulted in a significant reduction of toxicity to T-cells *in vitro*, and that this corresponded with a significant reduction in the expression levels

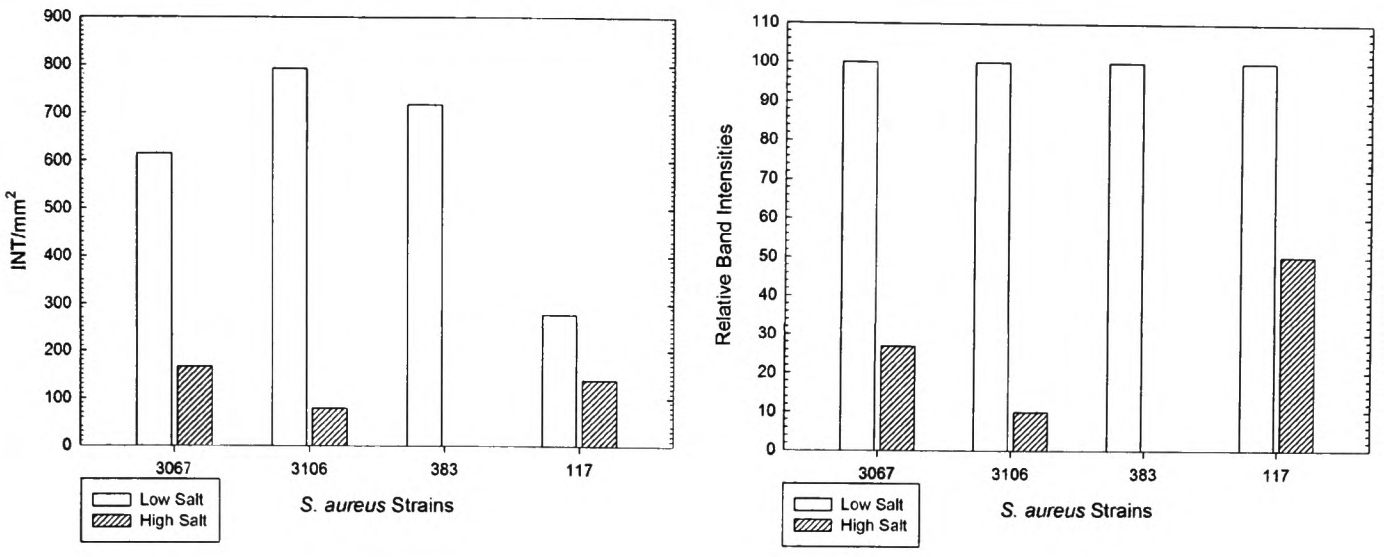

Figure 4.7: **NaCl significantly reduces *sec* expression.** *sec* band intensities, indicating different expression levels of *hla* for strains grown in BHI, and BHI supplemented with 1M NaCl. A Mann-Whitney test on the density intensity values shows a significant difference P=0.03.

of *hla* and *sec*. We next tested the effect of the emollient cream Oilatum® and Oilatum® supplemented with NaCl.

To determine whether the emollient was toxic to *S. aureus*, the lab strain N315 was grown in either brain heart infusion (BHI), or a 50:50 (v/v) mixture of BHI & Oilatum® for 17 hours, in triplicate. Samples were taken hourly and the cells enumerated by serial dilution and plating on TSA plates (Figure 4.8 on the next page). A normal growth curve was observed for both the BHI and BHI & Oilatum® (although the total bacterial numbers were lower in the presence of Oilatum®, possibly due to reduced nutrients and/or oxygenation). Oilatum® was therefore considered not to have a toxic effect on *S. aureus*. To confirm the findings N315 was grown overnight in BHI, harvested by centrifugation and 3 x 10^8 cells ml^{-1} resuspended either in tissue culture grade PBS or pure Oilatum® cream in triplicate, and incubated for 4 hours at 33°C. Cell numbers were enumerated by serial dilution and plating on TSA, no significant difference in cell numbers between the PBS and cream samples were observed (P=0.44,

t=0.88, DF=3) (Figure 4.9).

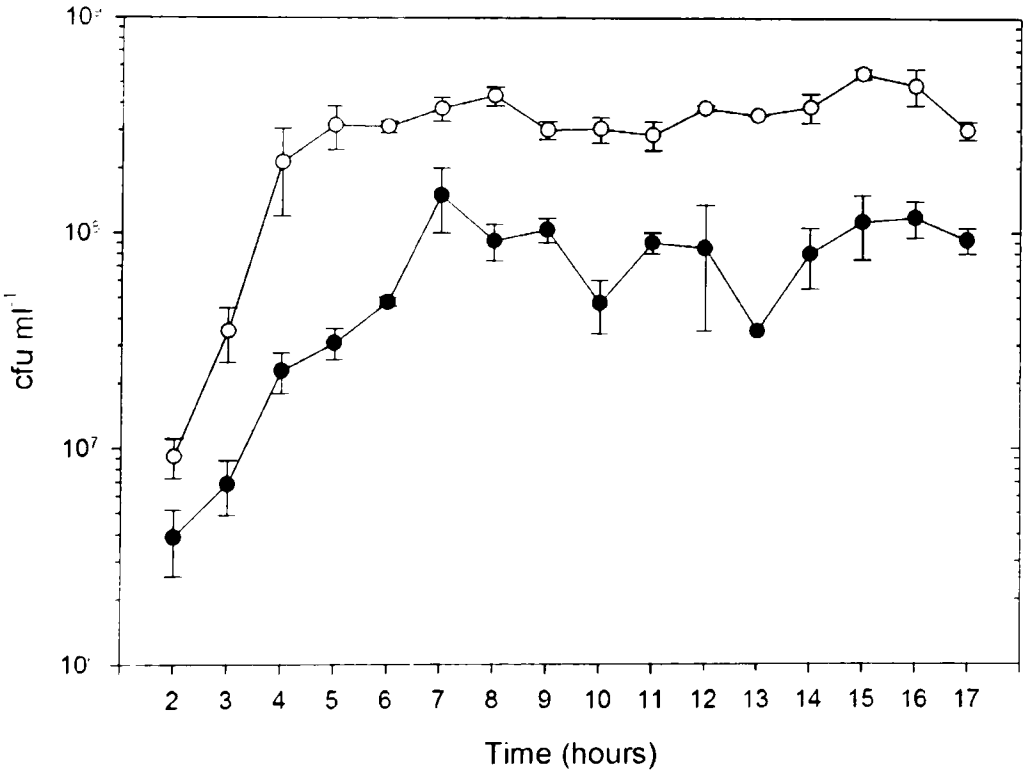

Figure 4.8: ***S. aureus* grows in the presence of Oilatum®.** Growth curve of lab strain N315 grown in triplicate. ○ grown in BHI. ● grown in a 50:50 v/v mix of BHI & Oilatum®. Error bars indicate SE.

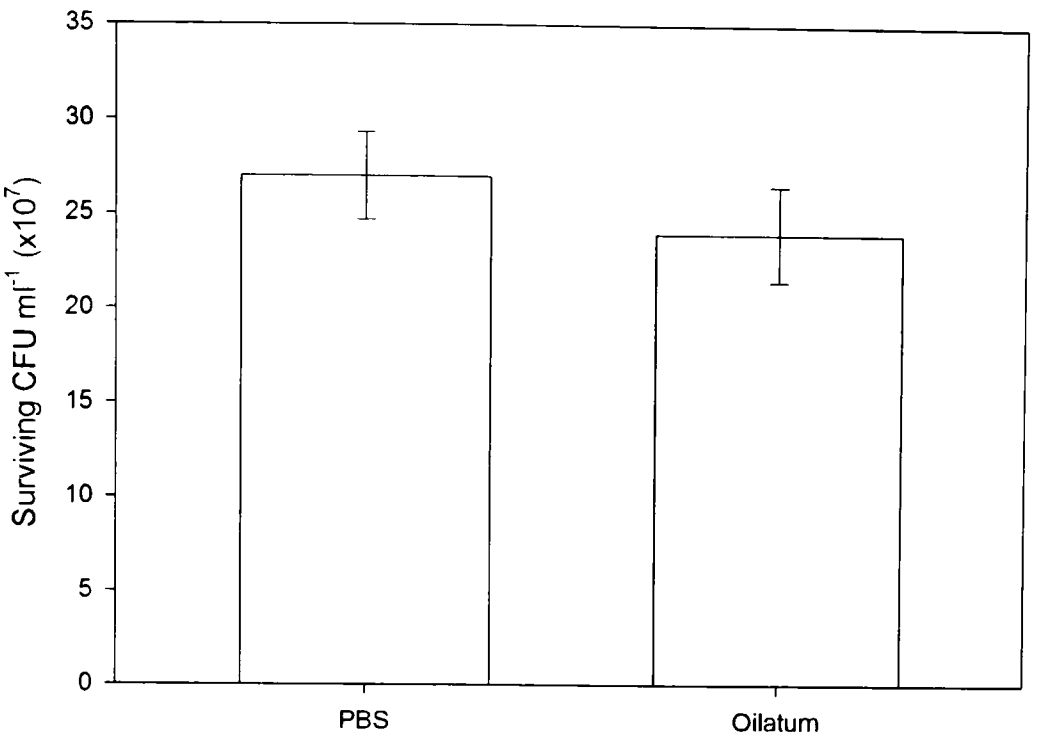

Figure 4.9: **Incubation of *S. aureus* in Oilatum® does not result in a significant decrease in viability in comparison to PBS.** Surviving CFU ml^{-1} *S. aureus* N315 when incubated in PBS or Oilatum® for 4 hours at 33°C. No significant difference between the recoveries was observed, P=0.44. Error bars indicate SE.

We wanted to determine whether NaCl would have an affect on toxicity when incorporated into an emollient used to treat eczema. To do this, strains were grown to an OD600 1.8 (a level previously determined to activate Agr expression under our growth conditions), harvested by centrifugation and resuspended in either Oilatum®, or Oilatum® supplemented with 1M NaCl. Samples were incubated for 4 hours at skin temperature (33°C) before being harvested and mRNA extracted for analysis of *hla* levels (Figure 4.10).

The band intensities were analysed using Quantity One and the values used to construct a graphical representation (Figure 4.11 on page 114). Interestingly, the analysis showed that the strains incubated with Oilatum® alone had a reduced expression of *hla* mRNA in comparison to Oilatum® supplemented with NaCl, P=0.01. Due to a lab move, a different gel documenting system

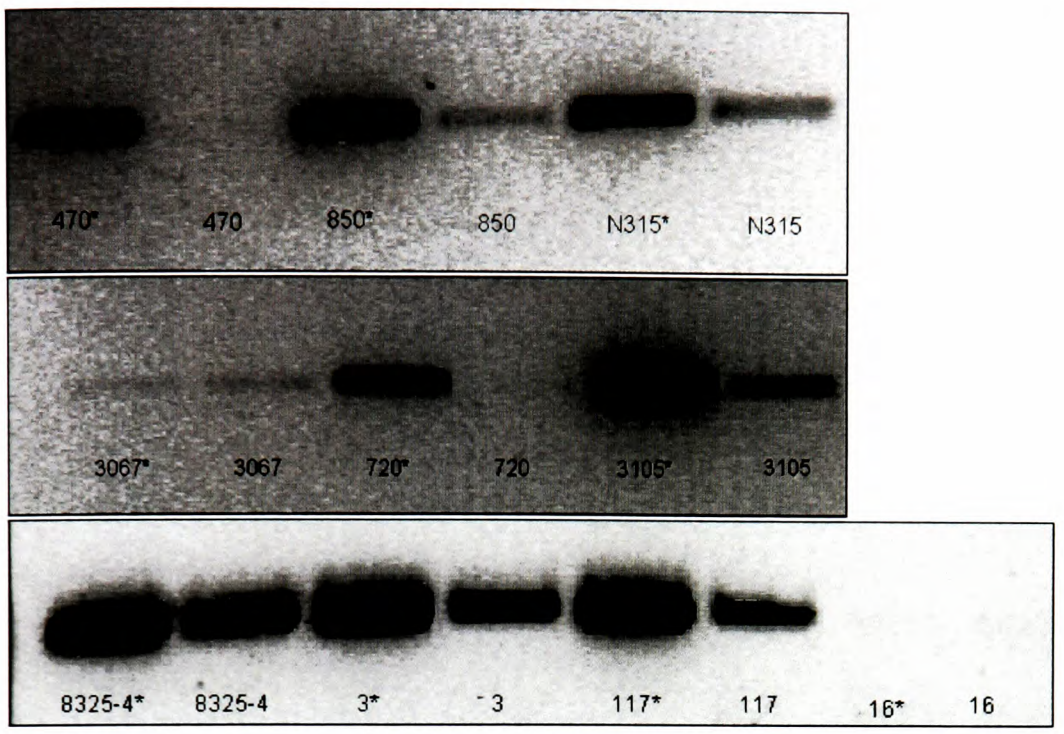

Figure 4.10: ***hla* expression is down regulated by Oilatum® relative to Oilutum® + Nacl**. *hla* mRNA RT-PCR product run on a 2% gel and stained with ethidium bromide. Cells were grown in BHI before being harvested by centrifugation and incubated in either Oilatum® cream or * Oilatum® supplemented with 1M NaCl for 4 hours prior to RNA purification. In all samples, apart from 3067 & 16 where both bands are very faint or absent, the band is visibly weaker in the sample incubated without NaCl; this is the opposite effect to samples grown in high salt media.

was used to visualise the gels, Grab-IT Annotating grabber 2.04.5. This system saves the files as Bitmap files rather than the raw image files used to calculate band intensities previously, accounting for the different y axis values. As we are not comparing the different images, but like for like, this does not cause a problem in this instance.

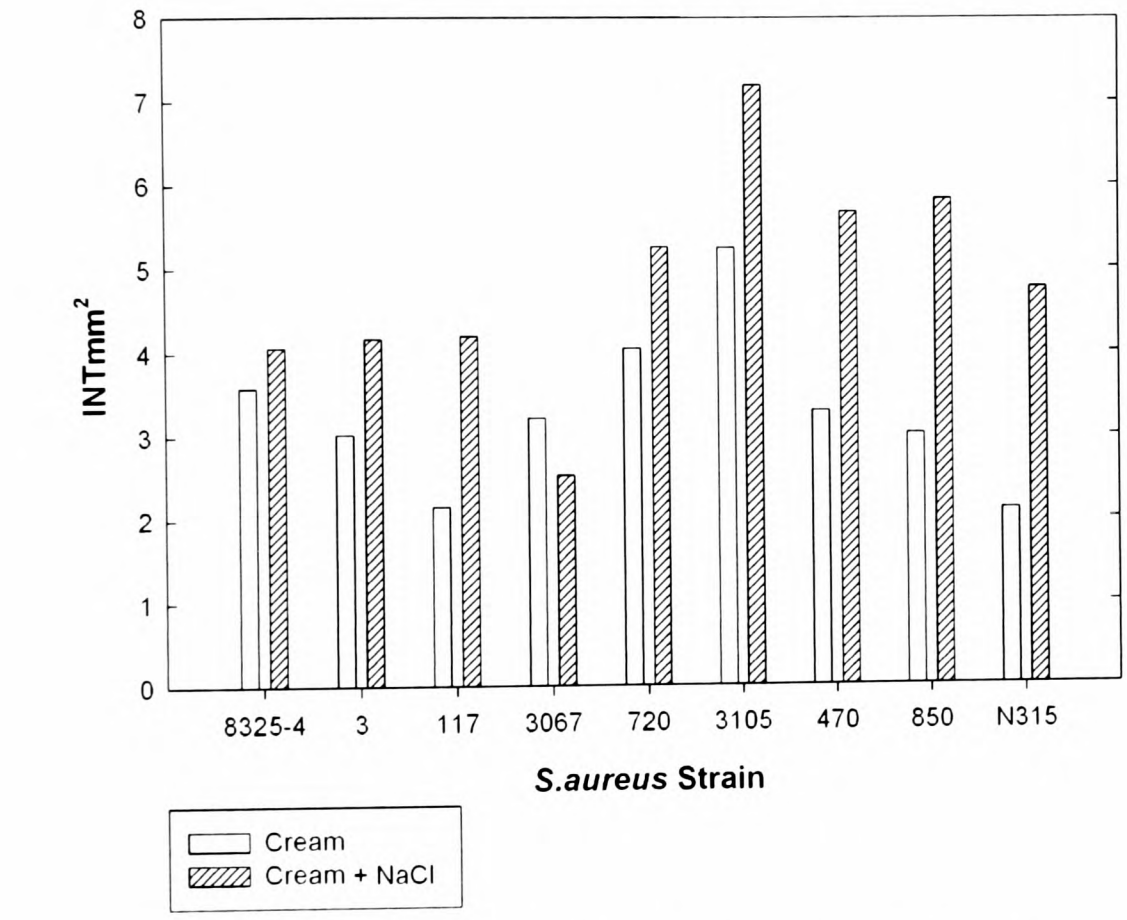

Figure 4.11: **Oilatum® down regulates *hla* expression in comparison to Oilatum® + NaCl**. Average band density intensity per mm^2 for each sample. A T-test on the density intensity values for incubation in Oilatum® Vs incubation in Oilatum® plus 1M NaCl samples shows the Oilatum® alone to be significantly lower P=0.01.

4.4 Discussion.

This chapter aimed to determine whether a common, inexpensive, chemical such as sodium chloride would have an effect on the toxicity of *S. aureus* and whether any reduction would be mediated via a decrease in toxin production, furthermore this chapter addressed whether the incorporation of NaCl into an emollient used for the treatment of eczema would reduce toxin production in *S. aureus* offering a potential therapeutic benefit.

The results of the T-cell assay show that a significant reduction in toxicity can be achieved by the addition of NaCl to growth media. Strains that initially showed little toxicity remained that way when grown with NaCl and the more toxic strains became significantly less toxic. The most toxic strain (850) showed a seven-fold decrease in toxicity (in low salt condition 9% of the T-cells survived, with high salt the T-cell survival increased to 64%). A significant drop in *hla* and *sec* expression levels encoding the pore-forming cytotoxin α-haemolysin and the superantigen like enterotoxin C was observed in the high salt treatment, and a corresponding drop in secreted toxins can be inferred from this. A limitation of the RT-PCR experiments is that no suitable housekeeping gene was used. As the RNA concentration was inferred solely by absorbance it is possible that small amounts of DNA contamination were present. By comparing levels of a suitable housekeeping gene you can ensure that no loss or degradation of RNA during extraction has occurred, there are no differences in the amount of total RNA added to the reactions and there are no variations in the efficiency of the reverse transcription or amplification steps.

The specific cause of the down regulation of toxins is not known, though a likely factor is the increased osmotic pressure, leading to increased metabolic costs (due to the need to maintain a constant internal environment). The

production of toxins mediated by the Agr system entails an enormous metabolic burden (Novick, 2003a), one which may not be feasible under more strenuous conditions.

It is possible that *S. aureus* is not only responding to osmotic pressure *per se*, but to cations present in NaCl, namely Na^+. A study examining the transcriptional regulation of the *dlt* operon (responsible for the modification of cell wall teichoic acids to D-alanine), found that cations such as Mg^+, Ca^+ and Na^+ (from 0.325 & 0.65 M NaCl) caused the down regulation of *dlt*, whereas sucrose (0.6 M) did not (Koprivnjak *et al.*, 2006), suggesting a response to cations rather than the osmotic pressure.

Far from there being one straight forward reason for the reduction in toxicity, and the mechanism in which that reduction is mediated, it is likely a number of interwoven factors are involved.

The addition of NaCl is not a viable option for any medication that is internalized. The inclusion of NaCl may however, have a beneficial effect if incorporated into creams applied topically. The effect of the salt on the skin would have to be considered, especially in the case of eczema where the skin may be badly broken.

When the strains were incubated in Oilatum® containing NaCl the expression levels of *hla* were higher than those incubated in Oilatum® alone. It is unknown whether the Oilatum® alone was causing a down-regulation of *hla* or that the Oilatum® in combination with the NaCl was causing an up-regulation. A possible explanation of this is the that the NaCl is having an unknown affect on the cream resulting in an up-regulation (or reduced down regulation) of *hla*. Further research using mRNA microarrays will give enable the change of all total gene expression to be examined.

Eczema is often treated with the narrow spectrum anti staphylococcal antibiotic fusidic acid, in combination with corticosteroids. The use of antibiotics applies a strong selection pressure for resistance, which in the case of fusidic acid can be acquired by mutation in the *fusA* gene, or by acquisition of the *fusB* plasmid (O'Neill *et al.*, 2004). The problem is magnified in cases such as eczema, as lesions readily become re-infected with bacteria when treatment is stopped, requiring re-application of antibiotics. Recent exposure to topical fusidic acid has been correlated with the presence of fusidic acid-resistant *S. aureus* (FRSA), and a significant trend towards increasing FRSA carriage with increased duration has been shown (Sule *et al.*, 2007).

4.5 Conclusion.

S. aureus has the ability to produce a comprehensive number of secreted and cell bound virulence factors. The inclusion of NaCl in growth media results in a significant down regulation of toxins and a reduced toxicity to T-cells. When Oilatum® and Oilatum® supplemented with NaCl were tested it was determined that Oilatum® alone was relatively more able to cause a down regulation of toxin expression. To examine the effect of Oilatum® in more detail, future work involving mRNA microarray technology will be used.

The targeting of toxins that are expressed, or the regulatory factors involved in their expression, is a highly attractive prospect due to the likelihood of weaker selection pressures. These data suggest that in addition to relieving the symptoms associated with eczema by moisturising the skin, the use of Oilatum® may further relieve symptoms by reducing lesion severity directly related to *S. aureus* toxin production.

"Any living cell carries with it the experiences of a billion years of experimentation by its ancestors."

Max Delbrück, 1949.

5

Mutation Rate.

