Phenotypic and Genomic Comparisons of Community-associated *Staphylococcus aureus* Clone ST93

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Abstract

Staphylococcus aureus is an opportunistic bacterial pathogen that primarily colonises the anterior nares of 30-50% of individuals at any one time, without causing disease. *S. aureus* does however cause a wide range of diseases including skin and soft tissue infections, pneumonia, osteoarticular infections and bacteraemia or septicaemia, frequently resulting in hospitalisation and a high mortality rate for invasive disease. *S. aureus* strains have acquired resistance to many classes of antibiotics, most importantly resistance to beta-lactam antibiotics (methicillin-resistant *S. aureus*, MRSA). While traditionally a health care associated issue, community-acquired MRSA (CA-MRSA) clones are increasing worldwide resulting in severe, antibiotic resistant infections occurring in patients without healthcare contact. The ST93 clone of CA-MRSA is essentially unique to Australia, and has been associated with severe, invasive *S. aureus* infections in otherwise healthy individuals. ST93 CA-MRSA is also the most common CA-MRSA clone in Australia. The overall aims of this study were to determine the virulence characteristics of a collection of ST93 *S. aureus* isolates from around Australia, and to uncover the molecular determinants of virulence in ST93 *S. aureus*.

Fifty-eight ST93 isolates were assessed for virulence using the *Galleria mellonella* invertebrate virulence model and by measuring expression of key virulence factors. Whole genome sequencing and genomic analysis of all isolates was used to uncover genetic differences that might account for differences in virulence characteristics.

In this study ~50% of isolates (n=28) were avirulent when compared to a virulent reference ST93 isolate JKD6159 using the *G. mellonella* model. This study also revealed that the *G. mellonella* model does not respond to exotoxin expression of isolates with no significant differences in *G. mellonella* mortality between culture supernatant of virulent and avirulent isolates used in the *G. mellonella* model. The expression levels of PSMα3 and δ-toxin varied significantly amongst the isolate collection, and the concentration of δ-toxin was found not to correlate with that of PSMα3, indicating that systems other than the quorum-sensing *agr* system must be controlling the expression of PSMα3 in ST93 *S. aureus*.

Using whole genome sequencing and phylogenetic analysis, the progenitor of ST93 isolates was predicted to be a methicillin-susceptible isolate of ST93 *S. aureus* from North Western Australia. The level of secreted virulence factors was found to decrease as ST93 isolates evolved. Mutations in a total of 20 genes of avirulent isolates were hypothesised to account for the loss of virulence in these ST93 isolates. Eighteen of the genes contained at least one non-synonymous SNP, with the remaining two genes containing a frame-shift mutation, in *agrA*, and a 356bp deletion, in *hld*, supporting previous studies in other *S. aureus* clones that demonstrated the key role of the *agr* system in *S. aureus* virulence. Future work, including genetic manipulation experiments to investigate the role of specific mutations in virulence attenuation, will determine the factors behind the rise, dominance and evolution of this clone.

Declaration

This is to certify that:

i. *the thesis comprises only my original work towards the MPhil except where indicated in the Preface,*

ii. *due acknowledgment has been made in the text to all other material used,*

iii. *the thesis is less than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices*

Signed:

Justin Stepnell

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The following are a list of publications the author contributed to during his candidature

Gao W, Cameron DR, Davies JK, Kostoulias X, **Stepnell J**, Tuck KL, Yeaman MR, Peleg AY, Stinear TP and Howden BP (2013) The RpoB $H_{481}Y$ Rifampicin Resistance Mutation and an Active Stringent Response Reduce Virulence and Increase Resistance to Innate Immune Responses in *Staphylococcus aureus*. *The Journal of Infectious Diseases*. 207:929-39.

Stinear TP, Holt KE, Chua K, **Stepnell J**, Tuck KL, Coombs G, Harrison PF, Seemann T and Howden BP (2014) Adaptive Change Inferred from Genomic Population Analysis of the ST93 Epidemic Clone of Community-Associated Methicillin-Resistant *Staphylococcus aureus*. *Genome Biology and Evolution* 6(2):366-378

Chua KYL, Monk IR, Lin Y, Seemann T, Tuck KL, Porter JL, **Stepnell J**, Coombs GW, Davies JK, Stinear TP and Howden BP (2014) Hyperexpression of α-hemolysin explains enhanced virulence of sequence type 93 community-associated methicillin-resistant *Staphylococcus aureus*. *BMC Microbiology* 14:31

The author contributed to these publications by quantification of δ-hemolysin and PSMα3 secreted from *S. aureus* samples.

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List of Abbreviations

Agr	Accessory gene regulator
BHI	Brain Heart Infusion
bp	Base pairs
CA	catalytic ATP-binding
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CFU	Colony Forming Units
DHp	dimerization histidine phosphotransfer
DNA	Deoxyribonucleic Acid
gDNA	Genomic DNA
HA-MRSA	Hospital-acquired methicillin resistant *Staphylococcus aureus*
HPK	histidine protein kinase
HK	histidine kinase
hVISA	Heterogenous vancomycin *Staphylococcus aureus*
IS	Insertion sequence
MGE	Mobile genetic element
ML	Maximum likelihood
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSD	Membrane-spanning domain
NBD	Nucleotide-binding domain
NJ	Neighbour-joining
Nt	Nucleotide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PPR	Pattern recognition receptors
PSM	Phenol Soluble Modulin
PVL	Panton-Valentine leukocidin
RP-HPLC	Reversed-Phase High Pressure Liquid Chromatography
SNP	Single nucleotide polymorphism

SSTI	Skin and soft tissue infection
ST	Sequence type
TCRS	Two-component regulatory system
TFA	Trifluoroactic acid
TLR	Toll like receptor
TMD	Transmembrane domain
Tn	Transposon
TSB	Tryptic Soy Broth
TSS	Toxic shock syndrome
VISA	Vancomycin intermediate *Staphylococcus aureus*

Chapter One: Introduction

Chapter 1: Introduction

Staphylococcus aureus, a Gram positive coccus, is arguably one of the most important causes of bacterial diseases in humans. It is both an asymptomatic coloniser and opportunistic pathogen of humans [1]. It has the capacity to cause a wide spectrum of clinical diseases including; skin and soft tissue infections (SSTIs), bone and joint infections, toxic shock syndrome, bacteraemia, septicaemia and necrotizing pneumonia [2-4].

1.1 Taxonomy

S. aureus is a member of the genus *Staphylococcus*, family Staphylococcaceae [1]. Forty-seven species have been assigned to the genus *Staphylococcus* established by 16S rRNA gene sequence comparisons, with further classification into subspecies. Whilst *S. aureus* is primarily an asymptomatic coloniser, it can be an opportunistic pathogen in humans. Other species associated with human infection include *S. epidermidis*, *S. lugdunensis*, *S. saprophyticus* and *S. schleiferi* [5-8].

1.2 Epidemiology and Clinical Manifestations

S. aureus is frequently isolated from healthy individuals as part of the normal human microflora. Common asymptomatic colonisation sites include the skin and the back of the throat, with the primary site being the anterior nares. It is estimated that 20% of individuals permanently carry *S. aureus* in their anterior nares, 30% carry it sporadically and 50% never carry *S. aureus* [9]. Although carriage rates in normal healthy individuals are common, *S. aureus* is a potentially significant pathogen causing a range of acute, chronic, or recurrent infections that often require hospitalisation, and are in some cases life threatening. Being the most common bacterium isolated from hospitalised patients, *S. aureus* poses a substantial global health burden [10].

Amongst the diverse range of diseases caused by *S. aureus* skin and soft tissue infections (SSTIs) are most common, while more invasive diseases such as bacteraemia, endocarditis, necrotizing pneumonia, meningitis, bone and joint infections, sepsis, toxic shock syndrome (TSS) and

metastatic infections are less common but more severe. Different clones of *S. aureus* can demonstrate different disease manifestations or severity. The clone ST93, the subject of this project, has been associated with severe SSTIs, fatal necrotising pneumonia, bacteraemia and bone and joint infections [11-15].

In Australia *S. aureus* bacteraemia rates are estimated at 35/100,000 population [16], while *S. aureus* bacteraemia mortality rates are estimated to be between 15%-60% [17]. Diseases caused by *S. aureus* occur not only in hospitalised patients, but also in individuals in the community [18]. Treatment of diseases caused by *S. aureus* requires the administration of antibiotics and in some cases surgery [19].

1.2.1 Antibiotic Resistance

Antibiotics are frequently administered to treat diseases caused by *S. aureus*. Resistance to one of the first antibiotics penicillin, introduced in the early 1940s, was reported in 1942 [20]. In 1959 methicillin was the second antibiotic to be used to treat diseases primarily caused by *S. aureus* and in 1960 the first isolates of methicillin-resistant *S. aureus* (MRSA) were identified [21]. Isolates resistant to methicillin and β-lactams have acquired the SCC*mec* mobile genetic element in the chromosome. SCC*mec* contains a gene, *mecA*, which encodes an altered penicillin binding protein, known as either PBP2a or PBP2' [22]. This altered penicillin binding protein has a low affinity for most penicillins and other β-lactams. Isolates that contain the SCC*mec* element are referred to as MRSA. A range of alternative antibiotics are currently available for treatment of infections caused by MRSA, however this is not sufficient as *S. aureus* is continually evolving, developing mechanisms of resistance to classes of antibiotics via specific mutations or acquisition of resistance genes, often carried on plasmids or transposons (Table1.1) [22-25].

The most recent antibiotic to be found ineffective against a small number of clinical *S. aureus* isolates is the glycopeptide vancomycin. Vancomycin, first released in 1958, is an antibiotic administered intravenously and has been regarded as the last line antibiotic treatment against severe *S. aureus* infection when first line antibiotic treatments fail [26-27]. Until 1997 there were no reported cases of *S. aureus* resistance to vancomycin. In 1997 two *S. aureus* isolates with

reduced susceptibility to vancomycin were reported from Japan and since resistance has been reported globally [28-29]. Isolates that have a reduced susceptibility to vancomycin are termed vancomycin intermediate *S. aureus* (VISA) or heterogenous-VISA (hVISA). Reduction of vancomycin susceptibility is usually caused by acquired mutations within the genome [27]. To date over 30 *S. aureus* isolates have been reported as fully vancomycin resistant (VRSA) from the USA, Europe and Asia [30-33]. High level resistance to vancomycin arises from the acquisition of a genetic locus that includes the *vanA* gene which is found in vancomycin resistant enterococci [25].

While resistance to β-lactams and vancomycin are the most important resistance issues in *S. aureus*, the organism has developed resistance to multiple other antibiotics, including macrolides, quinolones, tetracyclines, aminoglycosides, trimethoprim-sulphamethoxazole, rifampicin and linezolid [34].

Table 1.1. Changes in the genetic make-up of *S. aureus* isolates that result in resistance to the beta-lactam and glycopeptide antibiotic classes.

Class	Antimicrobial	Mode of Action	Molecular Changes Resulting in Resistance	Mechanism
β-lactams	Penicillin	Binding to PBPs, inhibiting cell wall assembly	*blaZ*	Hydrolysis of beta-lactam ring
	Methicillin		*mecA* found on SCC*mec*	Encoding PBP2a, transpeptidase subtstitute and low affinity for beta-lactams
Glyco-peptide	Vancomycin	Inhibition of cell wall synthesis	*walKR*, point mutations. Several other mutations in chromosome	VISA: Thickened cell wall
			vanA	VRSA: Change to the peptidoglycan terminal binding site

1.2.2 *S. aureus* Population Structure

Population genetics is a powerful tool used in the surveillance and detection of outbreaks of *S. aureus* isolates in hospitals and communities on both local and international scales. Molecular typing of staphylococci can allow investigators to trace back to the source and map the spread of isolates over a period of time. Techniques used to study population genetics may also be used to determine the location and time at which large changes in DNA structure have occurred; for example the acquisition of large foreign sequences of DNA such as the SCC*mec* element or the smallest of DNA changes such as a single nucleotide polymorphism (SNP) [35-36]. A SNP is a DNA sequence variation where a single nucleotide differs between biological species.

A number of typing systems and molecular techniques and methods have been developed and used over the past 60 years to characterise *S. aureus* isolates (Table 1.2). All typing methods have advantages and limitations. The first of the classification techniques developed was phage typing [37]. An international basic set of phages are individually used to infect *S. aureus* isolates. A number or 'phage type' is assigned to the isolate based on the susceptibility to that phage, if any, or combination of phages that infected the isolate. This technique however is not ideal as many isolates are nontypeable, with no phage infecting the isolate [38].

A more modern, DNA sequence-based technique that is commonly used is multilocus sequence typing (MLST) [18]. This technique involves the PCR amplification and sequencing of a segment of seven housekeeping genes. Each sequence is compared to a database of sequences and is assigned an allele number. A new sequence type (ST) is assigned based on a unique combination of allele numbers. The web-based MLST database is frequently updated as new sequences are entered. Closely related isolates that share at least five of seven identical alleles are grouped together as a clonal complex (CC) [39]. The CC is labelled by what is predicted to be the progenitor ST. Isolates that differ from every other ST in the database by three or more of the MLST alleles do not belong to a CC and are referred to as singletons [39]. Common *S. aureus* STs include; 1, 5, 8, 22, 30, 36, 45, 59, 93, 228, 239, 241, 247 and 250 [40-42].

Table 1.2. Common typing methods that have been used to classify *S. aureus* isolates.

Typing Method	Basis	Reference
Phage typing	Infection with phage	[37]
Capsular typing	Reaction to monoclonal antibody	[43]
Pulsed field gel electrophoresis	Digestion of DNA, visualize on gel	[44]
Multi-locus sequence typing	DNA sequence analysis of seven housekeeping loci	[45]
spa typing	DNA sequence analysis of the Protein A gene variable-number tandem repeat region	[46]
coa typing	DNA sequence analysis of the coagulase gene variable-number tandem repeat region	[47]

As previously stated the problem with all typing methods, such as the DNA sequencing typing methods, or a combination of MLST and *spa* typing is that closely related isolates cannot be distinguished from each other. Isolates with little time to diverge from a localized outbreak will likely present the same ST and/or *spa* type. To overcome this, investigators have analysed more fragments throughout the *S. aureus* chromosome, providing higher typing resolution. For example, one of the earliest studies that extensively studied the relatedness of *S. aureus* isolates beyond the depth of MLST and *spa* typing methods investigated the mutations in 108 loci (46 kb) of 135 ST5 isolates [48]. Whole genome sequencing is a method that provides the investigator with the ability to analyse genetic differences between isolates across the entire genome, providing unparalleled resolution. Next generation sequencing technologies produce reads with read lengths varying between 150 and 700 bp depending on the method used. The reads are assembled to create contigs or a complete genome, and a consensus sequence generated. The read mapping approach uses a reference sequence to align against for assembly and de-novo assembly is the assembly of all the reads with no reference. By using whole genome sequencing even the most closely related isolates that may only differ by a small number of SNPs can be distinguished [49-51].

A recent study reported the whole genome sequencing and analysis of 63 *S. aureus* isolates from one of the most important global MRSA clones, called ST239, using next-generation DNA sequencing [52]. Forty-three isolates were from a global collection spanning from 1982 – 2003 and the remaining 20 were from a single hospital in northeast Thailand collected within a 7-

month period. Read mapping against the reference isolate TW20 identified 6,714 SNPs and SNP based phylogeny clearly demonstrated geographic and temporal grouping of isolates. This was the first study in which such a large cohort of *S. aureus* isolates belonging to the same ST have been compared using whole genome sequencing technology.

Whole genome sequencing has also been employed to study genetic transfer, the evolution of virulence and antibiotic resistance of *S. aureus* [53-54]. Studies have used whole genome sequencing to identify and correlate SNPs to pathogenesis with unrelated isolates from the same ST to a paired set of asymptomatically carried to fatal isolates [55-56]. A study revealed SNPs involved in daptomycin resistance with an isogenic pair of USA800 isolates obtained before and after daptomycin treatment failure [57]. These studies show how powerful whole genome sequencing is as an investigative tool.

1.2.3 Global Distribution of Community-Associated MRSA

Cases of MRSA infections were once restricted to the hospital setting but are now frequently observed amongst individuals in the community. Therefore MRSA isolates have been classified into two categories, MRSA infections acquired from the hospital setting are referred to as healthcare-associated MRSA (HA-MRSA) and cases of MRSA infection that arise in individuals without health care contact are classified as community-associated MRSA (CA-MRSA). The first reported case of CA-MRSA was in the United States in 1980 [58]. Since that report CA-MRSA outbreaks have been observed around the world with Australia recording its first cases in the late 1980s amongst a population in the Kimberly region of Western Australia [59-60]. CA-MRSA strains are genetically distinct from HA-MRSA strains and as such are hypothesised to have arisen independently and not transmitted from a HA-MRSA infected person to individuals within the community [61].

In early studies CA-MRSA clones showed a limited geographic distribution but in a recent study with the USA300 clone, it is clear that intercontinental exchange of clones can occur [36, 62]. Global travel is thought to be the reason for this with individuals unknowingly carrying a CA-MRSA isolate and transmitting it to a previously uninfected population. Globally, within geographic regions, the emergence, distribution and dominance of CA-MRSA clones is

constantly changing over time [63-65]. The most dominant global clones are presented in Figure 1.1. The change in dominance and emergence of clones has been observed in Australia since the mid-1990s. Soon after the rise of the first CA-MRSA clone, WA-MRSA-1, a second, unrelated, CA-MRSA, ST30, emerged and spread throughout Eastern Australia [66]. ST30 is also commonly referred to as the South West Pacific (SWP) clone.

In 2000, a new clone of CA-MRSA, ST93 Queensland clone, was first observed in Ipswich, Queensland [67]. Initially spreading rapidly down the East coast of Australia, ST93 is now the predominant CA-MRSA in Australia accounting for 41.4% of all CA-MRSA isolates based on national surveillance data [65]. ST93 is not only the most dominant CA-MRSA clone in Australia but the increase of CA-MRSA infections in the country is due to this clone [40]. The other dominant CA-MRSA clones are ST1 (WA-MRSA-1) and ST30 SWP representing 15.5% and 13.0% of the CA-MRSA isolates in Australia, respectively [65]. ST93 has recently spread internationally with cases reported in New Zealand, Samoa, Italy and the United Kingdom [68-71].

This situation is not isolated to Australia, with an epidemiological rise and evolution of CA-MRSA clones occurring globally at a comparable rate. In the United States the most commonly isolated CA-MRSA clones are ST1, ST5 and ST8. USA400 (ST1) was previously the dominant clone but recently USA300 (ST8) emerged in 2000 and is now the most frequently reported clone of CA-MRSA in the United States [63]. The international spread of USA300 is even more widespread than ST93 with a greater number of reports of USA300 *S. aureus* infection in a number of countries across Europe, the Middle East and Western Pacific [72]. It has been suggested that the spread of USA300 will continue [62].
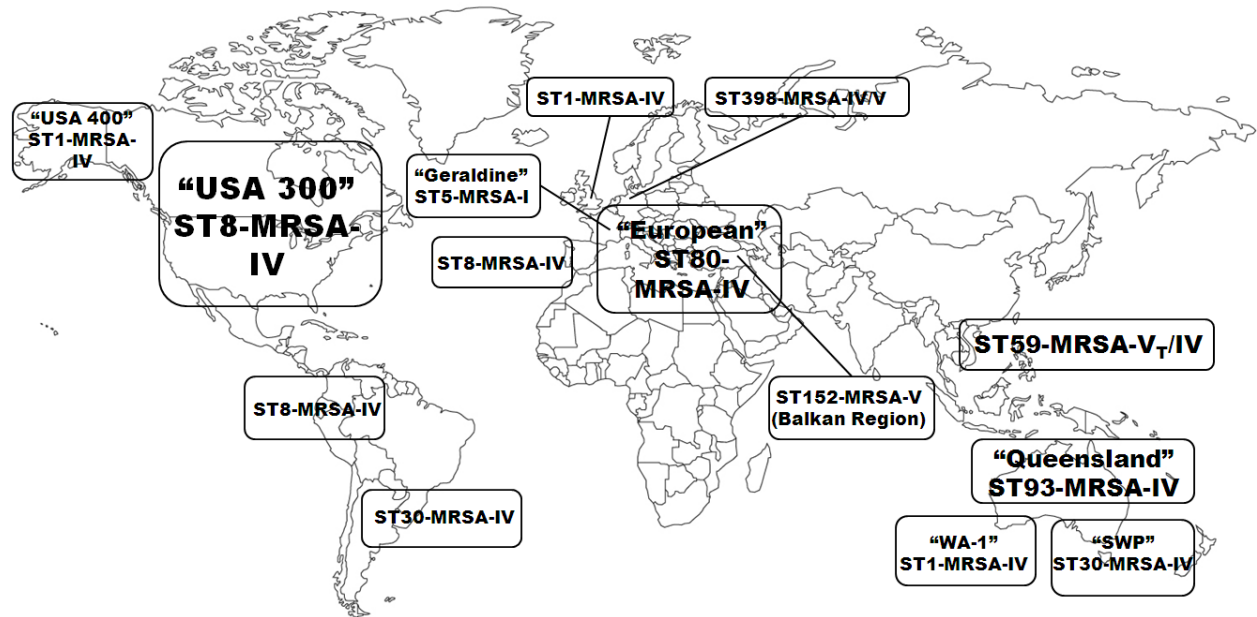
Figure 1.1. Global distribution of major MRSA clones. The major CA-MRSA clones are highlighted and their major geographic distribution shown. Nomenclature includes common names and formal typing results based on MLST, methicillin susceptibility and SCC*mec* typing. (Reproduced from [73]).