5.1 Abstract.

In vitro and *in vivo* studies of several pathogens have found that mutation rates vary in their association with antibiotic resistance depending on the types of infections caused. For this study we measured the mutation rates of 145 clinical *Staphylococcus aureus* isolates and found that MRSA strains had lower mutation rates than MSSA strains. Construction of a pair of isogenic MRSA and MSSA strains demonstrated that the insertion of the SCC*mec* element was sufficient to reduce mutation rates, and that the presence of the *mecA* gene alone could reduce rates. These finding suggest that this organism has evolved an antibiotic resistance mechanism that lowers mutation rates, which may contribute to this organism's evolution by preventing specialisation to individual niches.

5.2 Introduction.

Staphylococcus aureus is a major human pathogen. The emergence of antibiotic resistant strains (e.g. MRSA: **m**ethicillin **r**esistant ***S**taphylococcus **a**ureus*) in hospitals is well documented, but such strains are now emerging in community settings amongst previously healthy people. The rate of emergence of MRSA strains worldwide suggest that this organism is highly adaptable.

Mutation rates between and within species, and even genes within a strain of a single species, can vary significantly. Within a genome the housekeeping genes, involved in basic bacterial metabolism and structure, are found to mutate at a low frequency whilst the accessory genome, important for bacterial adaptability to changing environments, is generally found to be more mutable (Martinez & Baquero, 2000; Moxon *et al.*, 1994b). A comparison between the yeast *Saccharomyces cerevisiae* and its close relative *Candida albicans* revealed that the highly expressed genes have evolved at a reduced rate (Pal *et al.*, 2001). The environment in which the bacteria grows can also affect mutation rate. Stressful conditions including: starvation, oxidation, UV irradiation, and 'arms races' induced by the coevolution of bacteria with parasitic bacteriophage have all been shown to increase mutation rates (Tenaillon *et al.*, 2004; Pal *et al.*, 2007).

Elevated mutation rates are often brought about by mutations arising in DNA damage repair genes, especially the mismatch repair systems (MRS), and lead to hypermutator phenotypes (Prunier & Leclercq, 2005; O'Neill & Chopra, 2002). In *E. coli*, inactivation of any one of >20 genes including: *mutD* (replication fidelity), *mutS*, *mutL*, *mutH* and *uvrD* (*dam*-directed mismatch repair [DDMR]) and, *mutM*, *mutY* and *mutT* (repair of oxidized guanines), can confer mutator phenotypes of differing strengths (Horst *et al.*, 1999).

Hypermutators have been readily isolated from populations of a variety of human pathogens at frequencies ranging from 2% to as high as 57% (Sundin & Weigand, 2007), with *S. aureus* hypermutators being found to make up almost 15% of populations isolated from the lungs of cystic fibrosis patients (Prunier *et al.*, 2003).

High mutation rates may increase adaptability to novel environmental conditions, though once they have adapted they may become less adaptable to new environments (Giraud *et al.*, 2001). They are also prone to the accumulation of deleterious mutations, and may impede cooperation in pathogenic bacteria (Harrison & Buckling, 2005). The mutation rate within a population is therefore not static but a fluctuating feature dependent upon a multitude of parameters including population size and environment (Denamur & Matic, 2006; Tenaillon *et al.*, 1999).

Most bacteria are haploid for the vast majority of their genes, which coupled with typically short generation times allows mutations to emerge and accumulate, rapidly creating significant phenotypic changes (Woodford & Ellington, 2007). Mutational resistance to antibiotics such as rifampicin and fusidic acid can readily arise, limiting their use in monotherapy (Mason *et al.*, 2003). This feature can be exploited to make an estimation of the mutation frequency or mutation rate (See Method 2.11 on page 65). Mutation frequency is simply the average fraction of mutant bacteria in a few replicate cultures. Mutation rate is the probability of a cell sustaining a mutation during its lifetime and if worked out correctly is more accurate and reproducible than the mutant frequency (Rosche & Foster, 2000).

In vitro and *in vivo* studies of several pathogens have found that mutation rates vary in their association with antibiotic resistance, depending on the types of infections caused. *Pseudomonas aeruginosa* (*P. aeruginosa*) isolated

from the lungs of cystic fibrosis sufferers shows a strong association between high mutation rates and increased antibiotic resistance, the same is true for *Escherichia coli* (*E. coli*) isolated from an experimental mouse model of infection (Oliver *et al.*, 2000; Giraud *et al.*, 2001). It was hypothesised that high levels of mutations increases the chance of antibiotic resistance mutations being introduced into the chromosome. It could also increase the rates of compensatory mutations being introduced that alleviate some of the costs associated with being resistant, making those with higher mutation rates more successful.

However, the converse has also been observed, where no association between high mutation rates and increased antibiotic resistance among *P. aeruginosa* isolates from acutely infected patients in intensive care units (ICU) was found (Gutierrez *et al.*, 2004). A study examining natural populations of *E. coli* found that despite the capacity of mutators (those with high mutation rates) to generate antibiotic resistant mutants *in vitro* and in experimental infections, that the highest frequency of antibiotic resistance was found amongst those with intermediate mutation rates (Denamur *et al.*, 2005). To explain these contrary findings for both *P. aeruginosa* and *E. coli* it has been hypothesised that bacteria with high mutation rates will rapidly specialise to one environment, and that this specialisation will render them less fit in another environment (Giraud *et al.*, 2001). So, although the rate at which antibiotic-resistance mutations arise might be higher, the strain will not necessarily be more fit when an infection involves movement through fluctuating environments.

The vast majority of mutations are deleterious, providing a selection pressure for lower mutation rates. This has lead to the theory that populations will evolve a mutation rate that is as low as possible (Drake, 1991; Radman *et al.*, 2000). It is not practicable, however, for mutation rates to evolve to zero. A

higher mutation rate increases the chance of acquiring beneficial mutations, this in turn allows mutators to hitchhike with the beneficial mutation and achieve fixation within a population (Maynard Smith & Haigh, 1974; Tenaillon *et al.*, 1999). Added to this is a limit to the fidelity of replication, constrained by the costs of fidelity (See Figure 5.1) (Sniegowski *et al.*, 2000).

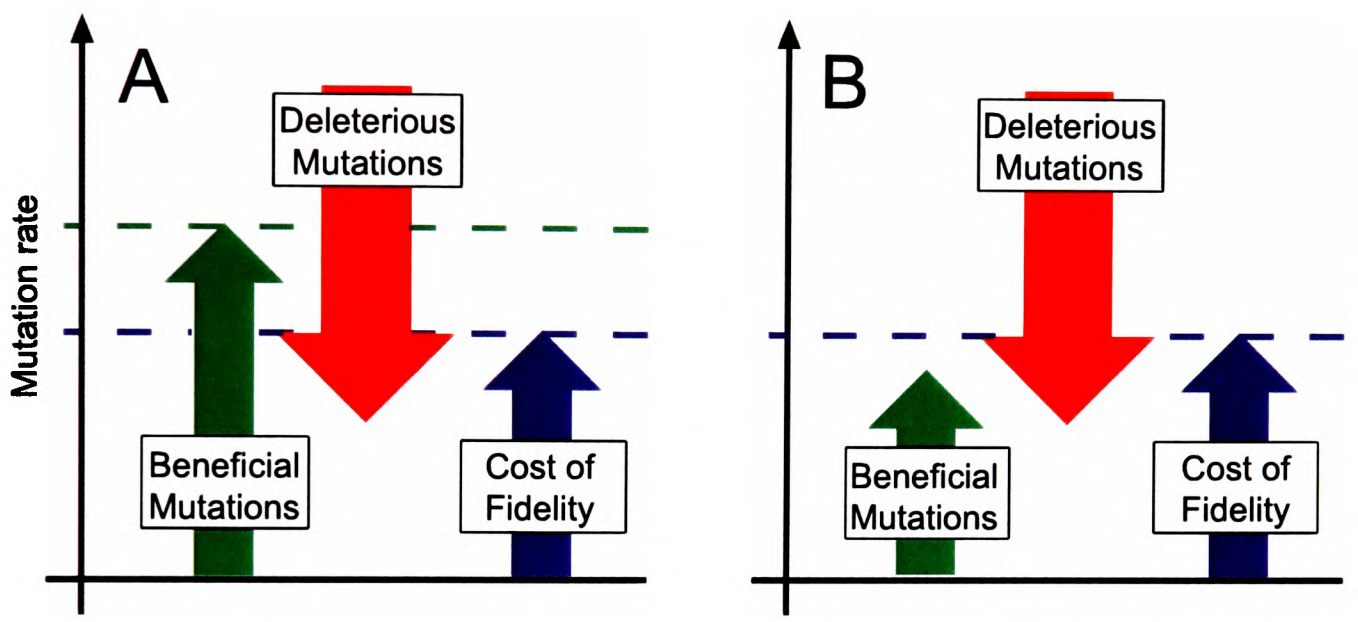

Figure 5.1: **Evolutionary forces affecting the genomic mutation rate.** Deleterious mutations (red arrow) decrease fitness, resulting in continual selection for a lower mutation rate. Rates are restrained from evolving to zero by two possible selective forces: (1) the increased probability of acquiring beneficial mutations under a higher mutation rate (green arrow), this provides a positive selective pressure on its own, but also allows mutators to hitchhike with the beneficial mutation and achieve fixation within a population, and (2) constraints on the fidelity of replication (blue arrow). **A:** The mutation rate is potentially set by a tradeoff between the effects of deleterious and beneficial mutations (green line), since mutation is required for long-term adaptation. Recombination, however, weakens the effect of beneficial mutations on mutation rate modifiers, decreasing the probability of hitchhiking, and thus the mutation rate in many populations may instead be set by a tradeoff between deleterious mutations and constraints on the fidelity of replication (blue line). **B:** Little is known about the cost of fidelity. In both sexual and asexual populations, this cost may be sufficient to set the prevailing mutation rate higher than the value that would be set by beneficial mutations, since decreases in mutation would be costly to individual fitness. Adapted from Sniegowski *et al.* (2000).

Here we sought to determine the role mutation rates might play in the biology of the human pathogen *S. aureus*. We measured the mutation rates for a large

(n=145), well-defined, collection of diverse clinical *S. aureus* isolates. These strains were isolated from the noses of healthy carriers, from hospital acquired infections and from community acquired infections. The genetic background and antibiotic resistance profile of each strain has been determined (Peacock *et al.*, 2002). Some of the strains in this collection are methicillin resistant, which in *S. aureus* is conferred by the acquisition of a mobile genetic element known as the SCC*mec*. This can vary in size and has been classified into at least seven groups depending on the size and genetic content of the element (types I–VII, Figure 1.7 on page 27). The collection of strains used in this study had types I, II and IV.

5.3 Results.

The 145 clinical strains were assayed for their mutation rate as outlined in Method 2.11 on page 65; briefly, samples were grown for a 24 hour period in BHI before aliquots were plated on TSA plates (for total cell count) and TSA plates containing 30 mg l^{-1} rifampicin to enumerate the spontaneous mutants giving rise to rifampicin resistance.

5.3.1 Is there a difference in mutation rates between MSSA and MRSA?

MSSA strains had a significantly higher mutation rate than MRSA strains ($P<0.0001$ $t=17.03$ $DF=143$, t-test outlined in Eq. 4 on page 67) (Figure 5.2).

To determine whether there were any differences between the different SCC*mec* elements the data were split by SCC*mec* type (Figure 5.3 on page 126). MSSA strains were significantly different from the MRSA type I, II & IV SCC*mec*

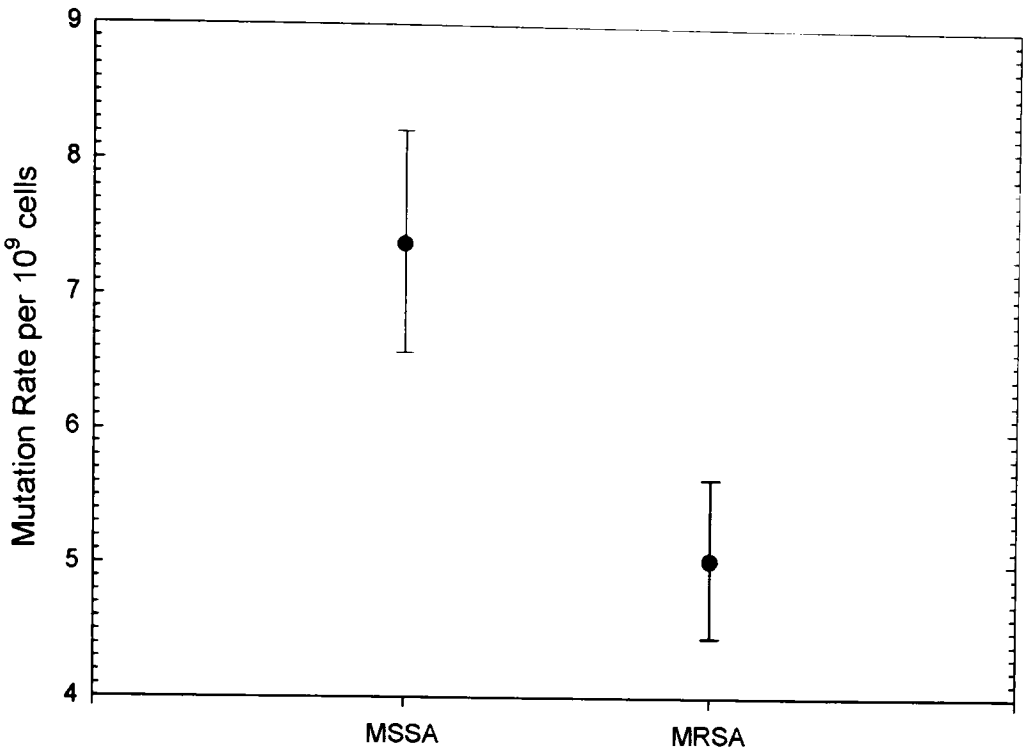

Figure 5.2: **MRSA strains have a lower mutation rate.** Mutation rate •, 95% confidence intervals whiskers. Derived from: MSSA (n=68) and MRSA (n=77) strains using MSS-MLE method (See § 2.11 on page 65). P<0.0001, using a two tailed t test.

elements in all cases. MRSA type I SCC*mec* was found to be significantly different from MRSA type IV SCC*mec*. No significant difference was found between the other comparisons (Table 5.1).

Test	**P Value**	**Adjusted α value**	**H_0 Rejected?**
MSSA Vs Type I	< 0.0001	0.0085	Yes
MSSA Vs Type II	< 0.0001	0.0102	Yes
MSSA Vs Type IV	< 0.0001	0.0127	Yes
Type I Vs Type IV	0.0036	0.0169	Yes
Type I Vs Type II	0.0267	0.0253	No
Type II Vs Type IV	0.2656	0.0500	No

Table 5.1: **Sequentially rejective Šidàk test comparison.**

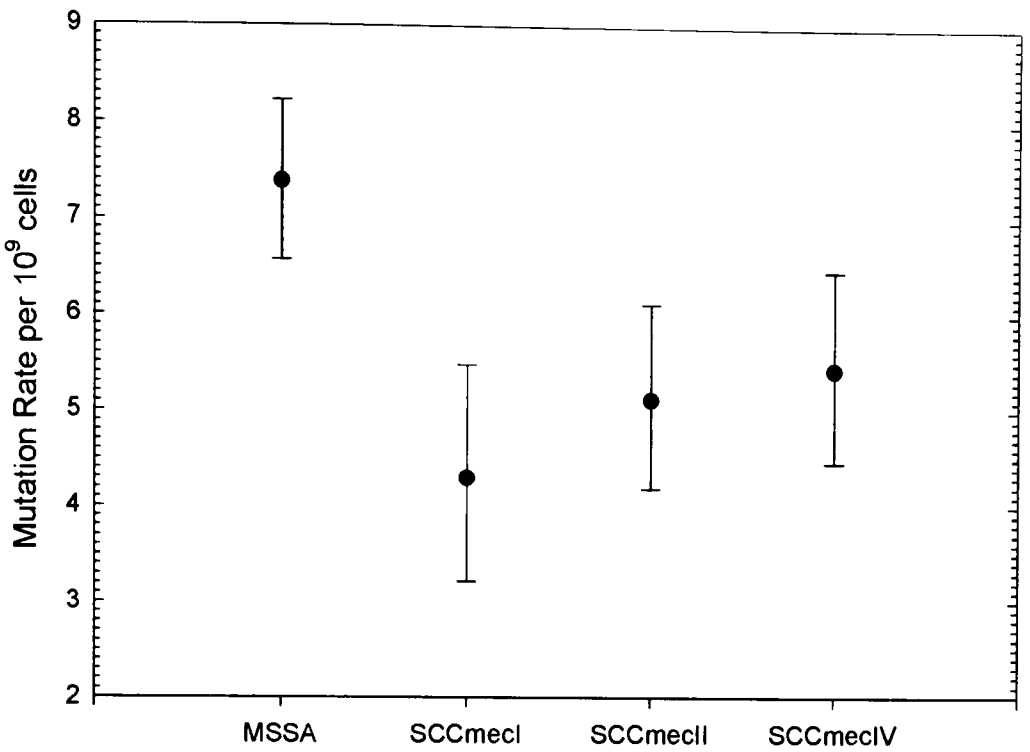

Figure 5.3: **MRSA strains have a lower mutation rate regardless of SCC*mec* type.** Mutation rates and 95% confidence intervals derived from MSSA (n=68), MRSA type I SCC*mec* (n=17), type II SCC*mec* (n=30) & type IV SCC*mec* (n=30) strains using MSS-MLE method (See § 2.11 on page 65).

5.3.2 Does the presence of SCC*mec* cause a reduction in mutation rate directly?

To determine whether the SCC*mec* element is causing an effect on the mutation rate, strains RN4220 and an isogenic SCC*mec* II bearing strain, BB4252, were compared (Figure 5.4 on the facing page).

BB4252 showed a significantly reduced mutation rate ($P<0.0001$, $T=6.32$, $DF=18$). To confirm the findings the experiment was repeated using fusidic acid rather than rifampicin. Again a significant reduction was seen ($P<0.0001$, $T=6.44$, $DF=18$), (Figure 5.5 on the next page).

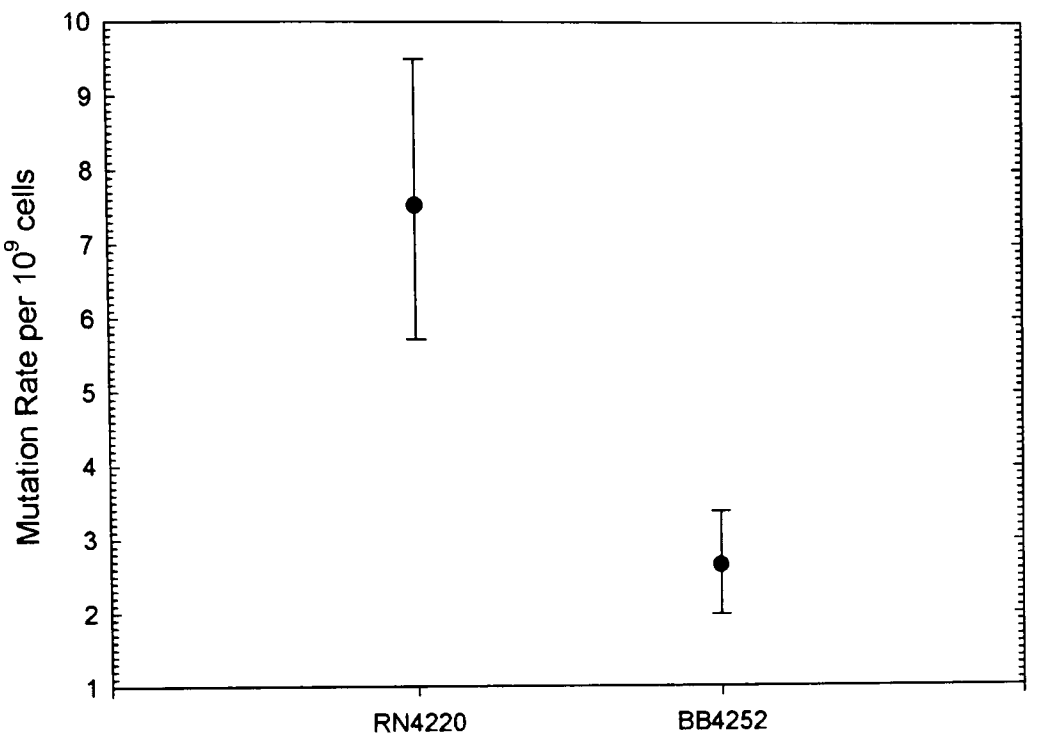

Figure 5.4: **Addition of type II SCC*mec* causes a reduction in mutation rate.** Mutation rates and 95% confidence intervals derived from RN4220 (n=10) & BB4252 (n=10) using the MSS-MLE method (See § 2.11 on page 65).

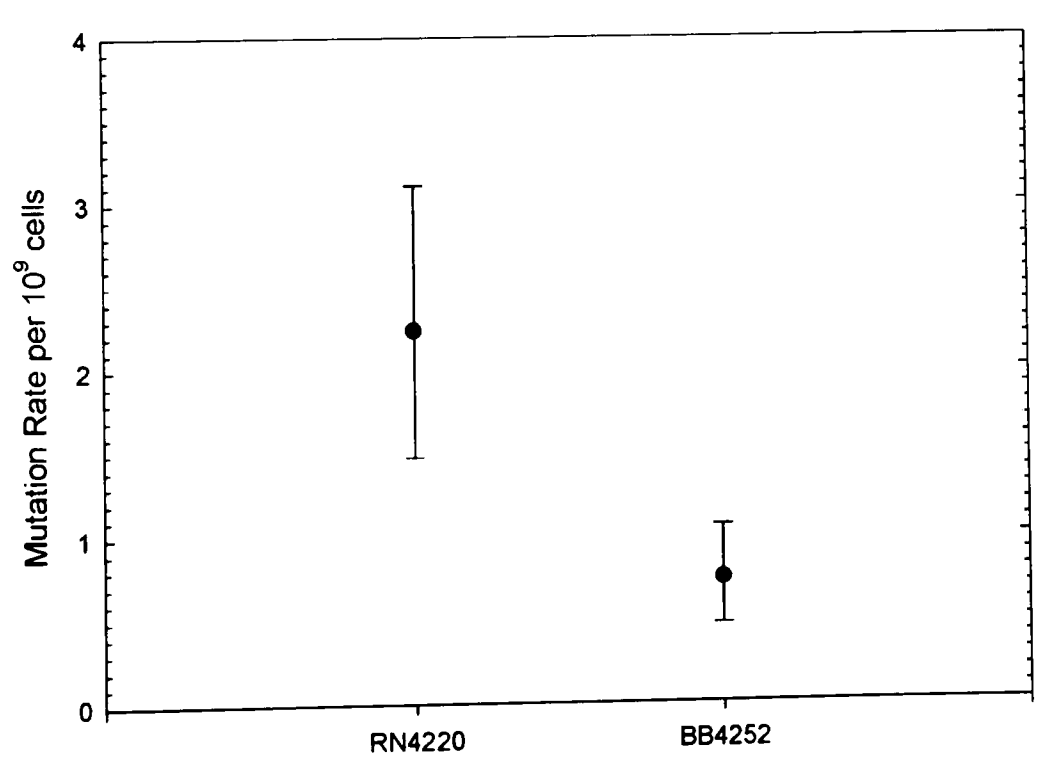

Figure 5.5: **Addition of type II SCC*mec* causes a reduction in mutation rate.** Mutation rates and 95% confidence intervals derived from RN4220 (n=10) & BB4252 (n=10) using the MSS-MLE method (See § 2.11 on page 65).

5.3.3 Is SCC*mec* as a whole responsible or is the *mecA* gene involved?

The SCC*mec* elements vary considerably in their size (20.9–66.9 Kb) and composition. Types I & IV–VII only confer methicillin resistance whereas types II & III also encode multiple resistance determinants. To test whether *mecA* affected the mutation rate a number of isogenic strains (kindly provided by Dr. Clarissa Pozzi & Dr. Jim O'Gara, University College Dublin) were tested (Table 5.2, Figure 5.6 on the next page).

Strain	**Description**
RN4220	Restriction mutant capable of excepting foreign DNA (Kreiswirth *et al.*, 1983).
RN4220 (pRB479)	RN4220 with expression vector pRB479 (Brückner, 1997).
RN4220 (p*mecA* Low)	RN4220 with expression vector pRB479 with 2867 bp EcoR1 fragment containing *mecA*.
RN4220 (p*mecA* High)	RN4220 with expression vector pRB479 with 2867 bp EcoR1 fragment containing *mecA* and selected for high methicillin resistance by serial passage on selective media.
BHICC	HA-MRSA isolate bearing type II SCC*mec*, CC8.
BHICCΔ*mecA*	BHICC with *mecA* gene inactivated by insertion.
BHICCΔSCC*mec*	BHICC with SCC*mec* excised.
BHICCΔSCC*mec* +*mecA*	BHICC with SCC*mec* excised and *mecA* introduced via an expression vector.

Table 5.2: Isogenic strains provided by Dr. Clarissa Pozzi & Dr. Jim O'Gara, University College Dublin.

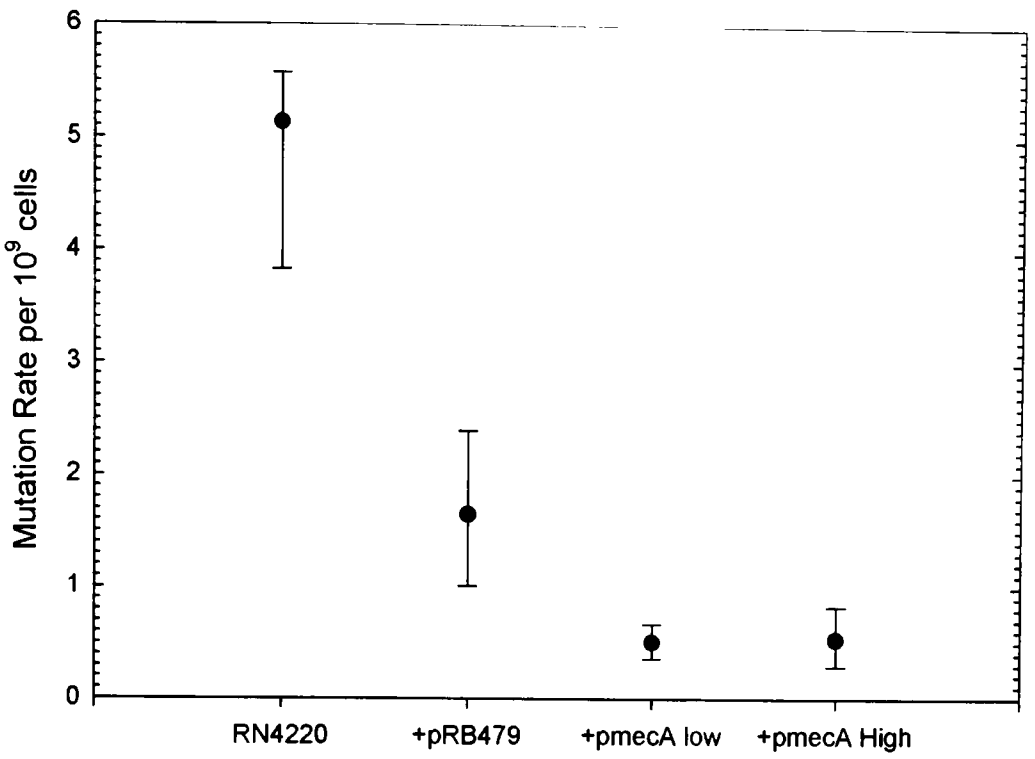

Figure 5.6: **Addition of the expression vector pRB479 and pRB479 + *mecA* causes a reduction in mutation rate.** Mutation rates and 95% confidence intervals derived from RN4220 (n=9), RN4220+pRB479 (n=8), RN4220+*mecA* low (n=9) & RN4220+*mecA* high (n=10) using the MSS-MLE method (See § 2.11 on page 65).

The addition of expression vector pRB479, pRB479+*mecA* (low), and pRB479+*mecA* (high) resulted in a significant reduction in mutation rate in comparison to RN4220. The difference between RN4220+pBR479 and the *mecA* expressing strains was also highly significant, showing a further reduction in mutation rates in the *mecA* bearing strains. There was no significant difference between the low and high strains (Table 5.3 on the following page).

To determine whether the excision of SCC*mec* or inactivation of *mecA* in a clinical HA-MRSA strain would result in a change in mutation rate, the clinical HA-MRSA strain BHICC and its set of isogenic strains were examined (Figure 5.7 on the next page, Table 5.4 & Table 5.2).

Test	P Value	Adjusted α value	H_0 Rejected?
RN4220 Vs pmecA low	< 0.0001	0.0085	Yes
RN4220 Vs pmecA high	< 0.0001	0.0102	Yes
RN4220 Vs pRB479	< 0.0001	0.0127	Yes
pRB479 Vs pmecA low	< 0.0001	0.0169	Yes
pRB479 Vs pmecA high	< 0.0001	0.0253	Yes
pmecA low Vs pmecA high	0.7000	0.0500	No

Table 5.3: **Sequentially rejective Šidàk test comparison of RN4220 & isogenic MRSA strains.**

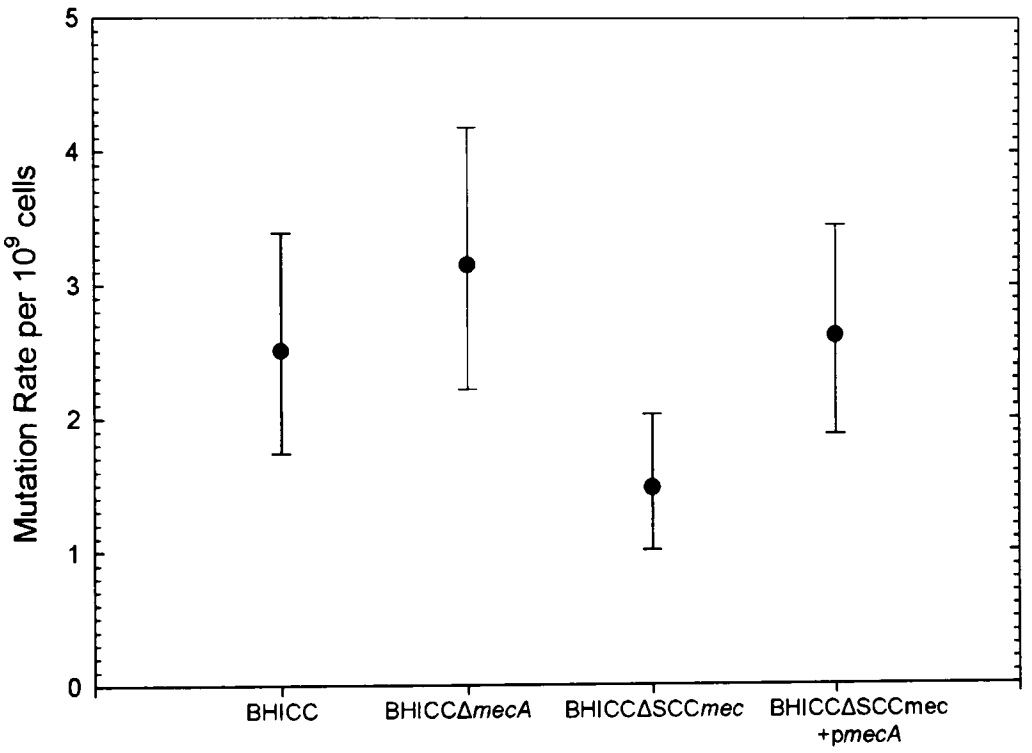

Figure 5.7: **Removal of SCC*mec* from BHICC results in a significant drop in mutation rate.** Mutation rates and 95% confidence intervals derived from BHICC (n=9), BHICCΔ*mecA* (n=10), BHICCΔSCC*mec* (n=10) & BHICCΔSCC*mec* +*mecA* (n=9) using the MSS-MLE method (See § 2.11 on page 65).

Test	P Value	Adjusted α value	H_0 Rejected?
BHICCΔ*mecA* Vs BHICCΔSCC*mec*	0.0003	0.0085	Yes
BHICCΔSCC*mec* Vs BHICCΔSCC*mec* +*mecA*	0.0034	0.0102	Yes
BHICC Vs BHICCΔSCC*mec*	0.009	0.0127	Yes
BHICC Vs BHICCΔ*mecA*	0.2177	0.0169	No
BHICCΔ*mecA* Vs BHICCΔSCC*mec* +*mecA*	0.2860	0.0253	No
BHICC Vs BHICCΔSCC*mec* +*mecA*	0.8120	0.0500	No

Table 5.4: **Sequentially rejective Šidàk test comparison of BHICC & isogenic MSSA strains.**

Inactivation of the *mecA* gene in strain BHICC resulted in an increase in mutation rate, though not a significant one. The removal of the SCC*mec* element caused a significant drop in mutation rate, the opposite of what would have been expected from our prior data. A number of potential reasons for this are considered in the discussion.

5.4 Discussion.

This chapter sought to determine the role mutation rates might play in the biology of the human pathogen *Staphylococcus aureus*. Clinical MRSA strains (SCC*mec* types I, II & IV) exhibited reduced mutation rates in comparison to MSSA strains. To further examine this, the MSSA strain RN4220 was compared with an isogenic MRSA strain BB4252 (type II SCC*mec*), with the isogenic MRSA strain exhibiting a reduced mutation rate. Incorporation of *mecA* into RN4220 reduced mutation rates. The incorporation of the expression vector pRB479 also resulted in a reduced mutation rate. These results indicate that methicillin resistance has an effect on the rate of mutation.

To see whether the excision of SCC*mec* or inactivation of *mecA* would result in an increase in mutation rate, the clinical MRSA strain BHICC and its isogenic derivatives were examined. Excision of SCC*mec* resulted in a significant drop in mutation rate — the opposite of what was expected from preceding results. Inactivation of *mecA* did result in an increase in mutation rate, but not a significant one. A number of reasons for this confounding result have been hypothesized:

- The strain BHICC has an alternative, unknown, element affecting mutation rates not present in other strains. Repeating the experiment with a

number of MRSA strains would resolve this.

- Manipulation of BHICC involved the addition of resistance genes within a vector. These resistance genes are absent in the wild type strain and may have an affect on mutation rate.
- Addition of plasmid borne recombinase to excise SCC*mec* may have affected mutation rates.
- Other pleiotropic effects resulting from the genetic manipulation of BHICC could be the cause of the mutation rate change.

5.4.1 Why Were Mutation Rates Reduced?

The ancestral mutation rate of the clinical MSSA strains is unknown (i.e. the mutation rate of the clinical MRSA strains before they became methicillin resistant), it is therefore not possible to say that the SCC*mec* element played a direct role in the reduction of mutation rates in these strains. Where isogenic strains were employed, a direct comparison can be made between the MSSA/MRSA phenotypes.

It is possible that strains with lower mutation rates were more successful following the acquisition of the SCC*mec* by not specialising to a specific niche too quickly (i.e. Computer simulations predict that weak mutators will be selected less rapidly than strong mutators but, once selected, will persist much longer than strong mutators (Taddei *et al.*, 1997)). Alternatively, mutation rates may have been lowered by a secondary event following acquisition of the SCC*mec* element, or a combination of processes may combine to reduce rates of mutation. These findings suggest that the nature of *S. aureus*' lifestyle is one where its environment fluctuates to such a degree that strains with lower mutation rates are favoured because they do not specialise to a niche as rapidly

as those with higher mutation rates.

Whereas the addition of SCC*mec* or *mecA* resulted in a significant drop in mutation rate, their excision or inactivation, in a clinical HA-MRSA strain, had little effect. This may indicate that although the presence of SCC*mec*/*mecA* causes an initial effect, the long term reduction in mutation rate is mediated by other factors, potentially compensating chromosomal mutations, located other than the SCC*mec* element.

Mutation rates are not homogenous throughout the bacterial chromosome, but differ depending on gene location. Mutation rates have been shown to increase in genes as distance from the replication origin increases (Sharp *et al.*. 1989; Mira & Ochman, 2002). These studies did not however, take into consideration the fact that different sets of genes were being compared. As such, inherent properties of the genes themselves, such as base composition and nearest neighbor frequencies, were not accounted for. A study by Hudson *et al.* (2002), using *lacZ* alleles inserted at four positions in the *Salmonella enterica* chromosome, showed mutation rates at an intermediate locus were significantly higher than those at loci nearer to, and farther from the replication origin. Insertion of the SCC*mec* element may move the genes for which we selected our mutations in (i.e. *rpoB* and *fusA* for rifampicin and fusidic acid resistance) far enough to affect mutations at these sites. These genes occur approximately 600,000 bp from the origin of replication—as determined by published strain sequences. SCC*mec* inserts into a specific site near the origin of replication, the result of which would move *rpoB* and *fusA* to a more central position on the chromosome where they would be subject to a higher mutation rate. The chromosomal location would also not account for the reduced mutation rate seen in the strains bearing plasmid borne *mecA*.

Bacterial mutation rates are controlled by mutator proteins (e.g. MutS/L)

and vary depending on which gene you select for mutations in, how much stress the bacteria is under (e.g. starvation) and the location of the gene on the chromosome. Although there are no genes on the SCC*mec* element with any homology to known mutator genes, it is possible that a novel regulator of mutations might be carried on the SCC*mec* element. Alternatively the SCC*mec* or a constituent gene or gene product may affect the expression of *mut* genes, either directly or indirectly. Increased expression of the MMR genes *mutL*, *mutS* and *mutH* in *E. coli* have been shown to result in a $\geq$twofold decrease in mutation rate (Galan *et al.*, 2007; Jacquelín *et al.*, 2008). It is not unknown for a protein with a known biological function to have other pleiotropic affects. The Toxic Shock Syndrome Toxin of *S. aureus* is able to down regulate exoprotein production to as little as 10% of that found in isogenic strains lacking *tst*, though the reason for this is unclear (Vojtov *et al.*, 2002).

Further work would include determining the expression levels of *mut* genes, such as *mutS* & *mutL*, in isogenic MRSA/MSSA strains.

5.5 Conclusion.

MRSA strains exhibit a reduced mutation rate when compared to MSSA strains. The reduction in rate can be induced by the addition of SCC*mec* to the chromosome, or *mecA* within a plasmid expression vector. The mechanism behind the reduction is unclear, though a regulator or pleiotropic affect may be responsible. By reducing the mutation rate, *S. aureus* may be able to prevent specialising to a specific niche too quickly, enabling it to thrive in a fluctuating environment.

"the time may come when antibiotics can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself, and by exposing his microbes to non-lethal quantities of the drug, make them resistant."

Alexander Fleming. Nobel Lecture, 1945.

6 Hospital Acquired MRSA.

6.1 Abstract.

In chapter 3 on page 71 an association between reduced toxicity and methicillin resistance was determined. Here, direct comparisons between clinical strains of the same clonal cluster were made. Hospital acquired MRSA (HA-MRSA) strains were found to be significantly less toxic and relatively 'less fit' than closely related MSSA strains. This association was confirmed by the construction of an isogenic HA-MRSA strain. The isogenic strain exhibited an immediate reduction in toxicity, indicating an as yet unidentified regulator, or pleiotropic effect as the cause of reduced toxicity.

6.2 Introduction.

Upon its inception in 1942, the use of penicillin to treat *S. aureus* infection was highly effective. Over 94% of *S. aureus* isolates were susceptible (Livermore, 2000),

allowing for the first time a comprehensive treatment of bacterial infection. By the late 1950s however, penicillin resistant bacteria had become numerous and widespread. The race was on to find a replacement, and in 1959 the semi-synthetic antibiotic methicillin was introduced. Methicillin (Figure 1.6 on page 26) is resistant to the β-lactamases employed by bacteria to degrade penicillin. Initial treatment with methicillin was successful, but only two years after its introduction the first case of methicillin resistance was reported in British hospitals (Jevons, 1961). Resistant isolates continued to emerge in Europe through the 1960s and the rest of the world throughout the 1970s. During the 1990s voluntary reporting by hospitals in England and Wales to the Health Protection Agency (HPA) showed that the proportion of methicillin resistant *S. aureus* (MRSA) isolates from blood culture rose from around 2% to a peak of 43% in 2002 before a slight decline thereafter (Johnson *et al.*, 2005). This situation was not repeated worldwide. Data collected from January 1999 to December 2002 involving 50,759 isolates from 495 hospitals in 26 countries showed that MRSA prevalence varied almost 100–fold, from <1% in northern Europe (Sweden, Denmark and the Netherlands) to >40% in Greece, Italy and the United Kingdom (Schito, 2006; Tiemersma *et al.*, 2004).

Methicillin acts by inhibiting the synthesis of bacterial cell walls. This is achieved by binding to and competitively inhibiting the penicillin-binding proteins (PBP, characterized by their affinity for and binding of penicillin). Penicillin binding proteins are used by Gram positive bacteria to cross-link the peptide (D-alanyl-alanine) used in peptidoglycan synthesis (Spratt, 1977). MRSA is able to resist methicillin due to an altered penicillin binding protein, penicillin binding protein 2′ (PBP2′, or PBP2a). PBP2′ has a low affinity for β-lactams which confers not only resistance to methicillin but to a broad spectrum of β-lactam antibiotics (including penicillin). Due to its low affinity

for β-lactams, PBP2′ enables cell wall synthesis to continue in the presence of β-lactam antibiotics (Lim & Strynadka, 2002b; Hartman & Tomasz, 1984; Reynolds & Brown, 1985).

PBP2′ is a product of the *mecA* gene, part of a mobile genetic element termed the 'Staphylococcal Chromosome Cassette *mec*' (SCC*mec*). The *mecA* gene is under the control of a repressor, MecI (though it can also be repressed by BlaI, the repressor of *blaZ* encoding β-lactamase the chemical inactivator of penicillin, Lewis & Dyke, 2000), and a trans-membrane β-lactam-sensing signaltransducer MecRI (Berger-Bächi & Rohrer, 2002).

MRSA strains can be divided into at least seven groups (I–VII) based upon the characterization of the SCC*mec* element they contain (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004; Oliveira *et al.*, 2006; Takano *et al.*, 2008). All SCC*mec* elements contain the methicillin resistance gene *mecA* as well as cassette chromosome recombinase genes (Katayama *et al.*, 2000). The recombinase genes are thought to be responsible for the excision and insertion of the cassette at a specific site located near the origin of replication, termed the 'open reading frame of unknown function' (*orfX*). Variation in SCC*mec* groups is due to additional genetic elements such as the linearised plasmids: pI258, pT181, and pUB110, encoding resistance determinants for erythromycin, arsenate & arsenite, tetracycline, kanamycin, tobramycin and bleomycin; the transposon, Tn554, containing resistance determinants to macrolides, clindamycin and streptogramin B (Deurenberg *et al.*, 2007); as well as 'junkyard' DNA (non-coding DNA or DNA that codes for genes without an obvious function). See Figure 1.7 on page 27 for a schematic of the seven SCC*mec* elements.

SCC*mec* types I (34.3 kb), IV (20.9-24.3 kb), V (28 kb), VI (20.9 Kb) and VII (35.9 kb) posses only the methicillin resistance determinate, *mecA*, whereas the larger types II (53 kb) and III (66.9 kb) contain multiple determinants for

resistance to non-β-lactam antibiotics (Figure 1.7 on page 27) (Deurenberg & Stobberingh, 2008). These extra resistance determinants are present on integrated plasmids and transposons that have been acquired through lateral gene transfer. The large size of the type III SCC*mec* element is the result of a fusion of two SCC*mec* elements, resulting in an additional truncated copy of the *ccr* complex and a 15 bp direct repeat between two IS431 elements (Chongtrakool *et al.*, 2006; Hiramatsu *et al.*, 2001).