1.3 Genomic Organisation of *S. aureus*

To date there are currently over 50 publically available draft and completed genomes of *S. aureus*. Based on these genomes the *S. aureus* genome varies in size from 2.67 Mbp to 3.04 Mbp (average ~2.8Mbp) with a G+C content of ~33%. Parts of the genome that are present in all isolates are termed the 'core genome' [74]. The *S. aureus* genome is highly conserved amongst isolates with the core genome making up ~75% (2.3 Mbp) of the total genome [74]. The core genome contains genes essential for growth and survival, virulence and attachment to host cells.

The rest of the genome, termed the accessory genome, is comprised of an array of mobile genetic elements (MGEs) that have been acquired via horizontal gene transfer [74]. MGEs are also lost by isolates via gene deletion [75-76]. Mobile elements include; chromosomal cassettes, bacteriophages, insertion sequence (IS) elements, pathogenicity islands and transposons (Tn) [49, 77-78]. Plasmids also contribute to the genomic content of *S. aureus,* with isolates containing between one and three plasmids. These plasmids are between 1.4kb and 37.1kb in

size. A range of genes are encoded on MGEs and plasmids including antibiotic resistance genes and genes involved in virulence mechanisms [79-81].

1.4 Pathogenesis

1.4.1 Virulence Factors

The interaction between *S. aureus* and the host is complex and not very well understood. *S. aureus* infection involves the initial attachment to the host, avoidance of the host immune system (innate and adaptive), growth and bacterial spread within the host, and the expression of toxins that cause specific tissue damage to the host [82-85]. Proteins required for initial attachment to host cells are collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and include clumping factor A and fibronectin-binding proteins [86-88]. Exotoxins expressed by *S. aureus* contribute to virulence by the formation of pores in a range of host cells. Known exotoxins include α-hemolysin, Panton-Valentin Leukocidin (PVL) and phenol-soluble modulins (PSMs) [84, 89-94].

The most extensively studied *S. aureus* clone in terms of virulence determinants is USA300 [95-96]. CA-MRSA clones however are diverse and therefore not all virulence determinants of USA300 are relevant to other CA-MRSA clones. A group of peptides called α-type phenol-soluble modulins (PSMs) are an example of this. α-type PSMs were previously shown to be a major virulence factor of a USA300 isolate, yet recently these peptides were proven to have no significant effect on the virulence of an ST93 isolate [84, 97]. Importantly ST93 has been shown to be the most virulent global clone of *S. aureus* using murine sepsis and skin infection models [12, 98].

Other virulence factors include the *S. aureus* Ess (ESAT-6 secretion system) pathway, biofilm formation and capsule expression. The Ess pathway has a role in chronic persistent infections [99]. Without the production of EsxA and EsxB virulence of murine abscess model of infection [100]. EssB is one of the proteins required for secretion of EsxA and EsxB across the staphylococcal envelope. As well as secreted proteins biofilm formation has an important role in virulence by protecting the bacteria from the host immune system [101]. The intercellular

adhesion locus (*ica*) is required for biofilm formation in *S. aureus* [102]. The *ica* locus is made up of five genes: *icaR*, *icaA*, *icaD*, *icaB* and *icaC* [102].

Capsule expression has a role in virulence by making *S. aureus* resistant to phagocytosis [103]. The number of putative capsular serotypes of *S. aureus* is 11 [104]. The most extensively studied capsular serotypes are 1, 2 and 5 and 8 [103]. Several studies using mice injected intraperitoneally have shown the importance of the type 1 capsule in staphylococcal virulence [103, 105-106].

1.4.2 Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

Attachment to host cells occurs due to the interaction of MSCRAMMs, in most cases anchored to the cell wall peptidoglycan of the *S. aureus* membrane, and the host's extracellular matrix including fibronectin and fibrinogen [107-109]. A total of 21 MSCRAMMs have been identified *in silico* by analysis of six finished and unfinished *S. aureus* genomes [110]. Characterized MSCRAMMs include fibronectin-binding proteins A and B (FnBPA and FnBPB), clumping factors A and B (ClfA and ClfB) and a collagen-binding protein, Cna [111].

FnBPA and FnBPB are key factors in the *in vitro* attachment and adherence of *S. aureus* cells to fibronectin [88]. Results from a study on FnBPA suggest that it is involved in the early stage of infection and required for pathogenesis [112].

ClfA promotes adherence of *S. aureus* to fibrinogen and fibrin and clumping of *S. aureus* after attachment [87, 113]. ClfB is not encoded on the same locus but named in succession to ClfA because it is a fibrinogen-binding protein and has similar structural properties to ClfA. ClfB however is only detectable in cells grown in early exponential phase unlike ClfA which is expressed at all stages of growth [114]. ClfB therefore is hypothesised to be involved in the initial attachment to host cells and does not have a role in the later stages of pathogenesis. Once the organism has successfully attached to and colonized host cells other virulence factors that are associated with pathogenesis (e.g. exotoxins) are expressed [112].

Cna is a collagen adhesion molecule, binding to collagen substrates and collagenous tissues [115]. Cna has been shown to be necessary in attachment of cartilage *in vitro* and furthermore has been shown to be a virulence factor in a mouse model of staphylococcus-induced septic arthritis [116-117].

With timely expression of MSCRAMMs during the course of infection crucial to the cell survival and spread within the host, the expression of these proteins must be tightly controlled. In *S. aureus* cells a number of two-component regulatory systems (TCRSs) and SarA homologues have been characterised that have a role in controlling the expression of such proteins [118-120].

1.4.3 Toxins

1.4.3.1 Alpha Hemolysin

The α-hemolysin (Hla) protein, first discovered in *S. aureus* in 1929, belongs to the β-channel pore-forming toxin (β-CFT) family [121]. Hla is a 33.25kDa protein encoded on all sequenced *S. aureus* genomes and is expressed by almost all isolates. Hla is an active pore forming toxin that targets a range of host cell types including endothelial cells, keratinocytes, epithelial cells, erythrocytes and lymphocytes [89, 122-125].

Hla was first shown to be an important virulence factor in a staphylococcal murine pneumonia model with comparisons between Newman wild type (WT) and Newman *hla* mutant strains demonstrating significant differences in post-infection time to death and total mortality [126]. Death occurred at 24-hours post-infection in mice inoculated with the Newman WT strain as opposed to death occurring 48 hours post-infection for only a small percentage of mice with the Newman *hla* strain. These experiments provided clear evidence of the role of Hla as a major virulence factor in the murine pneumonia model.

The role of Hla as a virulence factor in skin and soft tissue infections (SSTI) has been investigated using *S. aureus* strains LAC (USA300) and Newman (non-USA300 ST8) in a murine SSTI model [127]. Results indicated that the role of Hla as a virulence factor in SSTIs may be strain specific, with Hla in the LAC strain proving to be a major virulence factor as opposed to the Newman strain where there was no significant difference in lesion size between

wild-type and Hla deletion strains. It is clear from this study that other virulence factors contribute to the pathogenicity exhibited by SSTIs. Further investigations of Hla in SSTI murine models need to be conducted with other strains of CA-MRSA, including ST93, to determine the extent to which Hla contributes to virulence in other clones.

1.4.3.2 Panton-Valentine Leukocidin

Panton-Valentine Leukocidin (PVL) is a bi-component pore-forming toxin belonging to the same toxin family as Hla [128]. The two components of PVL, lukF-PV (38-kDa) and lukS-PV (32-kDa) are encoded by co-transcribed genes, *lukF-PV* and *lukS-PV* [129-130].

The PVL locus is acquired by *S. aureus* through phage transfection and is inserted into the host genome. Isolates carrying PVL are overrepresented in clinical isolates, with 20-80% carrying PVL compared to an estimated 0.6-2% in the whole *S. aureus* population [131-132]. The presence of PVL in CA-MRSA isolates is so high that it has been suggested that PVL be a genetic marker for CA-MRSA isolates [133-134]. With a significantly higher proportion of CA-MRSA isolates carrying PVL, the role of this toxin in the virulence and pathogenesis of CA-MRSA has been investigated in various animal models with conflicting results [135-140].

In a murine model of acute primary pneumonia PVL was identified to be a key virulence factor in pulmonary infections [135]. A later study using isolate LAC, a USA300-representative strain, in staphylococcal skin infection and pneumonia murine models determined that PVL does not contribute to *S. aureus* pathogenesis [138]. The authors concluded that different strains may be accountable for differences in results and PVL may even enhance the ability of the host immune system to recognise and clear the pathogen.

JKD6159 is a PVL positive ST93 isolate from Australia, and has demonstrated the highest virulence in both animal models (sepsis and SSTI) as previously stated. However, a PVL negative ST1 clone, JKD6272, was as virulent as PVL positive isolates in the same study [12]. The *in vitro* level of PVL expression of these isolates was not measured, but the results indicate that virulence may be PVL independent. However, murine models of staphylococcal infections

may not be a true representative of infection in humans as mouse neutrophils are less susceptible to PVL lysis than human and rabbit neutrophils [141].

1.4.3.3 Phenol Soluble Modulins

Phenol soluble modulins (PSMs) have been described in *S. epidermidis* as a major virulence factor [142]. Genes encoding PSMs have recently been identified in the *S. aureus* genome raising interest that PSM expression may also be a virulence factor in *S. aureus* [84]. PSMs are small secreted peptides and are thought to contribute to virulence by the lysis of human neutrophils. The *S. aureus* genome contains two types of PSMs, α- and β-type, and also a δ-toxin, similar to the α-type PSMs [84]. A study by Wang *et al.* [84] found that all MRSA genomes contain four α-type PSMs, each ~20 amino acids in length, and two β-type PSMs each ~40 amino acids in length and are found on pathogenicity islands υSaα and υSaβ respectively.

It was hypothesised that production of PSMs was a major virulence factor for *S. aureus* given that *in vitro* PSM production in CA-MRSA strains was found to be significantly higher than that of HA-MRSA strains [84]. Bacteraemia and SSTI murine models conducted with wild type and isogenic gene deletion strains and *in vitro* lysis of human neutrophils showed that α-type PSMs, in particular PSMα3, were essential in CA-MRSA-induced disease [84]. A later study also supported this hypothesis with PSM production in major CA-MRSA clinical isolates, USA300 (ST8) and USA500 (ST8), significantly higher than major HA-MRSA lineages, USA100 (ST59) and USA200 (ST36) [143].

Further investigation is needed to support the correlation of PSM expression of CA-MRSA strains and virulence as only one CA-MRSA strain was used for each murine model (MW2 [USA400] for bacteraemia and LAC [USA300] for SSTI).

1.4.4 Virulence Regulation by Two-Component Regulatory Systems

Gene expression is a complex process often involving a combination of transcriptional, translational and post-translational regulators. To date the most comprehensive core *S. aureus* virulence-regulatory network constructed consists of 20 regulators [144]. This is based on an extensive literature search and as such direct interactions between proteins within the system are

poorly understood or unknown [144]. This highlights how little is known about virulence regulation of *S. aureus*.