SCC*mec* types I, II & III have been traditionally restricted to hospitals or out patient settings such as nursing homes and as such are known as hospital acquired MRSAs (HA-MRSA) or health care associated MRSA (HCA-MRSA) (Rychlik *et al.*, 1990; Chambers, 2001; Maltezou & Giamarellou, 2006). The type IV & V SCC*mec*s are typically found in the emerging community acquired MRSA strains (CA-MRSA), types VI & VII are also considered CA-MRSA, although they are found infrequently (Udo *et al.*, 1993; Vandenesch *et al.*, 2003; Takano *et al.*, 2008).

For the remainder of this chapter HA-MRSA strains shall refer to strains carrying the type II SCC*mec* element.

In chapter three we found an association between reduced toxicity and methicillin resistance. This chapter aims to study this association and identify how and why toxin expression is being reduced in the HA-MRSA strains.

6.3 Results.

6.3.1 Are HA-MRSA Strains Less Toxic?

To verify our previous findings, that HA-MRSA strains were less toxic than MSSA strains (see Chapter 3 on page 71), a direct comparison between strains of the same clonal cluster was made. The T-cell assay was modified to use supernate rather than washed cells, resulting in a more sensitive assay (Method 2.4.2 on page 57). 13 HA-MRSA strains (Clonal Cluster 30 [CC30]) were compared with 14 MSSA strains (CC30 n=7, CC22 n=7) (Figure 6.1).

Due to a large proportion of zero T-cell survival instances the data could not be normalised. The non-parametric Mann-Whitney U test was employed for statistical analysis. The HA-MRSA strains showed a significantly reduced toxicity compared to MSSA strains ($P<0.001$), Figure 6.1.

6.3.2 Is there an Association Between Agr Inactivation/Delay and HA-MRSA?

A clear difference in toxicity was observed between the HA-MRSA and MSSA strains. Strains from clonal cluster 30 were analysed to determine if the Agr system, a global regulator of toxins, was involved in the reduced toxicity. A functioning Agr system was determined by Western blot of δ–haemolysin (Figure 6.2 on page 141), the product of RNAIII, and also the effector of the Agr system. Due to the confounding nature of TSST-1 these strains were removed from analysis (See § 3.3.5 on page 81 & Table 6.1 on page 141).

In total 56 strains from CC30 were analysed including 18 HA-MRSA and 38

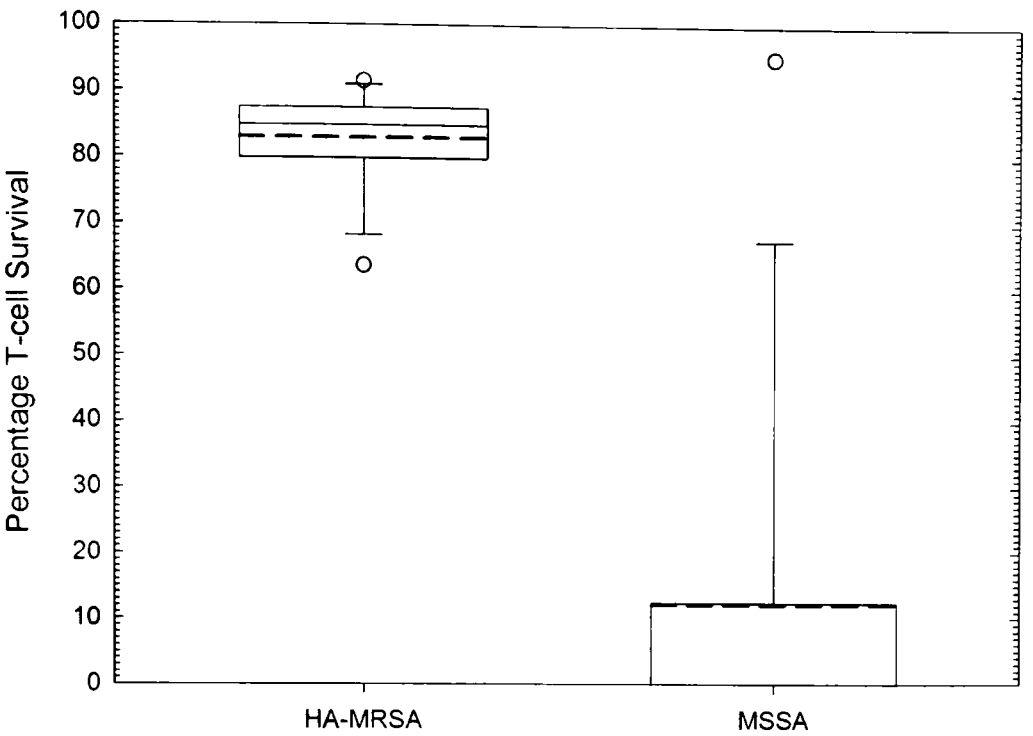

Figure 6.1: **HA-MRSA are less toxic than MSSA**. Percentage T-cell Survival by methicillin resistance. Boxplot shows: 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (o). MRSA SCC*mec* II (n=13), MSSA (n=14). MRSA SCC*mec* II are significantly less toxic than the MSSA strains ($P<0.001$) as determined using the Mann-Whitney test.

MSSA strains (Figure 6.1 on the next page). Where strains were designated HA-MRSA they were found to have a dysfunctional Agr system on 14/18 occasions (78%). MSSA strains were observed to have a dysfunctional Agr on 6/38 (16%) occasions. A statistically significant association between HA-MRSA and Agr dysfunction was found in CC30 strains, $P<0.001$ using Fisher's exact test. This demonstrates a clear association between being a HA-MRSA strain and a down regulation of toxicity in our clinical strain collection.

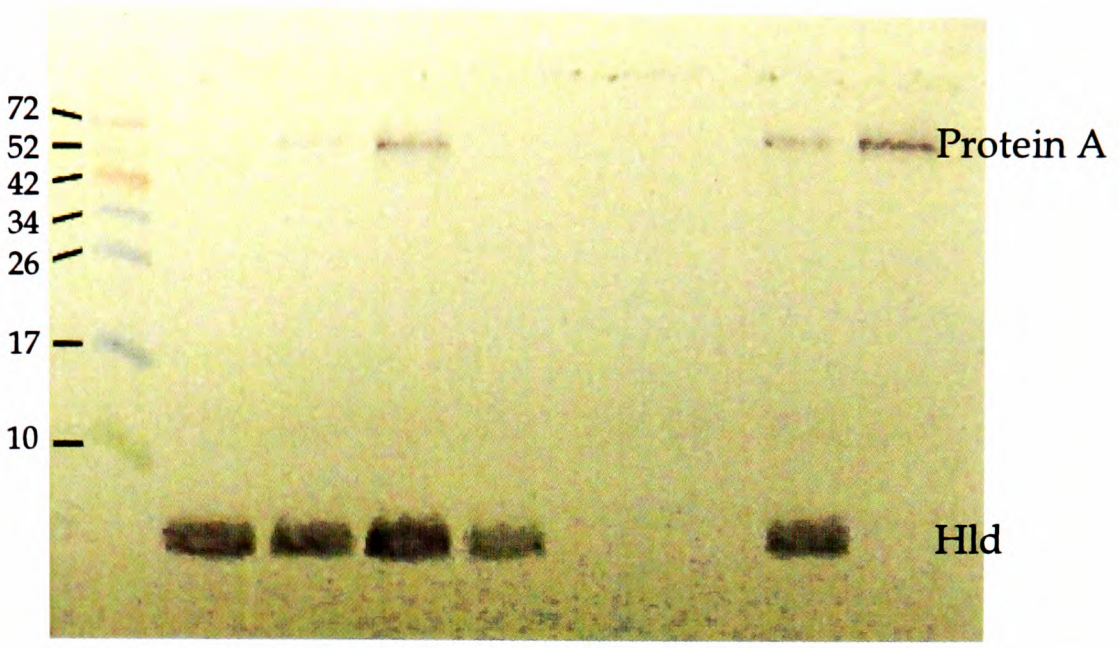

Figure 6.2: **Western Blot of δ–haemolysin (Hld).** Lower bands correspond to Hld and indicate a functioning Agr system. Upper bands indicate non–specific binding to protein A. Molecular weight (kDa) indicated on left.

Strain	Agr Function[a]	MRSA[b]	Strain	Agr Function	MRSA
21	-	+	507	-	-
41	-	+	509	-	-
45	-	+	620	-	+
69	-	+	721	-	+
73	+	-	736	+	-
119	-	+	739	+	-
123	+	+	906	+	+
133	+	-	908	+	-
137	-	+	3019	+	-
140	-	-	3033	+	-
144	+	-	3034	+	-
160	+	-	3037	-	-
209	+	-	3039	+	-
215	+	-	3050	+	-
252	-	+	3063	+	-
253	+	-	3066	+	-
307	+	-	3068	+	-
325	+	+	3078	+	-
354	-	+	3085	+	-
370	-	+	3097	+	-
378	-	+	3098	+	-
390	+	-	3100	+	-
393	-	+	3122	-	-
396	+	-	3127	+	-
399	+	-	3129	+	-
406	+	-	3182	-	-
448	+	+	3184	+	-
455	-	+	3185	+	-

Table 6.1: **Association between Agr function and MRSA.** [a] Agr function was determined by δ–haemolysin Western Blot. [b] All MRSA strains are CC30, HA-MRSA.

6.3.3 Does Producing Exotoxins Exert a Fitness Burden?

It was expected that producing toxins is costly. To show this, the clinical strain RN6390B and its isogenic Agr knockout RN6911 were competed against the marker strain, MSSA466 (Figure 6.3). A significant fitness cost was associated with toxin expression, using a two sample T-test (P=0.003, T = -5.74, DF=9).

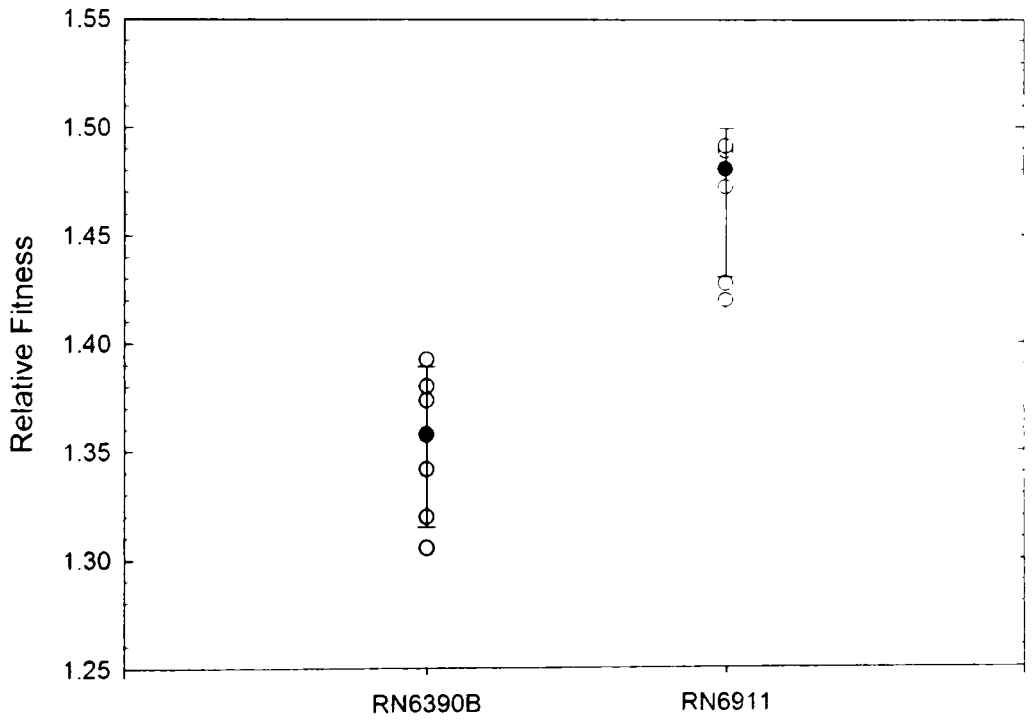

Figure 6.3: **Toxin Expression is Costly**. Strains RN6390B, a lab strain with functioning *agr* locus, and RN6911, an Agr^{-} isogenic strain, were competed against a marker strain for 24 hours in spent media, bacterial numbers were enumerated and the relative darwinian fitness calculated as per § 2.10 on page 63. ○ Data points, • Mean, Error bars indicate 95% confidence interval. RN6390B (n=6), RN6911 (n=6). A statistically significant difference in relative fitness was determined, P=0.003, T = -5.74, DF=9, using a Two Sample T-test.

6.3.4 Does SCC*mec*II Exert a Fitness Burden?

We hypothesized that the reduced toxicity exhibited in HA-MRSA strains was offsetting the costs of resistance. For this to be true there needs to be a cost associated with being HA-MRSA. We measured this with both clinical and isogenic strains. A collection of clinical strains of the same clonal cluster, thus sharing a similar genetic background, were used in a series of competition assays to compare their relative fitness (See § 2.10 on page 63).

A total of 67 strains including 44 MSSA and 23 HA-MRSA strains were competed against a tetracycline resistant marker strain (MSSA466). The resulting data were normally distributed (as indicated by an Anderson–Darling normality test) so a two sample T-test was used to compare relative fitness. The HA-MRSA strains showed a significantly reduced fitness when compared to MSSA strains ($P<0.001$, $T=6.08$, $DF=57$), Figure 6.4 on the following page.

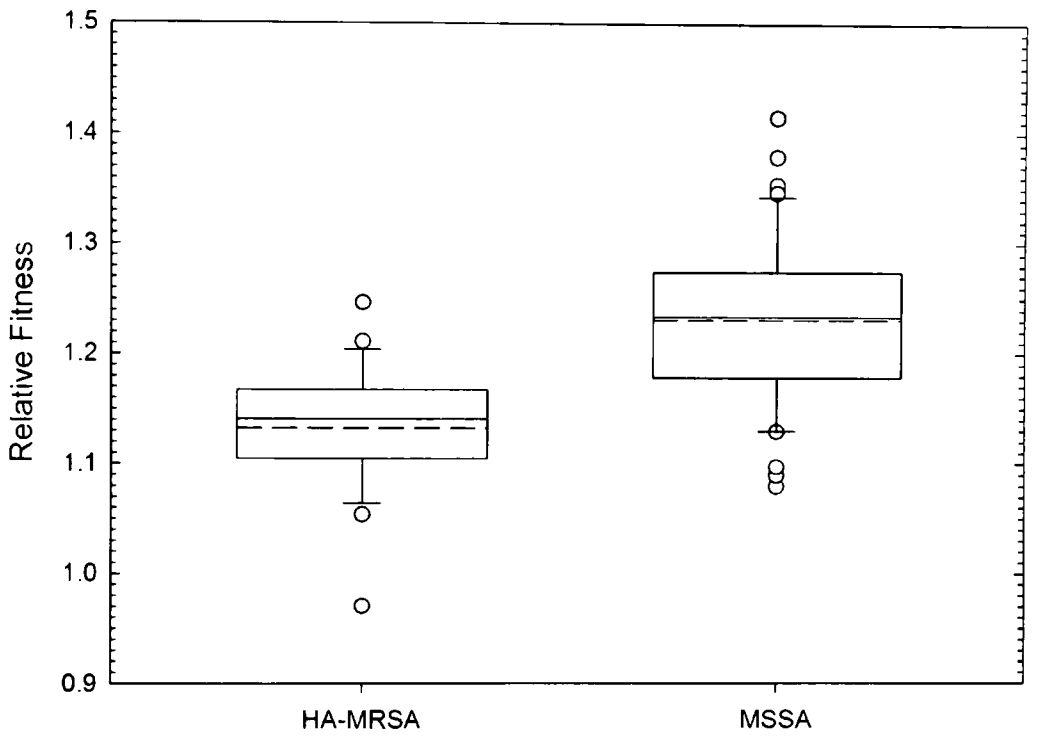

Figure 6.4: **HA-MRSA strains are less fit than MSSA strains.** Relative fitness of HA-MRSA strains compared to MSSA strains of the same clonal cluster, CC30. Strains were grown with a marker strain (MSSA466) for 24 hours in reduced nutrient media (0.25 x BHI), bacterial numbers were enumerated and the relative Darwinian fitness calculated as per § 2.10 on page 63. 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (o). HA-MRSA (n=23), MSSA (n=44). HA-MRSA strains exhibit a significantly reduced fitness compared to MSSA strains from the same clonal cluster ($P<0.001$, $T=6.08$, $DF=57$) using a Two Sample T–Test.

6.3.5 Construction of an Isogenic HA-MRSA Strain.

To confirm the results of the clinical strains an isogenic HA-MRSA strain was constructed (designated BB4252). The restriction mutant RN4220 (Kreiswirth *et al.*, 1983) was used to accept DNA from the clinical MRSA252 strain (Holden *et al.*, 2004) containing type II SCC*mec*. Phage transduction using multiple phage types and $CaCl_2$ transformation were employed to attempt to transfer the SCC*mec* element, though due to its large size this was a time consuming and highly inefficient process. The SCC*mec* element was eventually transferred using a $CaCl_2$ transformation (Method 2.13 on page 70) and confirmed by antibiotic resistance profile and PCR detection of the type II SCC*mec*-chromosome junction point (Figure 6.5).

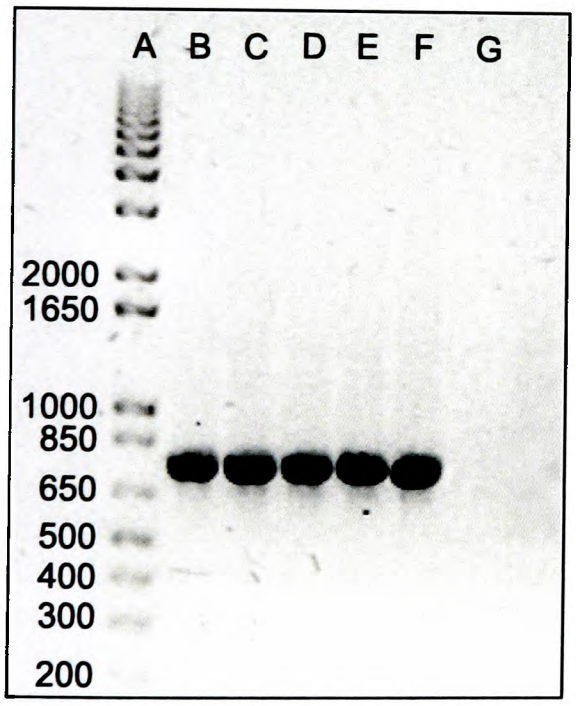

Figure 6.5: **Gel of type II SCC*mec*-chromosome junction point PCR product.** **A**: Molecular weight marker. **B–F**: 764 bp PCR fragment of type II SCC*mec*-chromosome junction point, indicating successful addition of SCC*mec* to RN4220 to create BB4252. **G**: PCR product of RN4220 DNA (negative control), no band present as RN4220 is an MSSA strain.

Isogenic HA-MRSA Strain Fitness Cost.

Having established an association between a reduced relative fitness and the type II SCC*mec* element in clinical strains we wanted to confirm that the fitness effect was due to the SCC*mec* element alone. To do this the newly constructed

MRSA isogenic strain, termed BB4252, and the parent strain RN4220 were competed against a tetracycline resistance marker strain (MSSA466) and their relative Darwinian fitness calculated. A statistically significant reduction in relative fitness of the isogenic MRSA strain (BB4252) was found using a two sample T-Test (P=0.032, T=2.59, DF=8). (Figure 6.6). In conclusion the presence of the type II SCC*mec* element significantly reduces the relative fitness of a strain.

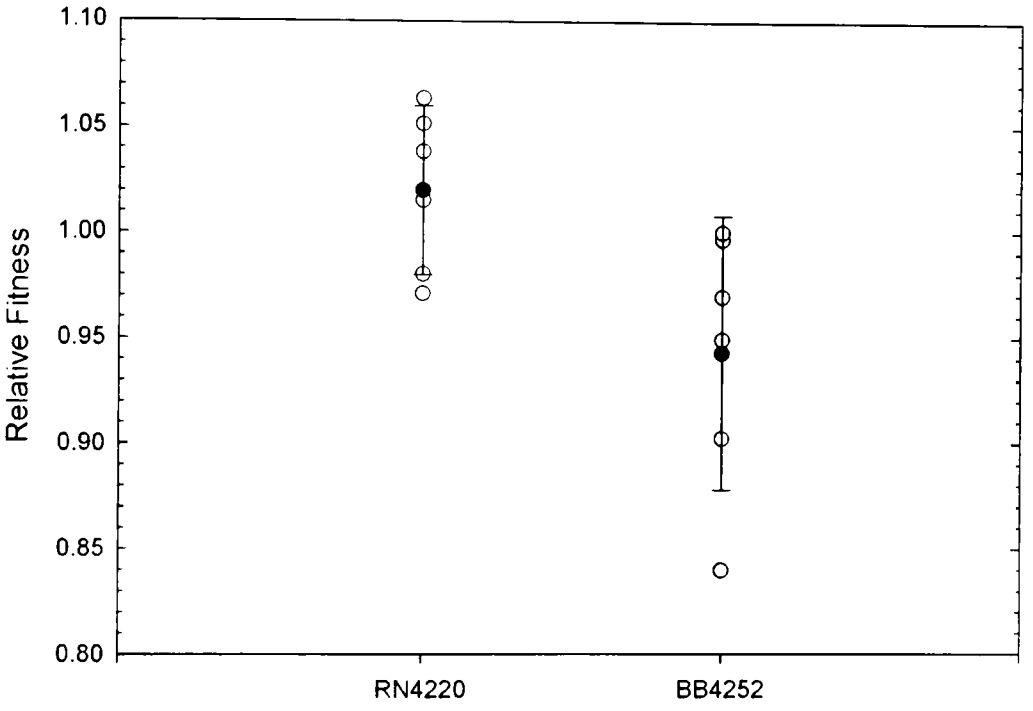

Figure 6.6: **Isogenic HA-MRSA (BB4252) is less fit than parent MSSA strain (RN4220).** Strains were grown with a marker strain for 24 hours in spent media, bacterial numbers were enumerated and the relative Darwinian fitness calculated as per § 2.10 on page 63. ○ Data points, • Mean, Error bars indicate 95% confidence interval. BB4252 (n=6), RN4220 (n=6). BB4252 exhibited a statistically significant difference in relative fitness compared to RN4220 (P<0.032, T=2.59, DF=8) using a Two Sample T-test.