The level to which virulence genes are expressed in the cells is dependent on the surrounding environment, and bacterial density. During exponential growth phase *S. aureus* genes encoding MSCRAMMs are up-regulated [145]. Up-regulation of cell surface proteins enables *S. aureus* to attach and colonise host cells [145]. During the post-exponential growth phase, these genes are then down-regulated and expression of exotoxins up-regulated by a quorum sensing pathway [145].

Upon changes in environmental conditions global regulators within *S. aureus* control the expression of genes either directly or through a cascade of events. Global virulence regulators recognised in *S. aureus* have broadly been categorised into two major families, two-component regulatory systems (TCRSs) and SarA homologues. Sequence analysis of *S. aureus* genomes has revealed that there are an estimated 16 TCRSs present including the accessory gene regulatory system (*agr*), *Sae*, *Arl* and *Srr* [145-146]. The *agr* system is the best characterised of the TCRSs with relatively little known about the other TCRSs. The SarA homologues include *SarS*, *SarT* and *SarR* [146]. Based on genomic and structural data the SarA homologues can be subdivided into three subfamilies; (1) single domain proteins; (2) double-domain proteins; and (3) proteins homologous to the MarA protein family [145]. Other proteins are also involved in the response to environmental changes including σ^B factors. The role of TCRSs, SarA homologues and other proteins in the virulence of *S. aureus* remains relatively unknown and further investigation is needed.

1.4.4.1 Agr Quorum Sensing System

The *agr* system is a TCR quorum sensing system found in all *S. aureus* genomes [146]. This TCRS controls the expression of virulence genes and other genes within the core genome that contributes to virulence. *In vitro agr* activity is very low if active at all during lag phase and is highly active during exponential growth phase. Activity of the *agr* system correlates with the expression of virulence genes. This is also thought to be the same *in vivo* with low *agr* activity during early growth phase correlating with expression of surface proteins for attachment to host

cells and high *agr* activity during the exponential growth of cells expressing virulence factors during. Regulation of the *agr* system is complex, with other regulatory genes and systems including *SarA* and σ^B and environmental factors such as cell density directly up- and down-regulating this system [147-148].

The *agr* locus is comprised of five genes, *agrB*, *D*, *C* and *A* and *hld* (RNAIII) with two divergent promoters, P2 and P3 (Figure 1.2). The *agr* sequence is highly conserved between *S. aureus* isolates with allelic variation only documented in *agr B-C-D* from amino acids 34 of AgrB to 205 of AgrC [149]. Amino acid sequence variations of these regions are not random, with four characterised functional variations [149-150]. This highlights the importance the *agr* system has in the survival of this organism in the host in relation to the timely expression of adhesion molecules and exotoxins. The *agr* locus however is not necessary for cell survival with *agr* knock-out mutants successfully created [151-153]. Mutations within regions of the *agr* system may have dramatic impacts on the activity of this system and therefore expression of virulence factors.

AgrD encodes a peptide that is modified into a thiolactone (or, in one case, a lactone) ring and secreted into the extracellular environment via a transmembrane endopeptidase, AgrB [154]. This thiolactone ringed peptide is an autoinducing peptide (AIP) and binds to and activates AgrC. Activation of AgrC induces phosphorylation of the cytoplasmic domain and the phosphate is transferred to AgrA, activating AgrA [155]. The next step in the cascade of events is the activated AgrA activating its own promoter, P2, and promoter P3. P3 activation results in expression of an RNA molecule, RNAIII. This molecule is both the intracellular regulatory effecter of the *agr* system and also encodes δ-toxin, an extracellular toxin [156]. RNAIII has been shown to directly upregulate virulence factors, α-hemolysin, and downregulate surface proteins, protein A and fibronectin-binding proteins [119, 157].

Four different *agr* types have been identified in *S. aureus*, types I-IV with ST93 isolates belonging to *agr* group III [158-160]. The *agr* types are assigned based on the amino acid sequence of the AIP (Figure 1.3). The different groups have arisen due to changes in the Agr B-C-D amino acid sequence. The AIP binding to AgrC is specific therefore the sequence of AgrC

is also group dependent. The effect of specific AIPs acting on *agr* systems of different groups has been tested and generally inhibits the activation of the *agr* systems [158, 160]. The four *agr* types are thought to have evolved from one ancestor and with a gradual shift of amino acid sequence of all genes the four groups have evolved [161].
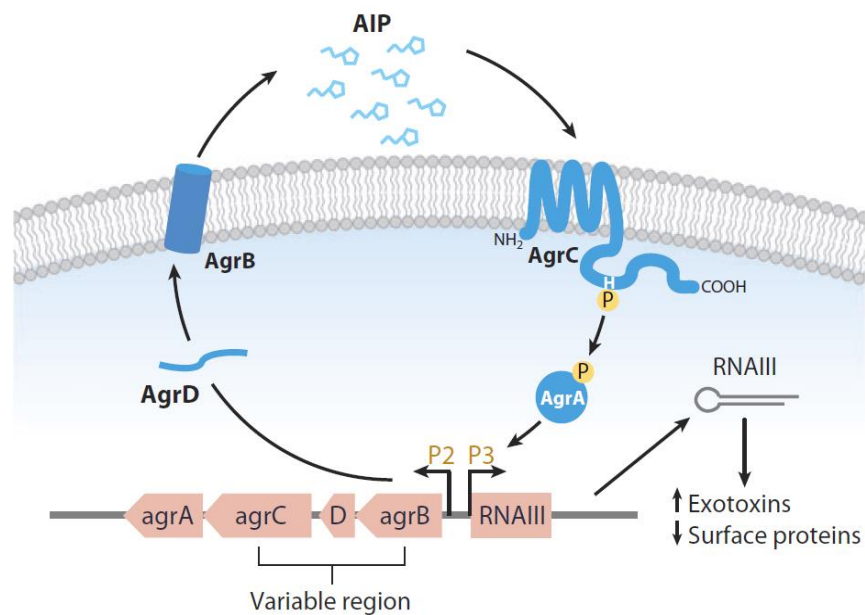
Figure 1.2. *agr* autoactivation (Reproduced from [162]). The AgrD peptide is processed and secreted by the transmembrane AgrB as a thiolactone (or, in one case, a lactone) ring (AIP). The AIP molecule binds to and activates AgrC inducing phosphorylation transferring the phosphate to AgrA. AgrA then activates promoters P2 and P3. The cycle repeats upon activation of P2. Activation of P3 transcribes RNAIII, a regulatory effecter molecule.
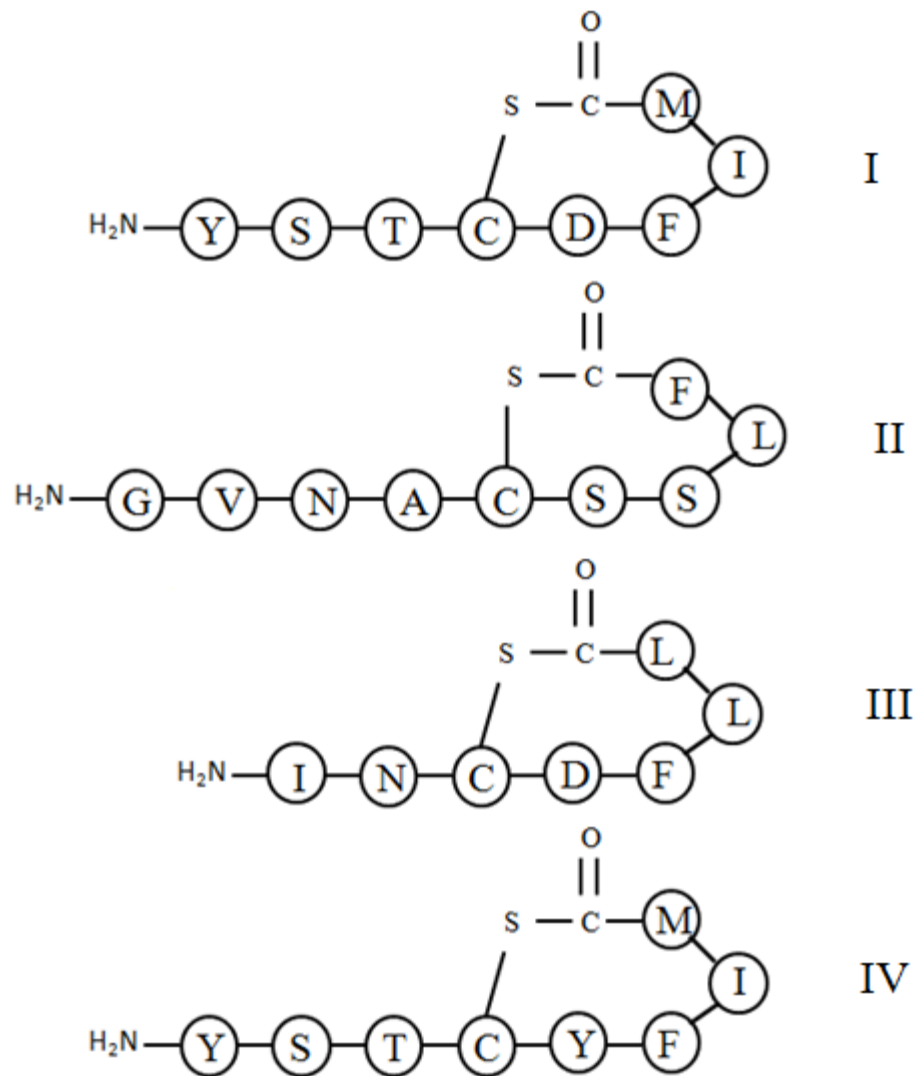
Figure 1.3. Structure of AIP for the four different *agr* groups, I to IV (Reproduced from [163]). Letters inside the circles represent amino acid residues.

1.5 The ATP Binding Cassette (ABC) Transporter Family

The ATP binding cassette (ABC) transporter family are one of the largest classes of transporters with almost 400 families and are found across all three domains of life, prokarya, eukorya and archea [164-166]. The ABC transporters are ATP-dependent membrane spanning proteins that import and export molecules ranging from ions such as Fe^{2+} and K^+ to macromolecules including B12 and haemin. Individual ABC transporters either transport a specific single molecule or a group of chemically related molecules across the cell membrane [167]. In bacteria the numbers of predicted ABC transport systems ranges from 4 to 189 with completely sequenced *S. aureus* isolate COL predicted to contain 71 ABC transporters based on whole genome sequence analysis [165].

The ABC transport systems are comprised of four protein domains, two hydrophobic membrane-spanning domains (MSDs) (or transmembrane domains, TMDs) and two hydrophilic nucleotide-binding domains (NBDs) [168]. The MSDs span the membrane multiple times in an α-helical confirmation and the NBDs are at the cytoplasmic surface interacting with ATP to supply the energy needed for the active transport [168]. The MSDs have specific substrate-binding sites therefore they determine the molecule that can attach and be actively transported. The number of ATP molecules hydrolysed per substrate transported is estimated to be two but has only been observed in one *in vitro* experiment in the osmoregulated ABC transporter (OpuA) from *Lactococcus lactis* [169].