Isogenic HA-MRSA Strain Toxicity.

The presence of type II SCC*mec* was shown to be costly in both clinical and isogenic strains. The clinical strains also exhibited a reduction in toxicity.

Producing toxins had also been shown to be costly (§ 6.3.3 on page 142), it was therefore hypothesised that the reduction in toxicity was related to the combined fitness costs of carrying the SCC*mec* element and expressing toxins.

Having established a fitness cost to both the presence of SCC*mec* and toxin expression, we wanted to determine if a reduction in toxicity would be seen as a direct result of having the type II SCC*mec* element, or if a secondary mutation is required. To examine this, we tested the toxicity of our isogenic strain BB4252 using the T-cell assay (Table 6.2, Figure 6.7 on the next page). BB4252 exhibited a reduced toxicity when compared to its parent strain, RN4220. T-cell survival 91.1% (mean) 92% (median) compared to 55.9% (mean) and 59.2% (median) for BB4252 and RN4220 respectively, P=0.0015 using a Mann-Whitney test. This indicates that the reduced toxicity is due to a regulatory effect of the SCC*mec*, either directly or pleiotropic, rather than by secondary mutations.

Strain	**RN4220**		**BB4252**		
Viable T-cells	**Dead T-cells**	**% Survival**	**Viable T-cells**	**Dead T-cells**	**% Survival**
235	95	71.2%	324	15	95.6%
365	121	75.1%	201	25	88.9%
119	117	50.4%	376	49	88.5%
124	294	29.7%	136	10	93.2%
146	77	65.5%	126	11	92.0%
250	53	82.5%	218	39	84.8%
44	176	20.0%	212	11	95.1%
148	132	52.9%			
Mean		**55.9%**			**91.1%**
Median		**59.2%**			**92.0%**

Table 6.2: T-cell survival when incubated for 10 minutes in supernate from strain RN4220 or BB4252, an isogenic MRSA strain containing type II SCC*mec*.

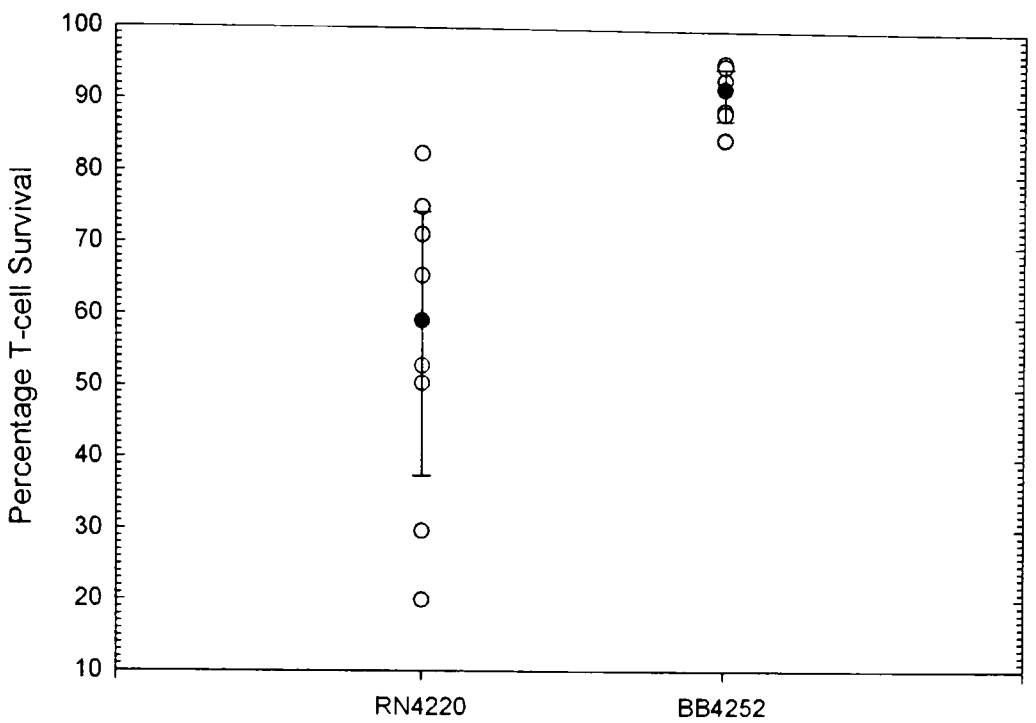

Figure 6.7: **Isogenic HA-MRSA strain is less toxic than its MSSA parent strain.** Percentage T-cell survival when incubated with supernate from RN4220 (n=8) and isogenic type II SCC*mec* strain BB4252 (n=7). • Median, ○ Data points, Error bars indicate 95% confidence level. The isogenic strain containing the type II SCC*mec* element was shown to be significantly less toxic (P=0.0015) using a Mann-Whitney test.

6.4 Discussion.

This chapter examined the association between reduced toxicity and methicillin resistance in HA-MRSA. Comparison of clinical HA-MRSA and MSSA strains from the same clonal cluster, thus being closely related, showed that clinical HA-MRSA strains exhibit a reduction in both toxicity and fitness. This association was confirmed by the construction of an isogenic HA-MRSA strain, BB4252.

A loss of Agr function has been shown to cause significantly reduced virulence in experimental animal infection models and *in vitro* experiments (Collins *et al.*, 2008; Abdelnour *et al.*, 1993; Cheung *et al.*, 1994; Gillaspy *et al.*, 1995; Novick, 2003a). Despite this, Agr^- strains are capable of colonising and

being transmitted among healthy individuals (Shopsin *et al.*, 2008b), and of causing disease (Traber *et al.*, 2008). In a hospital setting; with constant access to immunocompromised patients, open wounds, and hospital staff acting as vectors (Albrich & Harbarth, 2008); the loss of a functional Agr system would not necessarily be detrimental to *S. aureus*. The reduction in toxicity (See Figure refagrcost) would compensate (at least partially) for fitness costs associated with a high level of multidrug resistance. Furthermore, the reduction in toxin related transmission pathways (e.g. pus formation) is compensated for by an increase in vector transmission (e.g. hospital staff, visitors). Outside of a hospital setting, where the use of antibiotics is not widespread, the trade off may not be beneficial and may explain why MRSA strains carrying the type II SCC*mec* element are rarely found outside of a health-care associated setting (See § 7.4.1 on page 170).

The type II SCC*mec* element is 53 kb in length, accounting for ~2% of the ~2.9 million-bp *S. aureus* chromosome. It encodes multiple drug resistance determinants, site specific insertion sequences, recombinases necessary for site-specific integration and excision, and open reading frames of unknown function. Its physical size therefore likely exerts a metabolic burden.

Within the literature numerous indicators can be found for a cost of SCC*mec*. The expression of *mecA* has been observed at detectable levels in HA-MRSA strains without exposure to β-lactam antibiotics. This suggests that even though these strains carry intact repressors, the repression of *mecA* transcription is incomplete (Noto *et al.*, 2008). Transformation of a type I SCC*mec* element into *S. aureus* has been shown to yield highly oxacillin-resistant transformants with a reduced growth rate. This could be reversed by excision of the SCC*mec* (Ender *et al.*, 2004). The examination of transformants bearing either type I or IV SCC*mec*, found that the type I element reduced the fitness of its host

in terms of growth rate and cell yield whereas the type IV element (from a CA-MRSA strain) did not impose an energetic cost to its host (Lee *et al.*, 2007). This chapter suggests that the type II element imposes similar fitness costs to the type I element, also found in HA-MRSA.

Expression of PBP2′ (about 800 molecules cell^{-1}) does not alter the expression levels of the other four native PBPs (comprised individually between 150 and 450 molecules cell^{-1}). Any expression of PBP2′ is therefore additive to the metabolic burden (Zapun *et al.*, 2008; Pucci & Dougherty, 2002). The *mecA* gene is not the only resistance determinate on the SCC*mec* element. Other resistance determinants found on linearised plasmids within the SCC*mec* element also exert a metabolic cost, potentially in much the same way as plasmids found outside of the chromosome (Andersson & Levin, 1999; Dahlberg & Chao, 2003). A recent study by Noto *et al.* (2008) showed that certain MRSA strains when passaged to vancomycin resistance deleted portions of the SCC*mec*, providing a fitness advantage in mixed culture competition experiments. This supports the notion that multidrug resistance is costly and places a large burden on the finite resources of the cell.

Indication that a high cost is associated with the type II SCC*mec* element can be found in its limitation to a relatively small number of clonal clusters. Just 5 major pandemic clones of MRSA account for nearly 70% of all epidemic HA-MRSA strains. In contrast, the smaller type IV SCC*mec* associated with CA-MRSA is found in twice as many clones as any of the other SCC*mec* types (Aires de Sousa & de Lencastre, 2003; Robinson & Enright, 2004b). Strains from outside the major HA-MRSA lineages are resistant to transformation with plasmids encoding *mecA*, and do not express resistance. In comparison, strains from within the major HA-MRSA lineages are more transformable, readily excepting low copy number plasmids encoding *mecA* and expressing

resistance (Katayama *et al.*, 2005). This suggests that the presence of *mecA* within relatively few clonal complexes is partly due to genetic factors that are permissive of *mecA* and its gene product.

Although antibiotic resistance results in an initial fitness cost, empirical research has shown that fitness can be restored partially or fully by compensatory mutations, without the loss of resistance (Besier *et al.*, 2005; Kugelberg *et al.*, 2005; Andersson, 2003). The clinical HA-MRSA isolates examined in this study exhibited a reduced toxicity in the T-cell toxicity assay. A significant correlation was also found between the HA-MRSA strains (of CC30) and a dysfunctional/delayed Agr system. This correlation concurs with a recent study by Leonard *et al.* (2008) which examined 100 HA-MRSA (type II SCC*mec*) and 100 CA-MRSA (type IV SCC*mec*) isolates and found 41% of the HA-MRSA had a dysfunctional *agr* locus, while the CA-MRSA isolates all had a functional *agr* locus. Activation of the Agr system upon reaching quorum (See § 1.6.1 on page 32) entails a tremendous metabolic burden (Novick, 2003a). Inactivating part or all of the Agr system through mutation or regulation would compensate, at least partially, for the costs imposed by the type II SCC*mec* element.

A number of recent studies from Asia have shown a correlation between type II SCC*mec* HA-MRSA strains and the presence of *tst*, encoding the toxic shock syndrome toxin (Zaraket *et al.*, 2007; Hu *et al.*, 2008; Kim *et al.*, 2006). Strains containing *tst* produce exoproteins at very low levels, approximately 10% of that found with isogenic *tst* knockout strains (Vojtov *et al.*, 2002). The resulting low toxicity phenotype (Collins *et al.*, 2008) also results in apurulency, as observed in cases of post surgical toxic shock (Fast, 1988; Kreiswirth *et al.*, 1986) and murine subcutaneous abscess models (Vojtov *et al.*, 2002). The fact that exoproteins are down regulated in these strains may make them pre-adapted to bearing the large SCC*mec* element and associated fitness costs.

The isogenic strain showed an immediate reduction in toxicity in comparison to its parent strain. The initial cause of this is likely the metabolic burden caused by the SCC*mec* element, this cost would then be ameliorated by subsequent loss of Agr function (and a further drop in toxicity). A second possibility is that the large element contains an as yet unidentified regulator.

6.5 Conclusion.

Hospital acquired MRSA strains containing the type II SCC*mec* element exhibit a reduced relative fitness when compared to MSSA strains of the same genetic background. There is a significant correlation between the HA-MRSA strains and Agr dysfunction which may account for the observed reduction in toxicity. The results of the isogenic strain indicate that the SCC*mec* element causes a sufficient metabolic burden to down regulate toxin expression, that an as yet unidentified regulator is present or that a pleiotropic effect is occurring. This requires further research however, to be shown conclusively.

7

Community Acquired MRSA.

7.1 Abstract.

In chapter 3 on page 71 an association between reduced toxicity and methicillin resistance was determined. Here, direct comparisons between clinical strains of the same clonal cluster were made. Strains bearing the type IV SCC*mec* element, predominantly found in community acquired MRSA (CA-MRSA), were found to maintain a high degree of toxicity and were not restrained by the high fitness costs associated with the type II SCC*mec* element of HA-MRSA. The small fitness cost imposed by the type IV SCC*mec* is hypothesised to enable CA-MRSA to utilise their full arsenal of virulence factors, thriving within a community setting among otherwise healthy individuals.

7.2 Introduction.

Multiple definitions have been used to classify community acquired MRSA (CA-MRSA). A meta-analysis by Salgado *et al.* (2003) identified 8 different

definitions within the literature including isolates identified within 24–72 h of hospital admission, with or without other exclusions often including recent admission to hospital or long-term care facility or previous history of MRSA colonization. The Centers for Infectious Disease Control and Prevention (CDC) defines CA-MRSA as: 'identification of MRSA in a patient with signs and symptoms of infection, either in the outpatient setting or within 48 h after admission to a hospital, with no history of MRSA infection or colonisation, no history of admission to a hospital or a nursing home during the previous year, and absence of dialysis, surgery, permanent indwelling catheters or medical devices that pass through the skin to the body' (`http://www.cdc.gov/ncidod/dhqp/ar_mrsa_ca_clinicians.html`). It is this definition that is now commonly accepted.

During the 1980s cases of MRSA infection amongst out-patients with chronic conditions, residence in care facilities, and intravenous drug users were reported as CA-MRSA (Saravolatz *et al.*, 1982; Nelson & Wilson, 1985; Maltezou & Giamarellou, 2006). Although these cases affected people outside of a hospital setting, they are not now considered true CA-MRSA but rather healthcare-associated (HCA) MRSA due to the accompanying risk factors. In 1993, *ca.* 30 years after the report of methicillin resistance in hospitals (Jevons, 1961), the first true cases of CA-MRSA were described. The strains were isolated from patients in Western Australia with no prior hospitalisation, contact with hospital staff or associated risk factors (Udo *et al.*, 1993). Since 1993 the number of CA-MRSA cases have risen continually and are now reported worldwide (Vandenesch *et al.*, 2003). In the United States two clones, designated USA300 & USA400, are of particular concern having spread rapidly to become the predominant cause of CA-MRSA infections. The USA300 clone, isolated in September 2000, has since been implicated in outbreaks of skin and soft-tissue

infections in healthy individuals across diverse geographic regions of the United States (USA300 was found to have caused 87% of MRSA skin and soft-tissue infections at Grady Memorial Hospital, Atlanta, King *et al.*, 2006), Canada, and Europe (Ruppitsch *et al.*, 2007; Diep *et al.*, 2006; Tenover *et al.*, 2006; McDougal *et al.*, 2003).

Sequencing and analysis of the USA300 genome by Tenover *et al.* (2006) revealed mobile genetic elements encoding virulence and resistance determinants including the type IV SCC*mec*, the bicomponent cytotoxin Panton-Valentine leukocidin (PVL) and a 309 kb island of DNA not present in other *S. aureus* genomes termed ACME (encoding a complete arginine deiminase pathway that converts L-arginine to carbon dioxide, ATP, and ammonia). ACME is thought to have been acquired via horizontal transfer from *Staphylococcus epidermidis* and potentially enhances the capacity of USA300 to grow and survive within the host (Diep *et al.*, 2008c).

Despite the global spread of CA-MRSA and areas of high infection its general prevalence remains low. Tiemersma *et al.* (2004) puts the prevelance of CA-MRSA in Europe at 0.03–1.5%. Data from a meta-analysis concluded that the chance of carrying MRSA in the community was approximately 1.3%. When persons with health care contacts were excluded from analysis the prevalence of CA-MRSA dropped to $\leq$0.24% (Salgado *et al.*, 2003).

CA-MRSA differs from HA-MRSA in a number of important ways. Genotypically CA-MRSA strains are almost exclusively associated with the type IV SCC*mec* element (Maltezou & Giamarellou, 2006), which at between 20.9–24.3 kb (the size difference is due to variations in the junkyard regions) is two thirds the size of the smallest HA-MRSA element (type I SCC*mec*, 34.3 kb) and significantly smaller than the type II and III (53–66.9 kb) elements (Figure 1.7 on page 27). Type IV SCC*mec* does not contain resistance genes other than *mecA*

(Ma *et al.*, 2002). In contrast, HA-MRSA associated *mec* cassettes (II & III) frequently contain additional genetic elements such as pI258, pT181, pUB110 (linearised plasmids with resistance determinants for erythromycin, arsenate & arsenite, tetracycline, kanamycin, tobramycin and bleomycin) and Tn554 (transposon containing resistance determinants to macrolides, clindamycin and streptogramin B) (Deurenberg *et al.*, 2007).

Although the traditional nosocomial associated strains bearing type II SCC*mec* have not been known to spread in the community amongst healthy individuals, community associated type IV SCC*mec* bearing strains have been found in European hospitals and more recently hospitals in the US (Denis *et al.*, 2004; Popovich *et al.*, 2008). For the remainder of this chapter CA-MRSA strains and type IV SCC*mec* strains that have taken hold in hospitals shall be collectively referred to as MRSA-IV strains.

MRSA-IV strains belong to a diverse number of clonal types. Pulse field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) analysis has shown that they differ by region, and are unrelated to those traditionally found in hospitals (Naimi *et al.*, 2003, 2001; Groom *et al.*, 2001; Okuma *et al.*, 2002; Enright *et al.*, 2002). The reason for greater clonal diversity has not been determined, though greater efficiency of transfer of the type IV SCC*mec* element and/or a lower fitness cost to the recipient due to its smaller size and lack of 'excess baggage' included in other SCC*mec* types may play a role (Deresinski, 2005). An indication of the ease of horizontal transfer is given by Wielders *et al.* (2001) who provide evidence for the transfer of *mecA* DNA from *Staphylococcus epidermidis* to *S. aureus*, *in vivo*, in an infant treated for MSSA bacteraemia.

MRSA-IV strains have been linked to specific high virulence determinants such as the bicomponent cytotoxin Panton-Valentine leukocidin (PVL). PVL

is known to cause skin and soft-tissue infections, as well as severe necrotising pneumonia (Linde *et al.*, 2005; Diep *et al.*, 2004; Etienne, 2005; Chambers, 2001). PVL is uncommon amongst clinical isolates as a whole, occurring <5% of the time (Yamasaki *et al.*, 2005) though it has been found in as many as 40–90% of CA-MRSA strains harboring the type IV SCC*mec* (Deresinski, 2005). The association with PVL however, is likely confined to specific geographical areas or to specific clonal groups. An analysis of representative MRSA-IV isolates belonging to 8 clonal clusters in Australia were shown to lack the *pvl* gene (O'Brien *et al.*, 2004).

HA-MRSA is linked with risk factors, including: hospitalisation, surgery, long term stay in care facilities, dialysis, and indwelling medical devices. Conversely MRSA-IV shows no links with risk factors and has been associated with an otherwise healthy population, particularly the young (Naimi *et al.*, 2003; Shahin *et al.*, 1999), athletes (Kazakova *et al.*, 2005; Nguyen *et al.*, 2005; Begier *et al.*, 2004; Lindenmayer *et al.*, 1998), male same-sex couples (Diep *et al.*, 2008a; Lee *et al.*, 2005) and prison inmates (Main *et al.*, 2005; Baillargeon *et al.*, 2004; van Hal & Post, 2004; Pan *et al.*, 2003).

The previous chapters have shown HA-MRSA to have a fitness burden and that this is offset by a reduction in toxicity. It is hypothesised that this fitness burden contributes to the confinement of HA-MRSA within a hospital environment. Here we examine MRSA-IV strains to determine if fitness and toxicity are similarly effected.

7.3 Results.

7.3.1 Are MRSA-IV Strains More or Less Toxic than MSSA?

The toxicity of 10 clinical MRSA-IV strains containing the type IV SCC*mec* element (Clonal Complex 30 [CC30] n=3, CC22 n=7) were compared with 14 MSSA strains (CC22 n=7, CC30 n=7) (Table 7.1 on the facing page, Figure 7.1 on page 160) using the T-cell toxicity assay (Method 2.4.2 on page 57). Due to a large proportion of zero T-cell survival instances the data could not be normalised so a non-parametric Mann-Whitney test was used. The strains containing SCC*mec* IV showed no difference in toxicity compared to MSSA strains (P=0.24) so the null hypothesis was accepted, this held true when strains from the same clonal complex were compared (P=0.56).

7.3.2 Does SCC*mec*IV Exert a Fitness Burden?

Many MRSA-IV strains need to compete and grow outside of a healthcare associated environment. We hypothesise that the size of the type IV SCC*mec* element causes only a negligible metabolic burden in comparison to the larger SCC*mec* elements. To test this hypothesis a collection of clinical strains were used in a series of competition assays to measure their relative fitness (See § 2.10 on page 63). A total of 41 strains isolated from around the world, including 7 MSSA and 34 MRSA-IV were competed against a tetracycline resistant marker strain (MSSA466). A GLM analysis was used to determine any significant differences in relative fitness between MSSA Vs MRSA-IV strains and between clonal clusters.