The ABC transporters are involved directly with many cellular processes both essential for prokaryotic cell survival and other processes that are non-essential to *in vitro* growth but are important in survival in specific environments [164]. Essential cellular processes include those that are for cell metabolism and, in the case of exposure to antimicrobial agents, the efflux of these compounds [170]. Non-essential functions of the cell that involve ABC transporters include the excretion of toxins produced by the cell [171]. The ABC systems are also involved in DNA repair [172].

Inactivation of ABC transport systems of *S. aureus* has been experimentally shown to reduce the virulence of the isolate. An ABC transporter required for the transport of iron (III)-hydroxymates

in *S. aures* encoded by genes *fhuC*, *fhuB* and *fhuG* that are present in all sequenced *S. aureus* has been demonstrated to have a significant role in the increased morbidity of mice when compared to the Δ*fhuCBG* isolate [173]. Reduced virulence has also been demonstrated in other bacteria with a *sfbA* gene knock-out constructed in *Salmonella enteritidis* avirulent in BALB/c mice with oral administration when compared to the wild-type isolate [174].

1.6 Host Factors

The immune response to microbial pathogens is made up of a combination of innate and adaptive immune responses. The innate immune system is the first line of defence in the host's response to the invasion of foreign materials including microbial pathogens such as *S. aureus*. White blood cells including neutrophils and macrophages form much of the innate immune system response to the earliest stages of infection.

The innate immune system differentiates self from non-self and can recognise pathogens as an infectious agent that it has never had previous exposure to. Immune cells can do this as they have evolved to recognise highly conserved motifs that are common to, and have essential functions, in a diverse range of pathogens. Therefore, the motifs recognized are both expressed by a range of pathogens and have low mutation rates. The innate immune system differentiates between self- and non-self cells as the motifs recognised are not present in higher eukoryotic (self) cells. These motifs have been collectively termed pathogen-associated molecular patterns (PAMPs) and include the bacterial cell wall components, lipopolysaccharide, peptidoglycan, lipoteichoic acid and bacterial DNA [175]. Receptors that recognise PAMPs are called pattern recognition receptors (PRRs) [176].

Toll like receptors (TLRs) are a class of PRRs that recognise distinct PAMPs [177]. To date the number of members in the mammalian TLR family is ten (TLR1-10) [178]. Each TLR specifically detects a PAMP. For example, TLR4 detects lipopolysaccharide as shown in TLR4 knockout mice (C3H/HeJ) where lipopolysaccharide was not detected [179]. *Escherichia coli* lipopolysaccharide is detected by TLR4 however the severity of disease caused by this bacteria is not increased in TLR4 knockout mice compared to wild-type mice. Observations such as this one provide evidence that microbes are detected by a number of TLRs and other innate recognition

systems. This redundancy in the immune system greatly increases the chance of a pathogen being detected early by a number of systems, activating further pathways to eliminate the pathogen before any onset of infection to the host.

1.7 *In vivo* Animal Models

Animal models have been used to investigate a range of diseases associated with *S. aureus* including; SSTIs, septic arthritis, endocarditis, bacteraemia and pneumonia [12, 135, 180-183]. Ideally animal models selected for various diseases should be comparable to disease presentation in humans. Mammalian models have traditionally been used in the study of specific *S. aureus* diseases but recently non-mammalian models including the fruit fly (*Drosophila melanogaster*), silkworm larvae (*Bombyx mori*), greater wax moth larvae (*Galleria mellonella*) and the nematode (*Caenorhabditis elegans*) have been used for virulence analysis *in vivo* [184-187]. The advantages of non-mammalian models over mammalian models are substantial, including low cost, ease of infection, ability to study a large number simultaneously and the fact that ethical approval is not required.

1.7.1 Greater Wax Moth (*G. mellonella*) Model

The Greater Wax moth (*G. mellonella*) model is an invertebrate animal model that has been used for host-pathogen interactions of a range of bacteria including, *S. aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and pathogenic fungi [12, 186, 188-195].

The utility of *G. mellonella* as a model to assess the virulence of *S. aureus* was first demonstrated by comparing infection of an *agr* deletion mutant with its *agr* positive wild type, with the *agr* locus known to be important in virulence of mammalian models [186]. Results correlated with that of mammalian models with the *agr* mutant killing significantly less *G. mellonella* than wild type. This study suggested doses of $1 \times 10^5 - 1 \times 10^6$ cfu/larva and incubation temperature of 37°C were the most suitable parameters for future research using this model. This was because the killing of *G. mellonella* was dependent on the number of *S. aureus* cells injected and the temperature at which the *G. mellonella* were maintained [186]. More recently, another study presented results showing that the *G. mellonella* killing assays were comparable to BALB/c mouse skin and soft tissue assays [12].

Although the exact mechanism of *G. mellonella* killing is unknown the insect immune system is similar to that of the mammalian innate immune response, with pathogen killing occurring by similar mechanisms [196-197]. The insect has cellular and humoral immune response pathways that are mediated by hemocytes and antimicrobial peptides [186]. It is known, however, that live bacteria are needed to kill *G. mellonella* as injection of heat-killed bacteria and cell-free culture filtrates has no significant effect on larval survival [186, 188]. Two factors have been hypothesised to be involved in the death of *G. mellonella* in the *S. aureus* model, the bacteria divides in the host during infection reaching a threshold level or the bacteria remain static and produce virulence factors that cause death [188].

There are a number of advantages for the use of this invertebrate model with the first being the simplicity of the model and the ability to keep and breed large numbers. The optimal temperature for *S. aureus* experiments is 37°C and this is the temperature that *G. mellonella* are kept at during the assay, mimicking that of the internal body temperature of a human. This is important as a change of temperature can influence the expression of microbial virulence factors and reduce the rate of cell division. Arguably, the most important advantages are the consistency and reproducibility of results and correlation between virulence of the organism in the *G. mellonella* and animal models [12, 186]. Accurate inocula can be injected directly and precisely into the host's body including; live cells, heat-shocked cells, filter sterilised supernatant and antibiotics [188]. With the injection of antibiotics this model can be used to test the efficacy of antimicrobial agents and study therapeutics for infections [186]. The final advantage is that no ethical approvals are needed.

This *G. mellonella* killing model involves injecting the *G. mellonella* with the test organism and scoring the number of deaths daily. Survival curves are then constructed using the Kaplan-Meier method. This powerful and sensitive model of *in vivo* virulence of *S. aureus* allows for large numbers of isolates to be ranked based on virulence without the use of the mouse model. A subset of strains varying in virulence can then be tested in the mouse model to verify results obtained from the *G. mellonella* model. This method therefore also reduces the number of animals required.

1.8 Previous Work of ST93 Reference Isolate JKD6159

This current study is focused on 59 ST93 *S. aureus* isolates, including that of the completely sequenced isolate JKD6159. JKD6159 is a clinical isolate which caused septicaemia and multifocal pulmonary and musculoskeletal abscesses and also caused a familial outbreak of *S. aureus* skin disease [198]. In a previous study two *in vivo* virulence models, wax moth (*G. mellonella*) killing assay and mouse skin infection assay, showed that JKD6159 was significantly more virulent than other community and healthcare-associated *S. aureus* isolates including the USA300 strain, FPR3757, raising questions about the molecular mechanisms of apparent hypervirulence of this strain [12].

The whole genome of JKD6159 has previously been completed and consists of a 2.81Mbp genome with a 33% G+C content and a 20Kbp plasmid [199]. The sequence of JKD6159 has previously been compared against 19 completed *S. aureus* genomes from a number of sequence types [12]. Whole genome sequencing and comparisons to other sequenced genomes confirmed that ST93 is a distantly related clone, consistent with the fact that it is a singleton by MLST [12]. These sequence comparisons revealed only a small number of novel genes in JKD6159, none that were thought to explain the high virulence of the strain [12]. It was therefore hypothesised that small genetic changes, such as SNPs within genes encoding regulatory proteins, enhances the virulence of JKD6159. To date the cause of hypervirulence of isolate JKD6159 is unknown.

Given the apparent hypervirulence of JKD6159, and the paucity of data available to understand the determinants of virulence of this prevalent clone of ST93 CA-MRSA in Australia, this project was undertaken to enhance knowledge of the virulence mechanisms of ST93 *S. aureus*.

1.9 Project Aims

The overall aim of this study is to enhance understanding of virulence determinants of ST93 *S. aureus*. Specifically;

1. To compare the virulence profiles of ST93 isolates using the *G. mellonella* killing assay
2. To compare levels of expression of important virulence factors, PSMα3 and δ-toxin in ST93 isolates
3. To use comparative genomics to find mutations that correspond with differences between isolates as described in aims (1) and (2)

Chapter Two: Materials and Methods

2. 1 Bacterial Isolates

The bacterial isolates used in this study were 59 clinical ST93 *S. aureus* isolates obtained from patients between 1992 and 2009 throughout Australia, and one laboratory ST93 isolate TPS3202. TPS3202 was genetically identical to TPS3143, with the exception of the *agr* locus which had been genetically repaired to be functional, using the *agr* locus sequence from JKD6159. These are listed in Table 2.1. The fully sequenced isolate JKD6159 was used as the reference isolate for this study.

2.2 Animal Studies

2.2.1 Injection of *G. mellonella* (Wax moth) with Live *S. aureus* Cells

Live *S. aureus* cells of all 59 clinical ST93 isolates were used for injection of *G. mellonella*. A single colony of *S. aureus* was inoculated into a 10 mL heart infusion (HI) broth (Oxoid, Basingtoke, Hampshire, England) and incubated overnight at 37°C with constant shaking (180 rpm). From the overnight culture a 1:100 dilution was made into 20 mL HI in a 250 mL conical flask (Labserv, Thermo Fisher Scientific, Melbourne, Australia) and incubated overnight at 37°C with constant shaking (180 rpm). Then 1 mL of the 20 mL overnight bacterial culture was pelleted by centrifugation (13 000 rpm, 1 min, room temperature (RT)). Cells were resuspended in 1 mL phosphate-buffered saline, pH 7.0 (PBS) (Media Preparation Unit, The University of Melbourne, Victoria, Australia). Pelleting and resuspension of cells was repeated twice. A dilution was prepared for direct injection into *G. mellonella* larva ($1*10^8$ CFU/mL), and CFU enumeration performed to confirm the correct CFU injection on all occasions.

G. mellonella in their final instar larval stage were used. Ten µL of the $1*10^8$ CFU/mL bacterial suspension was directly injected into the first right proleg of the larvae using a high-performance liquid chromatography (HPLC) syringe (SGE Analytical Science, Melbourne, Australia). For each isolate at least eight *G. mellonella* were injected. The larvae were then stored in Petri dishes (eight per Petri dish) in the dark at 37°C for six days. Every 24 hr the larvae were checked for

life and considered dead when there was no response to stimuli. The number of deaths each day was recorded.