Strain	Clonal Complex	SCC*mec*	T-cell Survival (%)
98.4823.X	22	IV	0.0
AR0650784	22	IV	0.0
BTN170	22	IV	0.0
BTN1626	22	IV	1.3
EMRSA15	22	IV	3.9
WW2707/97	30	IV	8.2
C720	22	IV	14.3
SWEDAO17934/97	30	IV	26.8
WW2703/97	30	IV	45.4
BTN1916	22	IV	93.2
63	22	MSSA	0.0
40	22	MSSA	0.0
3040	22	MSSA	0.0
3021	22	MSSA	0.0
3038	22	MSSA	0.0
3001	30	MSSA	0.0
3033	30	MSSA	0.0
3019	30	MSSA	0.0
3026	30	MSSA	0.0
73	30	MSSA	8.9
101	30	MSSA	11.9
65	22	MSSA	13.7
133	30	MSSA	39.8
49	22	MSSA	95.2

Table 7.1: T-cell survival when incubated for 10 minutes in supernate from MRSA-IV strains (SCC*mec* IV), and MSSA strains.

Source	DF	Seq SS	Adj SS	Seq MS	F	P
MRSA	1	0.04741	0.06085	0.04741	2.07	0.161
CC	10	1.02439	1.02439	0.10244	4.47	0.001
Error	29	0.66406	0.66406	0.02290		
Total	40	1.73586				

The MRSA-IV strains showed no significant reduction in fitness compared to MSSA strains, P=0.161 (Table 7.2 on page 162, Figure 7.2 on page 161). A significant difference in fitness was found between the different clonal clusters.

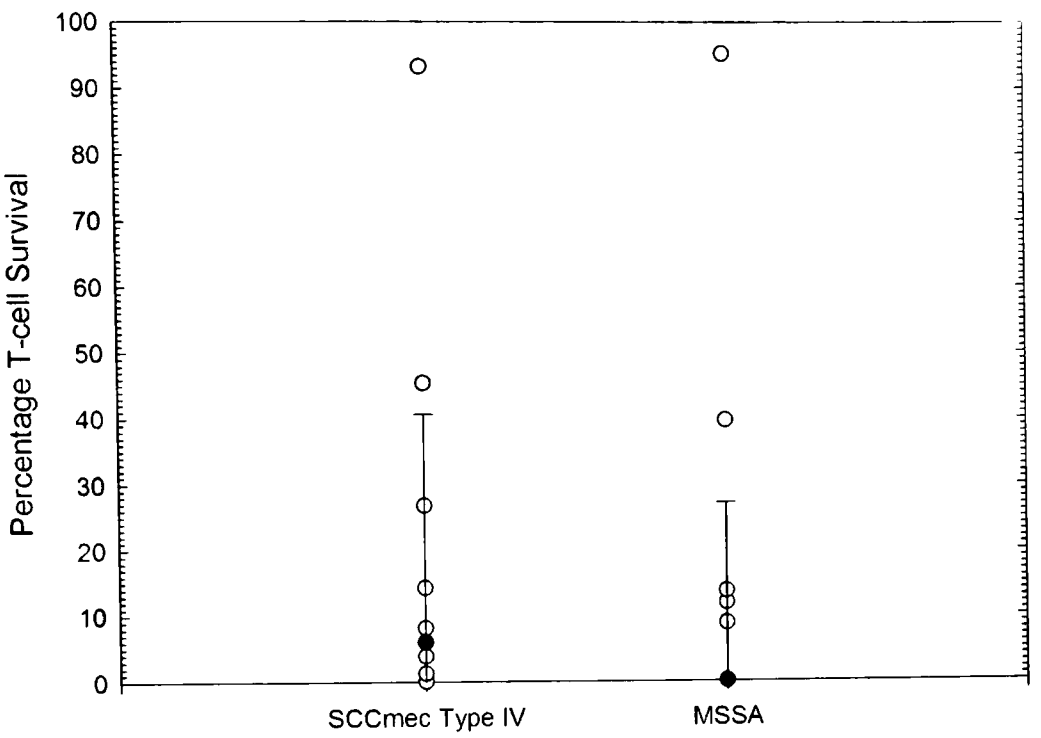

Figure 7.1: **MRSA-IV are as toxic as MSSA strains**. Percentage T-cell Survival by methicillin resistance. ○ Data points, • Median, Error bars indicate 95% confidence interval. MRSA SCC*mec* IV (n=10), MSSA (n=14). MRSA SCC*mec* IV are not significantly different to MSSA strains (P=0.24) using the Mann-Whitney confidence interval and test.

This was not surprising as MRSA-IV strains have been shown to have significant clonal diversity (Okuma *et al.*, 2002; Enright *et al.*, 2002).

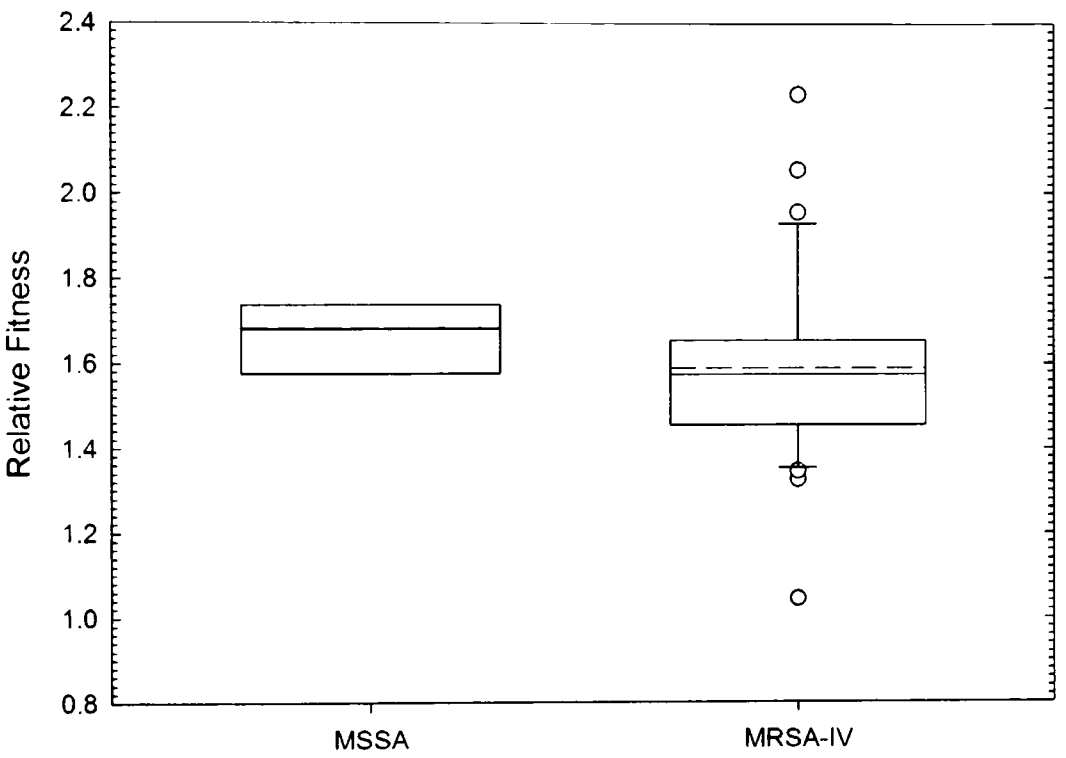

Figure 7.2: **MRSA-IV shows no significant difference in fitness compared to MSSA.** Relative Darwinian Fitness of MRSA-IV and MSSA strains. Strains were grown with a marker strain for 24 hours in a reduced nutrient media (0.25 x BHI), bacterial numbers were enumerated and the relative darwinian fitness calculated as per § 2.10 on page 63. 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (○). MRSA-IV (n=34), MSSA (n=7). No significant difference was found between MSSA and type IV SCC*mec* strains P=0.161 using a GLM.

Strain	MRSA/MSSA	Relative Fitness[a]	Clonal Cluster
BTN2445	MSSA	1.5193	22
E228	MSSA	1.6810	8
NL010548–1	MSSA	1.7329	8
NL011399–5	MSSA	1.9095	8
NOT110	MSSA	1.6273	22
NOT136	MSSA	1.5757	8
NOT160	MSSA	1.7384	22
98.4823.X	MRSA-IV	1.3268	22
98/10618	MRSA-IV	1.4404	22
99ST18131	MRSA-IV	1.5740	22
99ST7262	MRSA-IV	1.7206	22
AR0650784	MRSA-IV	1.5297	22
BTN1626	MRSA-IV	1.5067	22
BTN170	MRSA-IV	1.5536	22
BTN1916	MRSA-IV	1.6044	22
C720	MRSA-IV	1.5920	22
EMRSA15	MRSA-IV	1.5207	22
EMRSA2	MRSA-IV	1.5335	8
FIN62305	MRSA-IV	2.0552	296
FIN76167	MRSA-IV	1.4526	45
FRA97392	MRSA-IV	1.5756	8
Germany1442/98	MRSA-IV	1.0443	254
Germany825/96	MRSA-IV	1.6419	45
H182MRSA	MRSA-IV	1.6131	22
HT2001254	MRSA-IV	1.4551	1
HT2001634	MRSA-IV	1.4441	93
HT2001747	MRSA-IV	1.7756	1
HT2001749	MRSA-IV	1.9555	1
HT2002664	MRSA-IV	1.5554	80
NOT98–53	MRSA-IV	1.3458	280
NOTTMB5	MRSA-IV	1.5818	22
PHENOL27911	MRSA-IV	1.6281	22
SwedenAO17934/97	MRSA-IV	1.4031	30
SwedenAO9973/97	MRSA-IV	1.6542	22
SwedenCCUG41787	MRSA-IV	1.6715	45
Sweden8890/99	MRSA-IV	1.6539	80
WBG8343	MRSA-IV	2.2330	1
WBG8366	MRSA-IV	1.9138	255
WW2703/97	MRSA-IV	1.3591	30
WW2707/97	MRSA-IV	1.7499	30
WW844/96	MRSA-IV	1.4997	45

Table 7.2: **Relative Fitness of MRSA-IV and MSSA strains.** [a] Relative fitness is the ratio of the Malthusian parameters of the marker strain and the test strain (See § 2.10 on page 63).

7.3.3 Do Isogenic MRSA-IV & MSSA Strains Confirm These Findings?

The direct comparison of clinical MRSA-IV strains is made difficult by the fact that they belong to a diverse number of clonal types that differ by region and are unrelated to those found in hospitals. The GLM analysis indicated that the clonal type was a significant factor in strain fitness. To overcome this inherent variation, isogenic strains were used to confirm the findings of the clinical strains. The isogenic strains were kindly supplied by Dr. Berger-Bächi of the Institute of Medical Microbiology, University of Zürich, Switzerland and have been described previously (Lee *et al.*, 2007). The clinical MRSA-IV strain (ME1) was cured of SCC*mec* IV (ME230) and the isogenic strains competed (Figure 7.3 on the following page). An MSSA strain (BB255) was transformed with DNA containing the type IV SCC*mec* (ME2) and also competed against a common marker strain (Figure 7.4 on page 165). Strain details can be found in Table 7.3. No increase in fitness was associated with the excision of type IV SCC*mec*, P=0.56, T= -0.59, DF=26, using a two sample t-test. No fitness costs were associated with the insertion of type IV SCC*mec*, P=0.64, T= -0.47, DF=26, using a two sample t-test.

Strain	**Description**	**Oxacillin**[a]	**Cefoxitin**[a]
BB255	Clinical MSSA	0.25	4
ME1	Clinical MRSA-IV bearing type IV SCC*mec*	24	48
ME2	BB255 bearing type IV SCC*mec*	24	64
ME230	ME1 cured of SCC*mec*	0.38	4

Table 7.3: Description of Isogenic Strains. [a] Minimum Inhibitory Concentration (μg ml^{-1}) as determined by E-test.

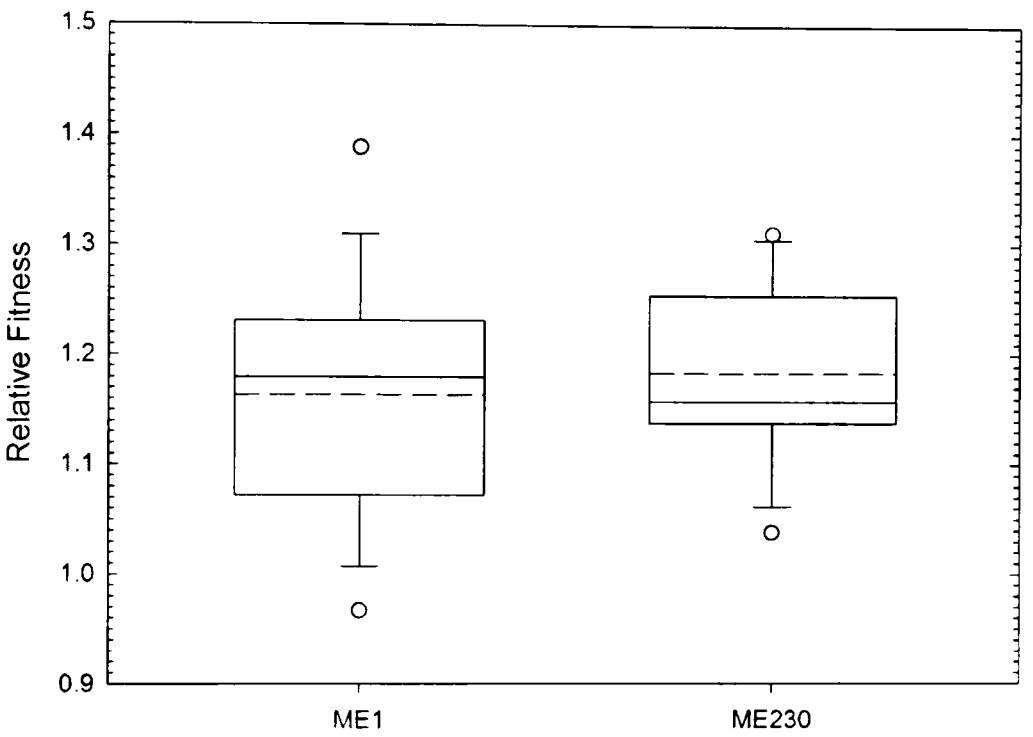

Figure 7.3: **No fitness gain associated with the excision of type IV SCC*mec*.** Relative Fitness of MRSA-IV (ME1, n=15) and isogenic strain cured of type IV SCC*mec* (ME230, n=15). Strains were grown with a marker strain for 24 hours in a reduced nutrient media, bacterial numbers were enumerated and the relative fitness calculated as per § 2.10 on page 63. 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (o). No significant difference was found between strains (P=0.56, T= -0.59, DF=26) using a two sample t-test.

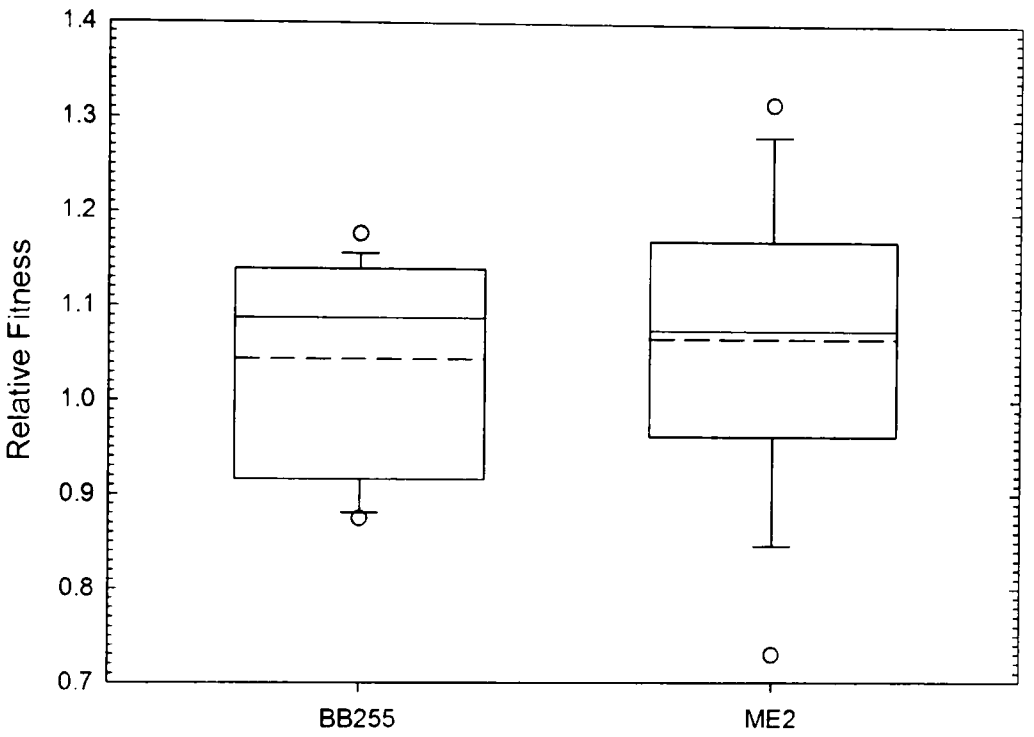

Figure 7.4: **No fitness costs associated with the insertion of type IV SCC*mec*.** Relative Fitness of MSSA (BB255, n=15) and isogenic strain with type IV SCC*mec* inserted (ME2, n=15). Strains were grown with a marker strain for 24 hours in a reduced nutrient media, bacterial numbers were enumerated and the relative darwinian fitness calculated as per § 2.10 on page 63. 25th & 75th percentile (box), 5th & 95th percentile (whiskers), median (solid line), mean (dashed line) and outliers (o). No significant difference was found between strains (P=0.64, T= -0.47, DF=26) using a two sample t-test.

7.4 Discussion.

The type IV SCC*mec* element typically found in CA-MRSA strains is between 20.9–24.3 kb in length, depending upon junkyard region variations. Open reading frames of known homologies encode only recombinase genes and the structural and regulatory genes for resistance to methicillin. This chapter aimed to elucidate whether this smaller SCC*mec* element exerted a significant metabolic burden when compared to MSSA strains and whether or not this resulted in a reduction in toxicity.

The previous chapter examined HA-MRSA isolates and found a significant fitness burden and reduction in toxicity associated with the type II SCC*mec* element. These results held true with a newly constructed HA-MRSA isogenic strain. No fitness burden or reduction of toxicity was found with the MRSA-IV strains in this study. These results confirm recent studies by Lee *et al.* (2007), whereby no fitness cost was associated with the introduction of type IV SCC*mec* into an MSSA strain and Karauzum *et al.* (2008), who, using both closely related clinical strains (ST8 & ST5) and an isogenic strain (ST30) cured of SCC*mec*, observed that the type IV SCC*mec* was not associated with a loss of virulence in a murine sepsis model.

Minimum inhibitory concentrations (MIC) of methicillin for MRSA strains varies widely, with a range from 3.1 μg to $\geq$1600 μg/ml frequently encountered (Parvez *et al.*, 2008). The expression of methicillin resistance within a strain can either be homogenous, where all cells in a population express high resistance, or heterogenous whereby the majority of cells express low-level resistance from which a sub population of highly resistant cells segregate (Berger-Bächi & Rohrer, 2002). The preponderance of strains isolated show a heterogenous phenotype (Berger-Bächi & Rohrer, 2002). CA-MRSA strains frequently exhibit

a lower MIC threshold than their HA-MRSA cousins (Kishii *et al.*, 2008; Ribeiro *et al.*, 2005). By their nature they are likely to encounter fewer antibiotics than HA-MRSA and as such the number of highly resistant strains within a population will likely remain lower. Any associated fitness costs of maintaining a high number of highly resistant sub clones will therefore be minimised in CA-MRSA strains.

There has been some debate as to whether a true distinction lies between HA-MRSA and CA-MRSA strains, a debate unhelped by the multiple definitions employed to classify CA-MRSA (Salgado *et al.*, 2003). Definitions based upon the SCC*mec* typing have helped clarify this, type IV being predominantly found in CA-MRSA strains (Maltezou & Giamarellou, 2006). HA-MRSA strains have been limited to a nosocomial environment, where they typically cause infections of the bloodstream, urinary and respiratory tracts. HA-MRSA strains are identified in a small number of clonal lineages and rarely cause disease among a healthy population (Diekema *et al.*, 2001). This contrasts starkly with the global spread of CA-MRSA strains found in diverse genetic backgrounds, unrelated to those found in hospitals (Naimi *et al.*, 2003, 2001; Groom *et al.*, 2001; Okuma *et al.*, 2002; Enright *et al.*, 2002) where they cause soft tissue infections such as abscesses, cellulitis, folliculitis, and impetigo, and severe diseases like necrotising fasciitis, necrotising pneumonia and even death in previously healthy patients (Kluytmans-VandenBergh & Kluytmans, 2006; Miller *et al.*, 2005; Hayani *et al.*, 2008). A clear epidemiological difference therefore exists between HA- and CA-MRSA strains though the genotypic difference may only be small.

A small genotypic difference may result in a large phenotypic difference where it ultimately matters the most, the manifestation of disease within the human or animal host. Well studied examples from other species have shown that a

small genetic divergence can be the difference between a harmless commensal or soil bacterium and a deadly pathogen. Examples include the presence of plasmid pXO1 in *Bacillus anthracis* encoding Anthrax toxin (Koehler, 2002; Okinaka *et al.*, 1999), β-prophage in *Diphtheria corynebacterium* responsible for Diphtheria and the prophage in *Vibrio cholerae* causing Cholera (Brussow *et al.*, 2004; Novick, 2003b). Within strains of CA-MRSA the *luk-PV* loci encoding the bicomponent cytotoxin Panton-Valentine leukocidin (PVL) has been identified as a similar virulence candidate. An association has been made between PVL and cases of severe necrotising pneumonia (Labandeira-Rey *et al.*, 2007b; Rubinstein *et al.*, 2008; Boyle-Vavra & Daum, 2007). This association is controversial. No significant difference was found between two prevalent CA-MRSA strains bearing *pvl* and their isogenic *pvl* knockouts in mouse models (Wardenburg *et al.*, 2008, 2007). Furthermore a significant number of cases of necrotising pneumonia are caused by methicillin susceptible *S. aureus*, and a large number of virulent CA-MRSA strains do not harbour *pvl* (Gillet *et al.*, 2008). It is likely, however, that PVL plays a part in the virulence of some CA-MRSA strains, though it is not the sole contributing factor (Labandeira-Rey *et al.*, 2007b; Diep *et al.*, 2008b).

The presence of a toxin regulator within the type II SCC*mec* element was indicated in the previous chapter (See § 6.4 on page 148). This was due to the direct reduction in toxicity observed with the isogenic HA-MRSA strain. This chapter has shown that no functioning regulator is present on the type IV SCC*mec*. A comparison of the type II & IV SCC*mec* elements reveals three transcription regulators on the type II element that could potentially play a role in toxicity regulation (Figure 7.5 on the facing page). There may also be further regulators that are as yet undiscovered.

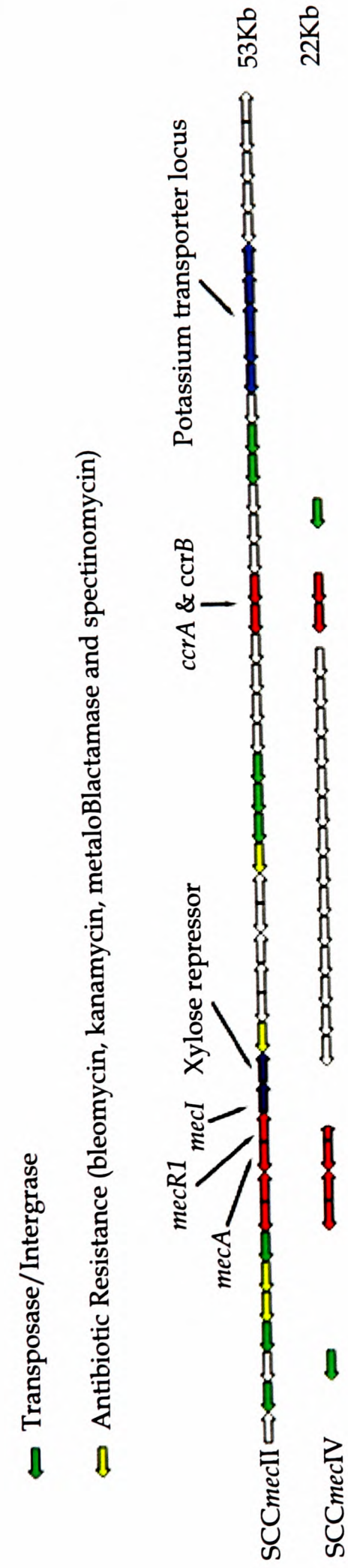

Figure 7.5: Comparison of open reading frames in SCC*mec* elements II & IV.

7.4.1 A Model of Infection.

CA-MRSA has been able to do what HA-MRSA has not, namely cause infection away from hospital or healthcare associated settings. Through this study we have been able to show that HA-MRSA strains are less toxic than MSSA and CA-MRSA strains and that this is associated with a higher fitness burden. It is not feasible to empirically test whether the factors found in this study are sufficient to cause the observed distribution of HA- & CA-MRSA within the environment, they can however be tested *in silico*. To do this, a colleague, Dr. Mario Recker of Oxford University, was asked to produce a model drawing assumptions based upon the findings of this thesis and prior published material.

- Both toxin production and antibiotic resistance lower the pathogen's fitness by a factor: $(1 - c_t^i)$ and $(1 - c_r^i)$ respectively.
- The propensity of MSSA to cause transmissible disease is greater than for HA-MRSA as these have a reduced toxin production, which is reflected in a reduced fitness cost, i.e. $\beta_t^{MSSA} > \beta_t^{HA-MRSA}$ and $c_t^{MSSA} > c_t^{HA-MRSA}$.
- CA-MRSA is more transmissible than MSSA, but at no extra fitness cost, i.e. $\beta_t^{CA-MRSA} > \beta_t^{MSSA}$ and $c_t^{MSSA} = c_t^{CA-MRSA}$.
- We assume that infection associated death (δ_i) is (slightly) higher in MRSA strains due to a reduced treatment success rate.
- We consider two routes of transmission: one via the toxin pathway, which will be more important in a community setting and the other via enhanced contact (and/or 'vectored' by health care workers and visitors) within a hospital setting.

The model competed three different strains:

- **MSSA**.
- **HA-MRSA**.
- **CA-MRSA**.

Within two different environments:

- **Community**: low antibiotic use, reduced vector transmission, healthy population.
- **Hospital**: high antibiotic use, high vector transmission via healthcare workers and visitors, compromised immune systems, but also a higher rate of treatment.

Furthermore it was assumed that susceptible hosts, S, can only be infected by one strain at a time, upon which they move into the infected classes, I_{MSSA}, $I_{CA-MRSA}$ or $I_{HA-MRSA}$; after successful treatment infected individuals return to the susceptible class, i.e. infection does not offer immunity to renewed infection.

For the rate of change in the proportion of the population susceptible or infected with MSSA, CA-MRSA or HA-MRSA the equations were defined as:

$$\frac{dS}{dt} = \mu - S\sum_i \lambda_i - \mu S + \sum_i \tau_i$$

$$\frac{dI_i}{dt} = \lambda_i S - (\mu + \delta_i + \tau_i)\, I_i$$

i = MSSA, CA-MRSA or HA-MRSA infection. The force of infection of strain i is given as:

$$\lambda_i = (\beta_t^i + \beta_h)(1 - c_r^i)(1 - c_t^i)$$

		MSSA	CA-MRSA	HA-MRSA
S	proportion susceptible			
I_i	proportion infected with type i			
μ	birth and natural death rate (we assume a constant population size)	0.013	0.013	0.013
β_t^i	transmissibility via toxin pathway of type i	0.03	0.04	0.02
β_h	transmissibility via 'vector' pathway	$0.1^{\star}/1^{\$}$	$0.1^{\star}/1^{\$}$	$0.1^{\star}/1^{\$}$
c_t^i	fitness cost due to toxin production	0.2	0.2	0
c_r^i	fitness cost due to antibiotic resistance	0	0.1	0.1
δ_i	mortality rate due to infection	0.01	0.02	0.02
τ_i	treatment rate	$0.2^{\star}/0.5^{\$}$	$0.1^{\star}/0.25^{\$}$	$0.1^{\star}/0.25^{\$}$

Table 7.4: Equation parameters. *in community setting, $^{\$}$in hospital setting.

The simulations started off with only MSSA being present in the population, and after it had reached a stable prevalence both CA-MRSA and HA-MRSA were introduced (at time t=0). Within the community (Figure 7.6 on the next page), the community-acquired MRSA quickly becomes the dominant strain because of increased toxicity and antibiotic resistance and despite a lower treatment success and increased lethality. In contrast, within a hospital setting (Figure 7.7 on page 174) the hospital-acquired form of MRSA outcompetes its community-acquired cousin because the now dominant secondary route of transmission (i.e. health care workers) disadvantages enhanced toxicity and its associated fitness cost.

There are undoubtedly multiple factors involved in the spread of *S. aureus* and as such the model is not intended to provide a complete or wholly explicit representation of infection. The simulations do however show that the factors identified are sufficient for the containment of HA-MRSA within the healthcare setting, and for CA-MRSA to predominate in the community. Manipulation of just one parameter (toxin-independent transmission) is needed to bring about these two behaviours.

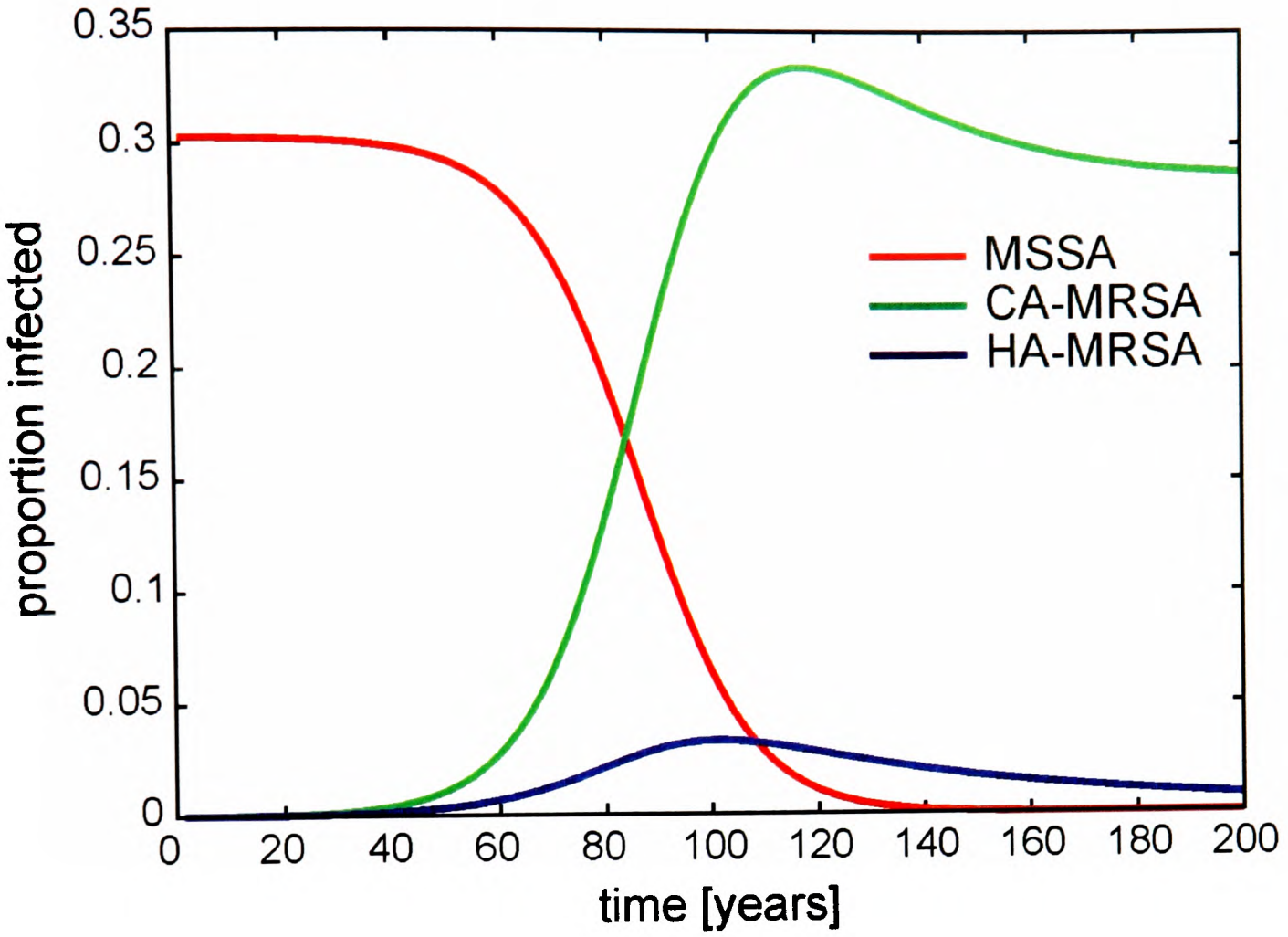

Figure 7.6: *S. aureus* infection within the community. CA-MRSA outcompetes other strains because of resistance to treatment and higher toxin related transmissibility compared to HA-MRSA.

One of the potential problems with the model is the recent findings of CA-MRSA spreading within healthcare settings (Popovich *et al.*, 2008; Benoit *et al.*, 2008; Maree *et al.*, 2007). This may be accounted for by at least two factors: (i) CA-MRSA strains causing disease are likely to be brought into a hospital environment, where initial treatment may be ineffective due to inappropriate antibiotic use e.g. β-lactams (Naimi *et al.*, 2003). (ii) As CA-MRSA strains proliferate in the healthcare setting they will be subjected to increased antimicrobial selection pressures, acquiring additional antimicrobial resistance elements and becoming more like traditional HA-MRSA strains (Gonzalez *et al.*, 2006).

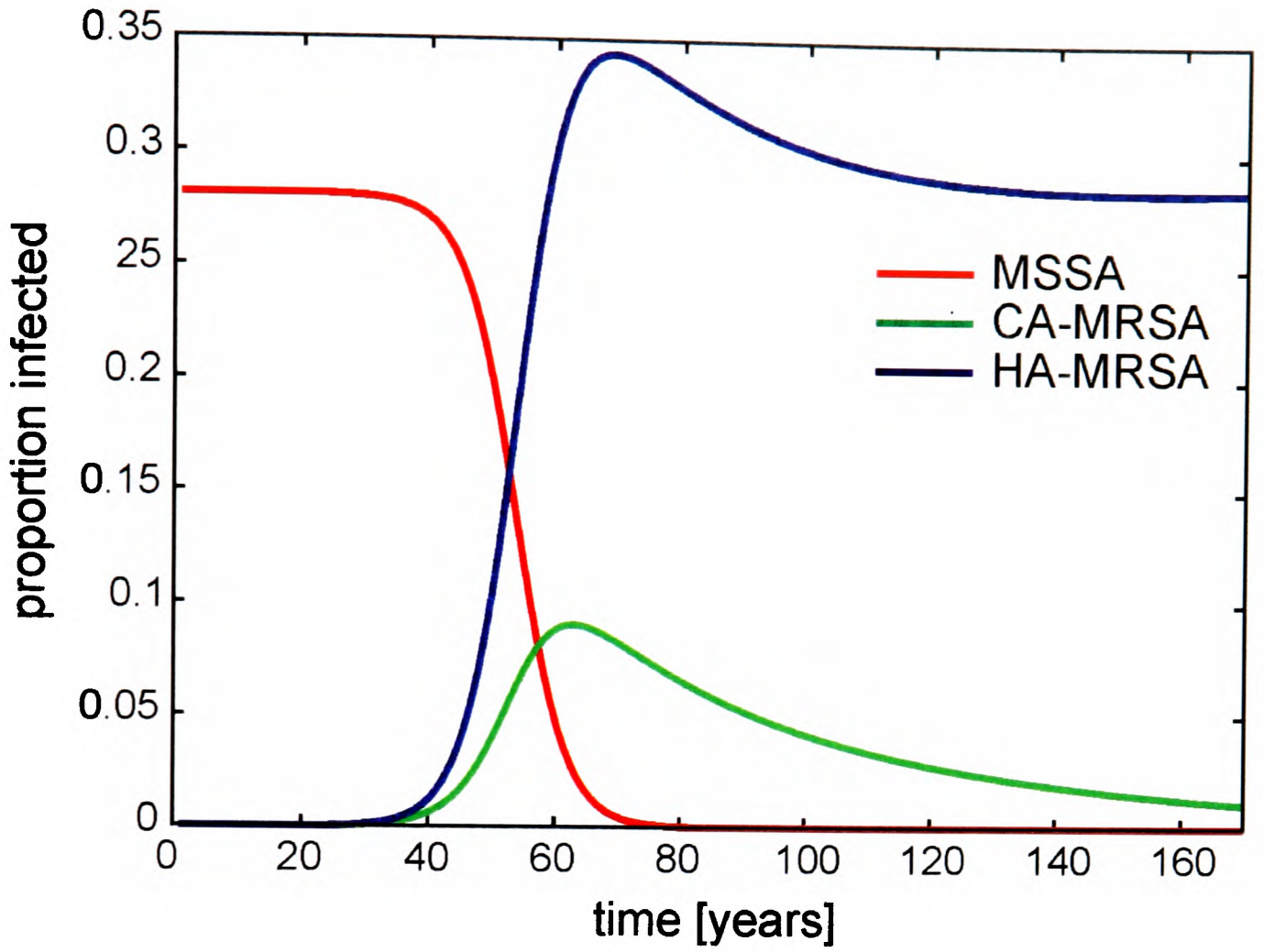

Figure 7.7: ***S. aureus* infection within a hospital.** HA-MRSA outcompetes other strains because of high resistance to treatment and reduced toxicity related death.

7.5 Conclusion.

Within the community the ability to infect otherwise healthy individuals requires a range of well adapted virulence factors. Clinical CA-MRSA isolates bearing the type IV SCC*mec* element have been shown to maintain a high degree of toxicity and are not restrained by the high fitness costs associated with the type II SCC*mec* element of HA-MRSA. This reduced fitness burden enables CA-MRSA strains to utilise their high degree of accessory genome plasticity, creating a selective advantage in the community. This advantage has enabled CA-MRSA to spread, and in certain areas replace HA-MRSA strains in the nosocomial setting (Strandén *et al.*, 2008; Moran *et al.*, 2006; Rybak

et al., 2006).

"Finally, in conclusion, let me say just this."

Peter Sellers. British Actor and Comedian, 1925–1980.

8

Conclusion.

Staphylococcus aureus is a common human commensal and opportunistic pathogen. At its disposal is a panoply of cell associated and extracellular virulence factors, and the ability to rapidly evolve resistance to novel antibiotics.

The latest figures from the Office for National Statistics (`www.statistics.gov.uk`) show that, in 2007, *S. aureus* played a role in the deaths of over 2,000 patients in England and Wales. In the United States, MRSA infection culminates in an estimated 18,650 deaths annually among hospitalised patients, likely costing billions of dollars (Klevens *et al.*, 2007; Noskin *et al.*, 2007).

The costs incurred both in monetary terms, and more importantly to health, combine to make *S. aureus* a highly studied pathogen. Using 'Staphylococcus aureus' as the search term in Scopus, an online journal database, returns almost 6,000 articles since the start of 2008. Google Scholar returns over 15,500. The majority of articles can be split into two broad categories; those that are epidemiologic in nature, examining rates of infection and mortality in relation

to a limited number of characteristics (i.e. MRSA/MSSA, presence or absence of PVL) and those endeavoring to elucidate the workings of *S. aureus* using well characterised laboratory strains.

Laboratory strains provide many benefits: they are well characterised — often with sequenced genomes, have established protocols, are amenable to genetic manipulation, and can be used at different locations and times. To this end they have been, and will likely remain, invaluable in the study of *S. aureus*. Laboratory strains are not without their pitfalls however, *in vitro* growth conditions are vastly different from those found *in vivo*, and laboratory strains adapt to these conditions accordingly. Depending on the magnitude of the selective advantage of adaptive mutations, the 10-20 generations that occur in the process of growing a bacterial culture are sufficient to create a heterogeneous population (Herring *et al.*, 2006). Sequential *in vitro* culture of *S. aureus* in rich media can select for mutations in the *agr* operon, decreasing the production of virulence factors whilst increasing growth yield (though not growth rate) (Somerville *et al.*, 2002). The repeated freeze thaw cycles, which many laboratory strains are subjected to, can lead to the mortality of large numbers of cells, leaving behind a genetically distinct population (Sleight *et al.*, 2008).

Many laboratory strains have been in use for years, maintained in what are now considered less than ideal conditions. The COL strain of *S. aureus*, first described in 1966 (Dyke *et al.*, 1966), provides an extreme example. Initially isolated from an operating theater in Colindale, it was maintained by serial passage for 10 years before stock solutions were frozen down. The COL genome was sequenced based on cultures from this stock (Fux *et al.*, 2005). Another frequently cited laboratory strain, 8325–4, has been maintained since at least 1967 (Novick, 1967). The strain 8325 and its derivatives (of which 8325–4

is one) have an 11-base deletion in *rsbU*, encoding a phosphatase activator of σ^B, an alternative sigma factor involved in the regulation of the virulon. This means that important differences in regulatory patterns among *S. aureus* strains exist. Strains lacking σ^B are also more likely to acquire spontaneous mutations in the *agr* loci, markedly altering their expression of virulence factors (Novick, 2003a). As laboratory strains are used globally by many groups the storage and growth conditions may not be the same, it is therefore possible for one laboratory strain to be genetically and phenotypically distinct from another of the same name.

The use of clinical isolates offers the chance to examine strains adapted to their host. Any one isolate however, cannot reveal the biological range within a species. Bacterial genomes show remarkable plasticity. Using a DNA micro array. Fitzgerald *et al.* (2001) examined 36 *S. aureus* strains, isolated from different human disease types, as well as bovine and ovine mastitis. Extensive genome diversity was discovered amongst the human disease causing strains, with approximately 22% of the genes representing nonessential genetic information. This information was determined to be strain-specific and organised through 18 large hypervariable regions, ten of which possessed genes encoding putative virulence factors or proteins mediating antibiotic resistance (Fitzgerald *et al.*, 2001).

Clinically relevant bacteria are under constant pressure from immune systems, antibiotics, and competition from other microbial species. It is therefore unsurprising that they differ from laboratory strains. The constant selective pressures maintained in clinical strains ensure that deleterious mutations, or those causing a disproportionate fitness disadvantage, are quickly removed from the population. Strains growing in rich media with little selective pressure are potentially capable of tolerating many more genetic alterations, over a much

shorter time span (Fux *et al.*, 2005); furthermore, many traits are not required in vitro, their continued expression becoming costly, so they are lost through mutation.

Throughout this thesis a diverse collection of clinical isolates and isogenic laboratory strains have been used to determine patterns and associations between genotypes and phenotypes of *S. aureus*. The use of a large number of clinical isolates enables the identification of factors that are likely to be clinically relevant from the noisy background found in natural samples. This reduces the number of findings that may be highly significant *in vitro*, but play a reduced role *in natura*. Once associations had been made with clinical strains they were confirmed via the careful use of appropriate isogenic laboratory strains, allowing single factors to be picked out. This approach allows the benefits of clinical and laboratory strains to be maximised, whilst minimising negative effects.

Utilising the T-cell assay, we were able to determine that a broad range of toxicities are present in clinical strains, that the presence of haemolytic toxins, and the ability of strains to co-ordinate the expression of their virulence factors with a functional Agr system is essential for high toxicity, and that negative associations between toxicity and *tst* positive strains, and HA-MRSA strains was present.