To confirm the concentration of bacteria injected into *G. mellonella* the CFU/ml was calculated as follows: A 100 µL volume of a 10^{-5} dilution of the bacterial inoculum was spread plated onto two HI agar plates. Plates were incubated overnight at 37°C. CFUs were counted on each plate and averaged to determine the concentration inoculated into *G. mellonella*. Final inocula of 0.5 – 1.5 x 10^6 CFU in *G. mellonella* were deemed acceptable. For any inocula outside this range the *G. mellonella* were discarded, and the experiment repeated.

2.2.2 Injection of *G. mellonella* with Culture Supernatant

Three isolates were used for injection of culture supernatants, TPS3105, TPS3202 and JKD6159. The number of *G. mellonella* for each isolate was; 30 TPS3202 and 24 for TPS3105 and JKD6159. A 20 mL overnight culture was prepared (section 2.2.1), and pelleted by centrifugation (13 000 rpm, 5 min, RT), then the supernatant filtered through a 0.2µM filter (Ministart, Sigma-Aldrich, St Louis, MO, USA). Ten µL was directly injected into the first right proleg of the larvae using a HPLC syringe (SGE Analytical Science). The larvae were stored in Petri dishes and checked for life every 24 hr (section 2.2.1).

2.2.3 *G. mellonella in vivo* CFU Counts

Three isolates were used, TPS3105, TPS3202 and JKD6159. *G. mellonella* were injected with ten µL live *S. aureus* cells and incubated at 37°C (section 2.2.1) with 24 *G. mellonella* injected per isolate. For the first two days if any *G. mellonella* died they were placed in an eppindorf tube and 1mL of PBS was added and then homogenised. At two days post injection if greater than half the total number of the *G. mellonella* were still alive then alive *G. mellonella* were homogenised with 1mL PBS until only half the *G. mellonella* remained. The remaining *G. mellonella* were homogenised in 1mL PBS at the day of death, or if alive for the duration of the experiment, day six. Serial dilutions of the homogenised *G. mellonella* were made and 100µL was spread plated on HI to determine the CFU/larvae.

Table 2.1. ST93 isolates used in this study.

Strain	Place of isolation	Year of isolation	Specimen or description	SCC*mec* Type	Reference
Clinical Isolates					
JKD6159	Vic	2004	Blood	IVa [2B]	[12]
TPS3104	WA	2009	Nose	IVa [2B]	This study
TPS3105	NSW	2005	Blood	IVa [2B]	This study
TPS3106	WA	2008	Nose	V [5C2&5]	This study
TPS3132	SA	2000	Unknown	IVa [2B]	This study
TPS3133	Qld	2000	Leg	IVa [2B]	This study
TPS3134	Vic	2000	Wound	IVa [2B]	This study
TPS3135	NSW	2000	Eye	IVa [2B]	This study
TPS3136	ACT	2000	Blood	IVa [2B]	This study
TPS3137	NSW	2000	Wound	IVa [2B]	This study
TPS3138	NSW	2000	Wound	IVa [2B]	This study
TPS3139	NSW	2000	Wound	IVa [2B]	This study
TPS3140	NSW	2000	Wound	IVa [2B]	This study
TPS3141	n/a	n/a	n/a	n/a	This study
TPS3142	Qld	2005	Blood	IVa [2B]	This study
TPS3144	Qld	2006	Abscess	IVa [2B]	This study
TPS3145	SA	2006	Abscess	IVa [2B]	This study
TPS3146	Qld	2006	Wound	IVa [2B]	This study
TPS3147	NT	2006	Wound	IVa [2B]	This study
TPS3148	NSW	2007	Blood	IVa [2B]	This study
TPS3149	NT	2007	Thigh	IVa [2B]	This study
TPS3150	NSW	2007	Sputum	IVa [2B]	This study
TPS3151	Vic	2008	Wound	IVa [2B]	This study
TPS3152	Qld	2008	Aspirate	IVa [2B]	This study
TPS3153	Qld	2008	Foot	IVa [2B]	This study
TPS3154	SA	2008	Boil	IVa [2B]	This study
TPS3155	Qld	2008	Forearm	IVa [2B]	This study
TPS3156	NT	2008	Leg	IVa [2B]	This study
TPS3157	NSW	2008	Ulcer	IVa [2B]	This study
TPS3158	WA	2008	Wound	IVa [2B]	This study
TPS3159	ACT	2008	Boil	IVa [2B]	This study
TPS3160	NSW	2008	Wound	IVa [2B]	This study
TPS3161	NSW	2008	Wound	IVa [2B]	This study
TPS3162	WA	2003	Axilla	IVa [2B]	This study
TPS3163	WA	2005	Abscess	IVa [2B]	This study
TPS3164	WA	2008	Lip	IVa [2B]	This study

TPS3165	WA	2008	Nose	IVa [2B]	This study
TPS3166	WA	2008	Thigh	IVa [2B]	This study
TPS3167	WA	2008	ETT	IVa [2B]	This study
TPS3168	WA	2008	Abscess	IVa [2B]	This study
TPS3169	WA	2008	Wound	IVa [2B]	This study
TPS3170	n/a	n/a	n/a	n/a	This study
TPS3171	WA	2008	Nose	IVa [2B]	This study
TPS3173	WA	2009	Leg	IVa [2B]	This study
TPS3174	WA	2009	Wound	IVa [2B]	This study
TPS3176	WA	2009	Abscess	IVa [2B]	This study
TPS3177	WA	2008	Blood	NA	This study
TPS3178	WA	2008	Pleural Fluid	NA	This study
TPS3179	WA	2008	Buttock	NA	This study
TPS3180	WA	2008	Blood	NA	This study
TPS3181	WA	2008	Heel	NA	This study
TPS3182	Qld	2008	Unknown	NA	This study
TPS3183	Vic	2007	Unknown	NA	This study
TPS3184	WA	1995	Skin	NA	This study
TPS3185	WA	2003	Throat	NA	This study
TPS3186	WA	2003	Hands	NA	This study
TPS3187	WA	1996	Skin	NA	This study
TPS3188	NT	1992	Unknown	NA	This study
TPS3189	NT	1992	Unknown	NA	This study

Laboratory Isolates

TPS3105r		TPS3105 with repaired *agrA* from JKD6159 (wild-type sequence)	IVa [2B]	[97]

2.3 Growth Curves

A single colony of *S. aureus* was inoculated into a 10mL HI broth and grown overnight at 37°C with constant shaking (180 rpm). A 1:100 dilution was made into 10 mL HI and 200 μL inoculated into a well of a 96 well plate (Costar, Coming Incorporated, Coming, NY, USA). Three wells were inoculated per isolate and one well was inoculated with plain HI as a control. The plate was then placed in a FLUOstar Omega microplate reader (BMG Labtech, Ortenburg, Germany), incubated for 9 hr 50 min at 37°C with constant shaking (180rpm) with the OD_{600} measured every 10 minutes. This was repeated a total of three times for each clinical ST93 isolate.

2.4 Detection and Quantification of Phenol Soluble Modulins and δ-Toxin

2.4.1 Supernatant Collection

A single colony of *S. aureus* was inoculated into 10 mL tryptone soya broth (TSB) (Oxoid) and grown overnight at 37°C with constant shaking (180 rpm). A 1:100 dilution of overnight culture was made into 3 mL TSB and incubated for 24 hr at 37°C with constant shaking (180 rpm). After incubation bacterial cultures were placed on ice for 10 min to stop growth. Cultures were then centrifuged (13 000 rpm, 5 min, 4°C) and supernatant filtered through a 0.2µM filter (Ministart) and stored at 4°C. This was done in triplicate for each clinical ST93 isolate.

2.4.2 Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

100µL of supernatant underwent chromatography on an Eclipse XDB-C18 5-µm particle size 150 mm x 4.6 mm column (Agilent Technologies, Santa Clara, California, USA) equipped to a Series 1200 HPLC (Agilent Technologies). The mobile phase consisted of solution A [Millipore filtered water (mqH$_2$O)/0.1% trifluoroacetic acid (TFA)] and solution B (acetonitrile/0.1% TFA). PSMα1 formylated, PSMα3 formylated and non-formylated and δ-toxin were eluted with a gradient of solvent A and B (0-100% solvent B) over 27 minutes with a total run time of 31 minutes. The solvent flow rate was 1mL/min. Peaks were detected by their absorbance at 214nm. Peaks of interest were collected and further analysed by MS analysis.

Solutions of the authentic synthetic peptides, PSMα3 formylated and non-formylated (Genscript, Piscataway, New Jersey, USA) were made by dissolving 1.0 mg of peptide in a known volume of Millipore filtered water (mqH$_2$O). Standard curves of synthetic PSMα3 formylated and non-formylated were constructed in the concentration range of 6.0 to 100 µg/mL with the conditions as described above. Both curves were found to be linear over this range.

The presence of PSMα3 formylated and non-formylated in the extract was confirmed by direct comparison of their retention time to the authentic standard as well as confirmation by MS analysis (section 2.4.3). Baseline separation was not achieved for PSMα3 and hence the purity of this peak was determined by MS analysis. All fractions at PSMα3 formylated HPLC chromatogram peak were collected and analysed by MS as this peak was not pure in isolate supernatant. PSMα1 formylated was a peptide present at this peak as determined by MS, mass to

charge (m/z) values 754 (3+ ion) and 1130 (2+ ion). HPLC chromatogram peak of δ-toxin formylated was identified by comparison of retention time to retention time of a peak fraction analysed by MS identifying the fraction as pure δ-toxin formylated, m/z values 752 (4+ ion), 1003 (3+ ion) and 1503 (2+ ion).

PSMα3 formylated, PSMα1 formylated and δ-toxin formylated are similar in length (22 resdues, 21 residues and 26 residues, respectively), the absorbance at this wavelength is comparable for the three peptides. Peptides of similar length is acceptable for concentration determination, therefore the PSMα3 formylated standard curve was used to determine PSMα1 formylated and δ-toxin formylated.

The retention times of PSMα3 formylated, PSMα3 non-formylated, PSMα1 formylated and δ-toxin were 22.5, 18, 22.5 and 26 mins respectively.

The area under the curve (AUC) for each concentration was determined and plotted against the known concentration. The standard curve for PSMα3 formylated was also used for PSMα1 formylated and δ-toxin formylated as both are formylated, therefore would behave similarly to PSMα3 formylated.

2.4.3. MALDI-MS Analysis of Peaks

Samples were analysed by MALDI-TOF-MS by direct injection. Mass spectra were acquired on a Q-TOF II mass spectrometer (Waters Corporation, Milford, Massachusetts, US) scanning over a mass/charge range of 400-1600 with an accelerating cone voltage of 35 kV. Synthetic PSMα3 formylated and non-formylated were used as standards. Known molecular weights of PSMα1 formylated and δ-toxin formylated were used to determine the presence of these peptides in samples [84].

2.5 DNA Isolation Techniques

2.5.1 Genomic DNA Extraction

1.5mL of an overnight 10mL bacterial culture was pelleted by centrifugation at 13,000xg for 1 min. The pellet was resuspended in 200µL of Gram positive lysis solution, 4.8µL of lysostaphin and 9mg of lysozyme (Sigma-Aldrich, St. Louis, Missouri, US). The cells were mixed thoroughly and incubated for 1 hr at 37°C. The GenElute Bacterial Genomic DNA Kit (Sigma) was then used to purify genomic DNA as per manufacturer's instructions. The genomic DNA was eluted off the spin column with 30µL of Elution Solution and centrifuged at 13,000rpm for 1 min. DNA was stored at -20°C.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Oligonucleotides

Synthetic oligonucletides used for PCR and sequencing were obtained from Sigma-Aldrich and are listed in table 2.2.

Table 2.2. Oligonucleotides used in this study.

Primer name	Sequence 5'-3'	Comment
3106 hld L2	TGGCCTATGTCACGACTTCA	Used for amplification and sequencing of *hld* region
3106 hld R2	GCTGGGATAGGCTTCTTCCT	Used for amplification and sequencing of *hld* region

2.6.2 PCR

PCR amplification was performed with Taq Polymerase (Roche, Penzberg, Upper Bavaria, Germany) in a reaction volume of 50µL using an Applied Biosystems Thermal Cycler (2720) (Foster City, California, United States). The PCR conditions consisted of initial DNA denaturation at 94°C for 2min, followed by 32 cycles of 94°C for 20s, 55°C for 30s and 72°C for 1min with a final extension step of 72°C for 7 min.

2.6.3 Agarose Gel Electrophoresis

DNA fragments were separated by gel electrophoresis in 1% (w/v) agarose gels (Lonza, Basel, Switzerland) using Tris-acetate-EDTA (TAE) buffer (2M Tris, 5.7% [v/v] glacial acid, 50mM EDTA) stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, US). DNA was

mixed with loading buffer (50% [w/v] sucrose, 0.05% [w/v] bromophenol blue and 60mM EDTA) before being loaded onto the gel. Gels were run at 110V for 25 min. A 1kb marker was used to determine DNA fragment size (New England Biolabs, Ipswich, MA, US). DNA was visualised using a G:BOX EF UV transilluminator (Syngene, Cambridge, England). Digital images were taken with GeneSnap (V7.12.02) (Syngene).

2.7 Genomics

2.7.1 Whole Genome Sequencing

The genome of ST93 reference isolate, JKD6159, has previously been completely sequenced and assembled and available at NCBI Genbank accession no. CP002114 and CP002115 [199]. The remaining clinical ST93 isolates underwent whole-genome shotgun sequencing using an Illumina HiSeq-2000 with 100-bp, paired-end TruSeq chemistry (Australian Genome Research Facility).

2.7.2 Sequence Comparisons of JKD6159 and Clinical ST93 Isolates

A read mapping approach was used to align the sequence reads from all sequenced isolates to the *S. aureus* ST93 JKD6159 genome using SHRiMP v2.0. Those positions in JKD6159 that were covered by at least three reads from every genome and with a minimum fragment length of 100bp defined a core ST93 genome. SNPs and indels up to ~10 bp and their predicted consequences on coding sequences (CDS) were identified using Nesoni v0.35, a Python utility that uses the reads from each genome aligned to the core genome to construct a tally of putative differences at each nucleotide position (including substitutions, insertions, and deletions) (www.bioinformatics.net.au).

2.7.3 Phylogenetic Tree Construction

Neighbour-joining (NJ) analysis was performed using uncorrected *P* distances as implemented in SplitsTree4 (v 4.13.1) [200]. A maximum likelihood (ML) tree was constructed using RAxML using the general time reversible (GTR) model of nucleotide substitution [201]. *Staphylococcus epidermidis* R6P2A was used as the outgroup. The inputs for each method were the nucleotide sequence alignments of the concatenated variable nucleotide positions for the core genome among all isolates, prepared using Nesoni as described above.

2.8 DNA Sequencing

2.8.1 Sequencing

Nucleotide sequencing of PCR products was performed using ABI PRISM Big Dye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer's instructions, in a reaction volume of 20µL, using a G-Storm Thermal Cycler (G482) (G-Storm, Somerset, United Kingdom). Reactions were analysed on an ABI 3100 capillary sequencer (Department of Pathology, University of Melbourne).

2.8.2 Bioinformatic Analysis of Sequence Data

Genome sequences were examined using the computer programme Artemis (Sanger Institute, Hixton, England). Sequenced fragments of isolate TPS3106 were compared to the genome sequence of JKD6159 by using the computer based sequence program Sequencher (Gene Codes, Ann Arbor, USA).

2.9 Statistical Analysis

Kaplan Meier plots of *G. mellonella* killing results versus JKD6159 were analysed using the Log-rank (Mantel-Cox) Test (GraphPad Prism 6). The null hypothesis (no difference between Kaplan Meier plots) was rejected for $P<0.05$. Isolates that were significantly less virulent that JKD6159 were deemed avirulent, isolates that did not differ significantly were deemed virulent (same as JKD6159), and isolates significantly more virulent than JKD6159 were deemed hyper-virulent.

Differences between mean *in vivo* growth rates of virulent and avirulent ST93 isolate groups were determined using the unpaired t-test. Differences were considered significant when the P-value was less than 0.05.

For the analysis of CFU/larva the unpaired t-test was used to determine if there was a significant difference between the dead and live *G. mellonella*. Differences were considered significant when the P-value was less than 0.05.

Differences between mean toxin expression levels of virulent and avirulent ST93 isolate groups were determined using the unpaired t-test. Differences were considered significant when the *P*-value was less than 0.05.

The correlation between δ-toxin and phylogeny was analysed by using the nonparametric Spearman correlation analysis (GraphPad Prism V6) and Felsenstein's phylogenetically independent contrasts (PIC) method [202]. The PIC test was performed using the R+ package "ape" (http://ape-package.ird.fr/). Correlation was considered significant when the *P*-value (two-tailed) was less than 0.05.

Chapter Three: Assessment of Virulence

3.1 Introduction

It has previously been suggested that isolates that belong to the same *S. aureus* MLST may have differences in virulence due to diversity within the genomes of isolates even within the same ST [203]. The virulence of a large cohort of ST93 isolates was therefore assessed, to determine if this is also true in this clone. The animal model used was the invertebrate *G. mellonella* killing assay, a model that has been used for several studies of *S. aureus* virulence [12, 186, 188-189, 204].

Isolates of *S. aureus* are virulent due to a range of proteins expressed by the cell. These include the expression of a variety of cell surface proteins that aid in colonisation by adhesion to host cells or hinder phagocytosis by leukocytes and secreted toxins and enzymes [205]. Exotoxins known to contribute to virulence of mammalian models include; α-hemolysin, PSMs and PVL, although the role of the latter is controversial [84, 126-127, 135]. To date only two isolates, Newman and BB270, have been used to investigate if secreted proteins are a factor in the death of *G. mellonella* by injection of cell-free culture filtrate [188]. This had no significant effect on the survival of *G. mellonella*. More studies are therefore needed to support these observations with the use of cell-free culture filtrate from different isolates.

The exact mechanism of how the immune system of *G. mellonella* responds to live *S. aureus* cells remains unknown, however the death of *G. mellonella* is hypothesised to occur as a cause of cell division within the host, secretion of an overwhelming volume of virulence factors produced or a combination of both. Only one study has previously performed *in vivo* CFU counts on individual larvae after post-injection with data presenting an increase in CFU/larva over a 48hr period [189].

The aim of this study was to investigate the virulence status of individual ST93 isolates using the *G. mellonella* invertebrate animal model and to investigate what factors caused death of the host. The virulence status of isolates was determined by direct injection of live *S. aureus* cells into *G.*

mellonella and recording death over a six day period. To test if any molecules secreted by *S. aureus* cells contributed to the death of *G. mellonella* cell free culture filtrate of avirulent, virulent and hypervirulent isolates, as determined by the *G. mellonella* killing assay, was directly injected into larvae. CFU counts were conducted on dead and live *G. mellonella* injected with *S. aureus* cells at varied time points.

3.2 Results

3.2.1 Assessment of ST93 Virulence Using *G. mellonella*

For each clinical isolate and the laboratory isolate TPS3202, 10µL of live *S. aureus* cells were injected using a HPLC syringe into the proleg of *G. mellonella* larvae that were in their fourth instar.

Compared to isolate JKD6159 a total of 28 isolates were avirulent, 30 virulent and only one isolate was hypervirulent; the laboratory isolate TPS3202 (Figure 3.1 and Table 3.1). These data demonstrate that there is great virulence diversity amongst isolates that belong to a single ST, in this case ST93. To further investigate the factors causing the larvae to die *in vitro* growth rates of the clinical isolates were first determined.
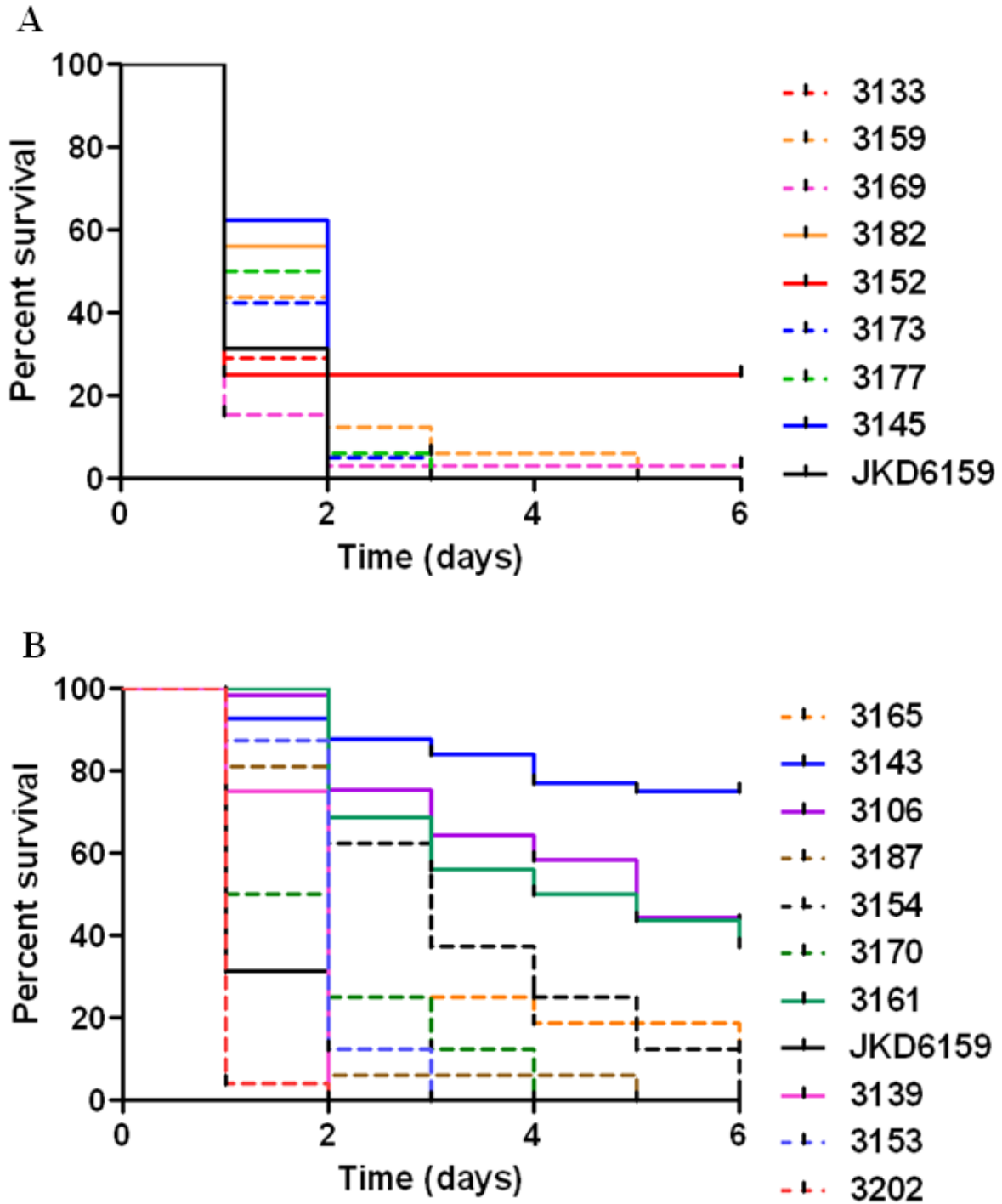
Figure 3.1. *G. mellonella* virulence assay. Kaplan Meier plots showing the percent survival curves of larvae injected with clinical and laboratory isolates up to 6 days post injection. JKD6159 is presented in each with a subset of isolates that did not differ significantly to JKD6159 (A) and a subset of isolates that did differ significantly to JKD6159 (p<0.05) (B).