The project was funded by Stiefel Laboratories®, a pharmaceutical company focusing on dermatology and skin care products. One of the main areas of skin care is the alleviation of symptoms of atopic dermatitis or eczema. *S. aureus* is found colonising the skin of >90% of eczema sufferers, where toxin production is directly related to eczema lesion severity. We wanted to determine if toxin production could be down regulated by the incorporation of NaCl into growth media. The toxicity of strains representing 9 dominant *S. aureus* clonal clusters

was examined. A significant reduction of toxicity was observed with those strains grown with NaCl. Reverse transcriptase PCR showed that the reduction in toxicity corresponded with a down regulation of both α-haemolysin and the superantigen, SEC. To determine if NaCl could be incorporated into an emoliant used to treat eczema, bacteria were incubated in either the emollient, Oilatum®, or Oilatum supplemented with NaCl. Interestingly, bacteria incubated in Oilatum alone showed lower *hla* expression levels. Further work is currently being undertaken, including a full mRNA array on bacteria incubated with and without Oilatum to, determine expression level changes.

Staphylococcus aureus is present as a commensal in the noses of approximately 30% of the human population, typically only causing disease in individuals with breaches in immunity. It is therefore largely considered an opportunist pathogen. A significant feature of *S. aureus* infections is the wide range in both the severity and body site in which infection occurs. What is clear is that during an infection *S. aureus* is exposed to widely fluctuating environments in which it must survive and grow to transmit to new sites and new hosts. We measured the mutation rates of clinical *S. aureus* strains and found that the *mecA* gene, conferring methicillin resistance, lowers mutation rates. By lowering the rate of mutation, *mecA* prevents *S. aureus* from adapting to a single niche too quickly. This allows MRSA strains to remain competitive, whilst moving easily through diverse and rapidly fluctuating environments.

The *mecA* gene is common to all MRSA strains, however, the SCC*mec* differs considerably between hospital- and community-MRSA strains. Hospital acquired MRSA strains, containing the type II SCC*mec* element, were found to exhibit a significantly reduced fitness and toxicity when compared to MSSA strains of the same genetic background. A significant correlation between HA-MRSA strains and Agr dysfunction was also observed. We hypothesised that

the reduction in toxicity was due to the metabolic burden imposed by the large SCC*mec* element. By modulating the toxin expression a significant metabolic burden can be reduced. Within a hospital setting the loss of virulence factors may be offset by the gain in antibiotic resistance and the increased vector transmission to compromised hosts from hospital staff and visitors. Compensatory mutations may play a role in the reduction in toxicity of clinical HA-MRSA strains, though the results from the isogenic HA-MRSA strain suggest that other factors, potentially a regulatory element, may be involved. Further work, including the examination of segments of the type II SCC*mec* for regulatory affects, is currently being undertaken.

Within the community the ability to infect otherwise healthy individuals requires a range of well adapted virulence factors. We found that clinical CA-MRSA isolates bearing the type IV SCC*mec* element maintained a high degree of toxicity and were not restrained by the high fitness costs associated with the type II SCC*mec* element. This reduced fitness burden enables CA-MRSA strains to utilise their high degree of accessory genome plasticity, creating a selection advantage in the community. This advantage has enabled CA-MRSA to spread and in certain areas replace HA-MRSA strains in the nosocomial setting (Strandén *et al.*, 2008; Moran *et al.*, 2006; Rybak *et al.*, 2006).

Cohen (1972) wrote:

> 'The staphylococci are somewhat of an enigma. They are easy to handle in the laboratory, capable of genetic study by phage-mediated transduction and lysogeny experiments, and often sensitive to many antibiotics and therefore useful for study of their modes of action. The staphylococci have been studied as much as any Gram positive bacterium, and yet the distinction between their interaction with man as a commensal and as a pathogen is still a mystery.'

Thirty-seven years have passed since those words were written, however, the sentiment remains true. This thesis has added to our understanding of *Staphylococcus aureus*, revealing new insights but also posing new questions.

A

Clinical Strain Collection & T-cell Assay Data.

Strain	origin	disease	CC	see	sec	sed	seb	sej	seg	seh	sei	eta	etb	clfa	clfb	spa	fnba	fnbb	cna	efb	ebps	ica	map	v8	coag	sdrc	sdrd	pvl	hlg	alpha	beta	delta	sdre	bbp	sea	tst	agr	pen	cip	fus	rif	tet	trim	ery	gent	net	amik	vanc	mrsa	alive	dead	alive	dead	T-cell %
2	1	1	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	4	48	4	48	8.94
3	1	1	51	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	4	1	0	0	0	0	0	0	0	0	0	0	68	42	25	65	63.81
13	1	1	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	25	29	37	32	28.21
19	2	1	16	0	1	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	37	62	33	1	63.91
21	2	1	30	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	33	25	18	71	54.26
25	1	1	25	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	31	32	14	43	29.71
38	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	31	33	14	62	50.00
39	2	1	39	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	14	32	43	58	36.27
40	2	1	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	43	62	58	51	42.62
65	2	1	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	58	1	38	8	92.37
67	2	1	8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	38	8	84	52	79.59
69	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	46	7	31	52	43.37
73	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	42	17	38	10	81.63
96	1	1	45	0	1	0	0	1	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	62	42	84	14	81.34
98	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	1	0	46	7	74	49	86.36
112	2	1	25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	42	42	46	27	42.59
114	1	1	39	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	62	17	57	72	81.21
116	2	1	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	41	40	50	39	53.53
117	2	1	12	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	47	19	32	13	71.17
118	2	1	25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	4	30	3	40	9.09
119	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	43	9	50	12	81.58
123	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	43	16	41	16	70.21
125	1	1	8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	41	16	81	16	77.67
126	1	1	12	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	44	14	44	14	79.05
140	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	0	1	0	0	0	0	0	0	0	0	0	0	86	17	86	17	81.00
144	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	83	22	83	22	79.05
148	2	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	130	6	103	3	96.28
150	2	1	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	30	55	42	52	40.22
154	1	1	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	27	46	32	62	35.33
155	1	1	5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	24	57	22	35	33.33
157	2	1	5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	72	40	49	50	57.35
159	2	1	25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	96	43	91	38	69.78
160	1	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	0	1	0	0	0	0	0	0	0	0	0	0	61	12	66	16	81.94
162	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	69	17	81	20	80.21
169	2	1	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	38	11	38	12	76.77
182	2	1	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	65	11	52	20	79.05
197	1	1	25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	46	49	30	31	48.72
205	2	1	25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	64	39	101	60	62.50
207	1	1	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	50	6	45	11	84.82
208	2	1	22	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	34	8	25	5	81.94
209	1	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	36	6	34	4	87.50
215	1	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	46	17	34	18	69.57
233	1	1	45	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	39	4	37	4	90.48
252	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	20	27	28	22	49.48
281	2	1	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	27	21	11	33	41.30

Figure A.1: Strain Data and T-cell Survival. **Origin:** 0=Carriage, 1=Community Acquired Disease, 3=Hospital Acquired Disease. **CC** = Clonal Cluster.

Strain	origin	disease	CC	spe	sec	sed	seb	sej	seg	seh	sei	eta	etb	cifa	clfb	spa	fnba	fnbb	cna	efb	ebps	ica	map	v8	coag	sdrc	sdrd	pvl	hlg	alpha	beta	delta	sdre	bbp	sea	tst	agr	pen	cip	fus	rif	tet	trim	ery	gent	net	amik	vanc	mrsa	alive	dead	alive	dead	T-cell %
283	1	1	25	0	0	0	1	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	15	82	17	86	16.00
291	2	1	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	58	7	46	11	85.25
295	2	1	45	0	1	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	68	65	66	62	51.34
303	2	1	12	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	78	25	78	13	80.41
307	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1	0	1	2	1	0	0	0	0	0	0	0	0	0	103	57	87	55	62.91
309	2	1	25	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	76	38	54	43	61.61
312	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	1	3	1	0	0	0	0	1	1	0	0	0	63	34	46	37	60.56
315	2	1	8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	68	17	68	53	66.02
316	1	1	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	76	33	39	19	68.86
321	2	1	45	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	62	35	76	50	61.88
325	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	0	0	1	0	0	0	1	76	48	106	74	59.87
326	2	1	8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	164	20	115	15	88.85
352	2	1	25	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	182	42	179	30	83.37
354	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	3	1	1	0	0	0	1	0	0	0	1	202	36	198	24	86.96
357	1	1	15	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	2	1	0	0	0	0	0	0	0	0	0	118	55	54	32	66.41
364	1	1	39	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	0	0	1	3	1	0	0	0	0	0	0	0	0	0	75	65	53	62	50.20
370	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	3	1	0	0	0	0	0	1	0	1	1	143	33	101	30	79 48
378	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	3	1	0	0	0	0	0	1	0	1	1	136	34	138	39	78.96
382	2	1	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	2	1	0	0	0	0	0	0	0	0	0	99	23	98	34	77.56
383	2	1	22	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	90	45	80	56	62.73
390	1	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	1	0	1	3	1	0	0	0	0	1	0	0	0	0	82	54	103	74	59.11
393	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	0	3	1	1	0	0	0	1	1	0	0	1	188	45	197	54	79.55
396	1	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	1	3	1	0	0	0	0	0	0	0	0	0	63	101	47	74	38.60
399	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1	1	3	1	0	0	0	0	0	0	0	0	0	149	31	178	24	85.60
402	2	1	12	0	0	0	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	73	31	77	44	66.67
406	2	1	30	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1	1	3	1	0	0	0	0	0	0	0	0	0	35	36	46	63	45.00
410	2	1	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0			2	1	0	0	0	0	0	0	0	0	0	53	53	51	77	44.44
414	1	1	22	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	72	24	61	18	76.00
415	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	3	1	0	0	0	0	0	0	0	0	74	35	68	36	66.67
417	2	1	16	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	2	1	0	0	0	0	0	0	0	0	0	77	51	66	45	59.83
427	1	1	39	0	0	0	1	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	0	1	3	1	0	0	0	0	0	0	0	0	98	5	90	19	88.68
433	1	1	5	0	0	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	2	1	0	0	0	0	0	0	0	0	0	107	34	86	32	74.52
434	1	1	8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	74	91	54	108	39.14
437	1	1	25	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	41	102	29	69	29.05
442	2	1	45	0	1	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	84	9	74	13	87.78
448	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	1	3	1	1	0	0	0	1	1	1	0	0	150	39	159	40	79.64
449	1	1	25	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	0	0	1	1	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	57	65	52	53	48.02
451	2	1	5	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	2	0	0	0	0	0	1	0	0	0	0	49	47	69	49	55.14
455	2	1	30	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	1	3	1	1	0	0	0	1	1	0	0	1	146	46	133	34	77 72
456	2	1	45	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0	165	63	181	74	71.64
458	2	1	15	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	0	0	2	1	0	0	0	0	0	0	0	0	0	134	25	151	29	84.07
462	2	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	1	0	3	1	0	0	0	0	0	0	0	0	0	105	27	123	30	80.00
474	1	1	45	0	1	0	0	0	1	0	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	106	25	91	24	80.08
476	1	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	3	1	0	1	0	0	0	0	0	0	0	186	21	116	32	85.07
481	2	1	45	0	1	1	0	1	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	73	76	37	58	45.08

Figure A.2: Strain Data and T-cell Survival. **Origin:** 0=Carriage, 1=Community Acquired Disease, 3=Hospital Acquired Disease. **CC** = Clonal Cluster.

Strain	origin	disease	CC	see	sec	sed	seb	sej	seg	seh	sei	eta	etb	clfa	clfb	spa	fnba	fnbb	cna	efb	ebps	ica	map	v8	coag	sdrc	sdrd	pvl	hlg	alpha	beta	delta	sdre	bbp	sea	tst	agr	pen	cip	fus	rif	tet	trim	ery	gent	net	amik	vanc	mrsa	alive	dead	alive	dead	T-cell %
486	2	1	45	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	0	0	1	1	1	0	0	1	1	1	1	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	61	27	38	46	57.56
511	1	1	5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	122	30	124	43	77.12
513	2	1	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	3	1	0	0	0	0	0	0	0	0	0	0	140	55	116	42	72.52
560	2	1	51	0	0	0	1	0	1	0	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	0	0	2	1	1	0	0	0	0	0	0	0	0	0	83	61	81	44	60.97
591	2	1	8	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	80	28	91	47	69.51
593	1	1	25	0	0	0	1	0	0	0	0	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	133	42	124	56	72.39
597	2	1	25	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	131	64	130	87	63.35
599	2	1	39	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	0	0	3	1	1	0	0	0	0	0	0	0	0	0	107	51	116	59	66.97
617	2	1	45	0	0	0	0	1	0	1	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	97	58	71	38	63.64
620	2	1	30	0	0	0	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1	0	0	3	1	1	0	0	0	0	0	0	1	0	0	115	20	115	19	85.50
624	2	1	45	0	1	0	0	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	21	45	23	34	35.77
640	1	1	22	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	70	22	75	25	75.52
686	1	1	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	90	68	72	95	49.85
701	1	1	25	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	92	30	96	34	74.60
707	2	1	16	0	0	1	0	0	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	123	41	93	25	76.60
720	1	1	22	0	0	0	0	1	0	1	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	47	39	57	54	52.79
721	2	1	30	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	3	1	1	1	0	0	0	1	0	0	0	1	104	34	93	37	73.51
724	2	1	22	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	52	22	43	17	70.90
730	1	1	45	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	41	60	50	75	40.27
736	1	1	30	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	127	137	98	111	47.57
739	1	1	30	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	0	3	1	0	0	0	0	0	0	0	0	0	0	94	39	77	32	70.66
767	1	1	39	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	1	0	3	1	0	0	0	0	0	1	0	0	0	0	29	39	36	65	38.46
811	2	1	5	0	0	0	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	55	26	69	36	66.67
827	1	1	8	0	1	0	0	0	1	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	34	66	38	88	31.86
831	2	1	30	0	0	0	0	1	0	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	3	1	1	0	0	0	0	1	0	0	0	0	1	97	37	73	26	72.96
837	1	1	12	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	78	26	93	24	77.38
850	1	1	8	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	33	42	40	58	42.20
858	2	1	30	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	3	1	1	0	0	0	0	1	0	0	0	0	1	83	16	93	36	77.19
863	2	1	16	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	92	108	83	77	48.61
888	2	1	39	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	1	3	1	0	0	0	0	0	0	0	0	0	69	9	79	6	90.80
900	1	1	45	0	0	0	0	1	0	1	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	49	70	36	80	36.17
906	2	1	30	0	0	0	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	1	1	3	1	1	0	0	0	0	1	0	0	0	57	10	67	18	81.58
908	2	1	30	0	0	0	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	0	1	3	1	0	0	0	0	0	0	0	0	0	0	122	19	53	8	86.63
952	2	1	39	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	0	1	1	1	1	0	1	0	1	3	1	0	0	0	0	0	0	0	0	0	0	36	46	53	43	50.00
959	1	1	30	0	0	0	0	1	0	0	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	0	0	1	1	0	0	0	0	1	1	3	1	0	0	0	0	0	0	0	0	0	0	41	15	48	26	68.46
3001	0	0	22	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	19	69	13	61	19.75
3002	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	34	32	27	32	48.80
3003	0	0	16	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	40	38	35	55	44.64
3004	0	0	5	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0		1	0	0	0	0	0	0	0	0	0	0	19	61	23	34	30.66
3009	0	0	15	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	59	21	51	20	72.85
3011	0	0	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	1	1	3	1	0	0	0	0	0	0	0	0	0	62	9	40	6	87.18
3012	0	0	39	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	3	1	0	0	0	0	0	0	0	0	0	86	11	69	17	84.70
3013	0	0	45	0	0	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	60	22	41	20	70.63
3014	0	0	5	0	0	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	74	12	51	17	81.17
3015	0	0	51	0	0	0	0	0	1	0	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1	0	0	2	1	1	1	0	0	0	0	0	0	0	0	42	16	48	19	72.00

Figure A.3: Strain Data and T-cell Survival. **Origin:** 0=Carriage, 1=Community Acquired Disease, 3=Hospital Acquired Disease. **CC** = Clonal Cluster

Strain	origin	disease	CC	see	sec	sed	seb	sej	seg	seh	sei	eta	etb	ctfa	ctfb	spa	fnba	fnbb	cna	efb	ebps	ica	map	v8	coag	sdrc	sdrd	pvl	hlg	alpha	beta	delta	sdre	bbp	sea	tst	agr	pen	cip	fus	rif	tet	trim	ery	gent	net	amik	vanc	mrsa	alive	dead	alive	dead	T-cell %
3016	0	0	25	0	0	0	1	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	53	23	51	13	74.29
3017	0	0	25	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	69	11	79	14	85.55
3018	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	46	4	50	5	91.43
3020	0	0	39	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	27	31	26	33	45.30
3021	0	0	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	36	18	45	44	56.64
3022	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	108	41	119	43	72.99
3023	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	70	29	98	49	68.29
3025	0	0	8	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	106	11	114	47	79.14
3026	0	0	30	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	73	22	83	35	73.24
3027	0	0	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	18	65	17	87	18.72
3028	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	68	17	51	21	75.80
3029	0	0	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	92	6	69	4.17
3030	0	0	45	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	39	51	26	58	37.36
3031	0	0	5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	29	38	41	39	47.62
3032	0	0	39	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	42	19	29	26	61.21
3033	0	0	30	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	56	34	41	19	64.67
3034	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	72	22	58	12	79.27
3045	0	0	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	112	29	111	28	79.64
3046	0	0	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	98	36	63	30	70.93
3047	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	34	45	47	42	48.21
3050	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	77	38	92	49	66.02
3051	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	76	54	77	54	58.62
3052	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	63	55	30	30	52.25
3053	0	0	45	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	67	76	63	64	48.15
3054	0	0	45	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	45	55	33	53	41.94
3056	0	0	5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	58	17	46	14	77.04
3057	0	0	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	38	30	38	30	55.88
3058	0	0	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	51	36	44	28	59.75
3059	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	101	29	75	32	74.26
3060	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	94	19	107	23	82.72
3061	0	0	51	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	4	1	0	0	0	0	0	0	0	0	0	0	0	84	15	83	24	81.07
3062	0	0	22	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	63	32	67	68	56.52
3063	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	55	18	64	28	72.12
3064	0	0	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	82	109	79	79	46.13
3066	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	96	31	82	28	75.11
3067	0	0	45	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	146	21	115	16	87.58
3068	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	100	19	74	19	82.08
3070	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	56	49	67	42	57.48
3071	0	0	5	0	0	1	0	1	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	116	22	109	20	84.27
3076	0	0	8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	18	93	12	72	15.38
3077	0	0	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	81	57	101	58	61.28
3078	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	41	32	44	33	56.67
3079	0	0	30	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	42	24	63	29	66.46
3080	0	0	25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	98	15	85	18	84.72
3081	0	0	45	0	1	1	0	1	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	42	104	69	116	33.53

Figure A.4: Strain Data and T-cell Survival. **Origin:** 0=Carriage, 1=Community Acquired Disease, 3=Hospital Acquired Disease. CC = Clonal Cluster

Strain	origin	disease	CC	see	sec	sed	seb	sej	seg	seh	sei	eta	etb	clfa	clfb	spa	fnba	fnbb	cna	efb	ebps	ica	map	v8	coag	sdrc	sdrd	pvl	hlg	alpha	beta	delta	sdre	bbp	sea	ist	agr	pen	cip	fus	rif	tet	tsst	ery	gent	net	amik	vanc	mrsa	alive	dead	alive	dead	T-cell %
3082	0	0	45	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	0	1	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	25	63	24	56	29.17
3083	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	3	1	0	0	0	0	0	0	0	0	0	0	79	28	78	27	74.06
3085	0	0	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	57	25	59	30	67.84
3086	0	0	51	0	0	0	0	0	1	0	1	0	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	0	0	0	4	1	0	0	0	0	0	0	0	0	0	0	140	50	142	35	76.84
3086	0	0	51	0	0	0	0	0	1	0	1	0	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	0	0	0	4	1	0	0	0	0	0	0	0	0	0	0	63	63	73	59	52.71
3087	0	0	9	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	59	25	71	28	71.04
3089	0	0	16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	51	66	32	40	43.92
3090	0	0	45	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	31	13	33	15	69.57
3091	0	0	25	0	0	0	0	0	1	0	1	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	2	1	1	0	0	0	0	0	0	0	0	0	43	82	64	68	45.15
3092	0	0	15	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	2	1	0	0	0	0	0	0	0	0	0	0	42	14	47	19	72.95
3093	0	0	15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	56	25	69	27	70.62
3095	0	0	39	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	1	0	0	3	2	1	1	0	0	0	0	0	0	0	0	68	34	47	25	66.09
3096	0	0	5	0	0	1	0	0	1	0	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	25	71	26	88	24.29
3097	0	0	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	1	0	0	3	2	1	0	0	0	0	0	0	0	0	0	44	32	46	25	61.22
3098	0	0	30	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	1	0	0	3	1	0	0	0	0	0	0	0	0	0	0	38	23	52	13	71.43
3099	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	0	3	3	1	0	0	0	0	0	0	0	0	0	41	25	27	34	53.54
3100	0	0	30	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	3	3	1	0	0	0	0	0	0	0	0	0	27	40	39	44	44.00
3101	0	0	12	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	25	20	50	34	58.14
3102	0	0	30	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	35	39	34	13	57.02
3103	0	0	30	0	0	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	16	84	35	157	17.47
3104	0	0	45	0	1	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	37	133	28	122	20.31
3105	0	0	15	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	86	71	78	80	52.06
3106	0	0	25	0	1	0	0	0	1	0	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	8	160	5	158	3.93
3107	0	0	30	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	3	1	0	0	0	0	0	0	0	0	0	0	94	24	80	35	74.68
3185	0	0	30	0	0	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	0	1	1	1	3	1	0	0	0	0	0	0	0	0	0	0	35	16	38	20	66.97
3186	0	0	39	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	36	20	22	27	55.24

Figure A.5: Strain Data and T-cell Survival. **Origin:** 0=Carriage, 1=Community Acquired Disease, 3=Hospital Acquired Disease. **CC** = Clonal Cluster

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