3.2.2. *In vitro* Growth Rates of all Avirulent and Virulent ST93 Isolates

To investigate if growth rates are a contributor or key factor for the virulence profiles of the isolates *in vitro* growth curves in BHI were performed for each of the 59 clinical ST93 isolates using the OD_{600} reading. All isolates demonstrated a very similar growth pattern with all isolates reaching the start and end of exponential growth phase at approximately the same times, 1 hr and 4 hr 30min respectively. The maximum OD_{600} reached for all clinical isolates was between 1.3 and 1.6. Mean doubling times of isolates during exponential growth phase ranged from 30.51m for isolate TPS3147, to 37.31m for isolate JKD6159 (Table 3.1).

Overall there were no significant differences in doubling times between virulent and avirulent ST93 isolates ($p = 0.59$). These results show that all ST93 isolates have the ability to replicate at similar rates *in vivo* however other factors such as immune mechanisms of the host and the chemical environment could have an effect on growth rates. Further investigation of the bacterial determinants contributing to the virulence of ST93 isolate was therefore required. Injection of supernatant from *S. aureus* grown in HI broth culture was then conducted to determine what impact exotoxins, secreted peptides and other metabolites secreted by *S. aureus* had on *G. mellonella* larvae.

Table 3.1. Virulence and mean doubling time (mins, mean ± SD) comparisons of 60 ST93 *S. aureus* isolates.

Isolate (TPS)	Virulent Status	Doubling time	Isolate (TPS)	Virulent Status	Doubling time	Isolate (TPS)	Virulent Status	Doubling time
3104	Virulent	32.47 ± 0.99	3150	Avirulent	36.13 ± 4.92	3170	Avirulent	33.02 ± 0.79
3105	Avirulent	34.33 ± 0.98	3151	Virulent	33.66 ± 0.57	3171	Virulent	36.04 ± 1.38
3106	Avirulent	32.65 ± 0.52	3152	Virulent	35.17 ± 5.38	3173	Virulent	32.04 ± 1.05
3132	Virulent	36.62 ± 5.77	3153	Avirulent	34.55 ± 0.59	3174	Avirulent	34.19 ± 1.72
3133	Virulent	36.37 ± 5.20	3154	Avirulent	33.20 ± 2.72	3176	Virulent	33.73 ± 1.14
3134	Virulent	35.40 ± 6.53	3155	Virulent	32.10 ± 0.89	3177	Virulent	33.90 ± 1.60
3135	Avirulent	36.78 ± 4.83	3156	Avirulent	35.89 ± 6.03	3178	Avirulent	33.47 ± 1.66
3136	Avirulent	36.49 ± 5.03	3157	Avirulent	35.42 ± 5.19	3179	Avirulent	34.61 ± 5.24
3137	Virulent	35.39 ± 5.63	3158	Avirulent	36.01 ± 6.83	3180	Avirulent	34.8 ± 5.45
3138	Avirulent	36.25 ± 6.09	3159	Virulent	35.62 ± 5.65	3181	Virulent	35.04 ± 5.17
3139	Avirulent	32.99 ± 2.52	3160	Virulent	35.88 ± 5.53	3182	Virulent	34.78 ± 5.55
3140	Virulent	36.64 ± 5.74	3161	Avirulent	31.19 ± 1.56	3183	Avirulent	36.52 ± 5.62
3141	Virulent	36.28 ± 4.58	3162	Avirulent	35.52 ± 4.60	3184	Virulent	32.95 ± 1.64
3142	Avirulent	35.96 ± 5.35	3163	Avirulent	32.84 ± 1.79	3185	Virulent	31.68 ± 1.28
3144	Virulent	36.22 ± 6.07	3164	Avirulent	36.64 ± 4.93	3186	Avirulent	34.88 ± 5.42
3145	Virulent	35.43 ± 6.08	3165	Avirulent	32.34 ± 1.21	3187	Avirulent	34.15 ± 4.75
3146	Virulent	35.46 ± 6.40	3166	Virulent	31.84 ± 0.97	3188	Avirulent	34.89 ± 4.51
3147	Virulent	30.51 ± 0.92	3167	Virulent	32.56 ± 1.04	3189	Virulent	34.67 ± 6.13
3148	Virulent	33.62 ± 2.03	3168	Avirulent	31.99 ± 2.01	JKD6159	Reference	37.3 ± 1.61
3149	Virulent	32.55 ± 1.24	3169	Virulent	33.42 ± 3.64	3202	Hyper-virulent	-

3.2.3 Assessment of Exotoxins in the *G. mellonella* Animal Model by Direct Injection of *S. aureus* Culture Supernatant from Avirulent, Virulent and Hypervirulent ST93 isolates

S. aureus secretes numerous virulence factors and it is unknown what effects these have on the *G. mellonella* model. Experiments so far have shown that there is a difference in ST93 virulence profiles. Culture supernatants of three selected isolates, hypervirulent strain TPS3202, virulent strain JKD6159 and avirulent strain TPS3105, were directly injected into *G. mellonella* to determine if secreted factors in the supernatants accounted for the difference in virulence amongst isolates.

Out of a combined 78 *G. mellonella* larvae injected with *S. aureus* supernatant from isolates TPS3105, TPS3202 and JKD6159 only one died during the six day duration of the experiment; one larvae injected with TPS3202 supernatant (Figure 3.2). As only one *G. mellonella* died, and it was at day one, it is highly likely that it was due to the trauma of injection, rather than a direct effect of the culture supernatant. These results demonstrate that *S. aureus* supernatant, prepared as described in materials and methods, has no effect on the *G. mellonella* animal model. Live cells injected into *G. mellonella* may secrete toxins at higher levels than that injected from the prepared culture supernatant. The results lead to the hypothesis that it is expression of cell surface factors that determines the virulence profile in the *G. mellonella* model. This suggests that avirulent *S. aureus* isolates may be successfully cleared by *G. mellonella* as opposed to virulent isolates that may not be able to be cleared, and the bacterial load increases to levels that cause the *G. mellonella* larvae's death.
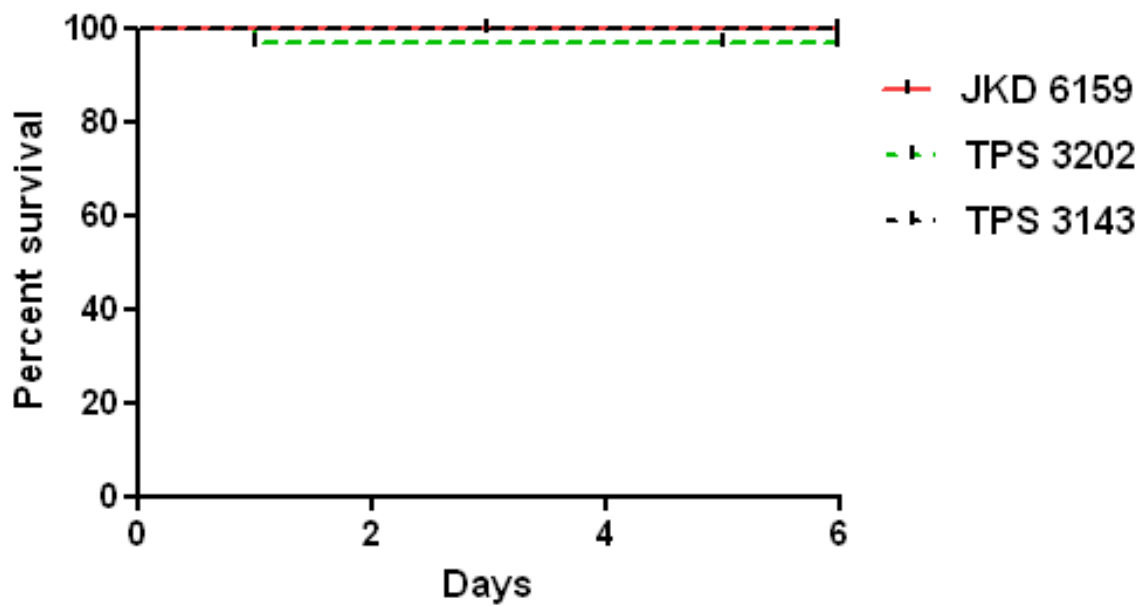
Figure 3.2. *G. mellonella* virulence assay. Kaplan Meier plot showing the percent survival curves of larvae injected with 10µL of supernatant of isolates JKD6159, TPS3105 and TPS3202. There was no significant difference between isolates.

3.2.4 *In vivo* End Point CFU Comparisons Between *S. aureus* Isolates via Injection of Live Cells in *G. mellonella* and Cell Counts within the Host Organism at Specific Time Points, Dead or Alive

There was a significant difference between the bacterial counts obtained from dead larvae injected with each of the three isolates and the live larvae injected with TPS3105 ($p = <0.01$) (Figure 3.3). The average bacterial count of all dead *G. mellonella* contained over a log fold increase compared to the bacterial count in live *G. mellonella*, 4.8 x 10^7 CFU/larva and 2.0 x 10^6.CFU/larva respectively. With initial inoculums of 0.5 – 1.5 x 10^6 CFU/larva there was an increase in *S. aureus* bacterial burden with all isolates. These data indicate that the death of *G. mellonella* is directly related to the bacterial load. A larger set of *S. aureus* isolates, both virulent and avirulent, is needed to support these claims. With a larger data set the minimum CFU/larvae that cause death to the *G. mellonella* larvae could be determined.
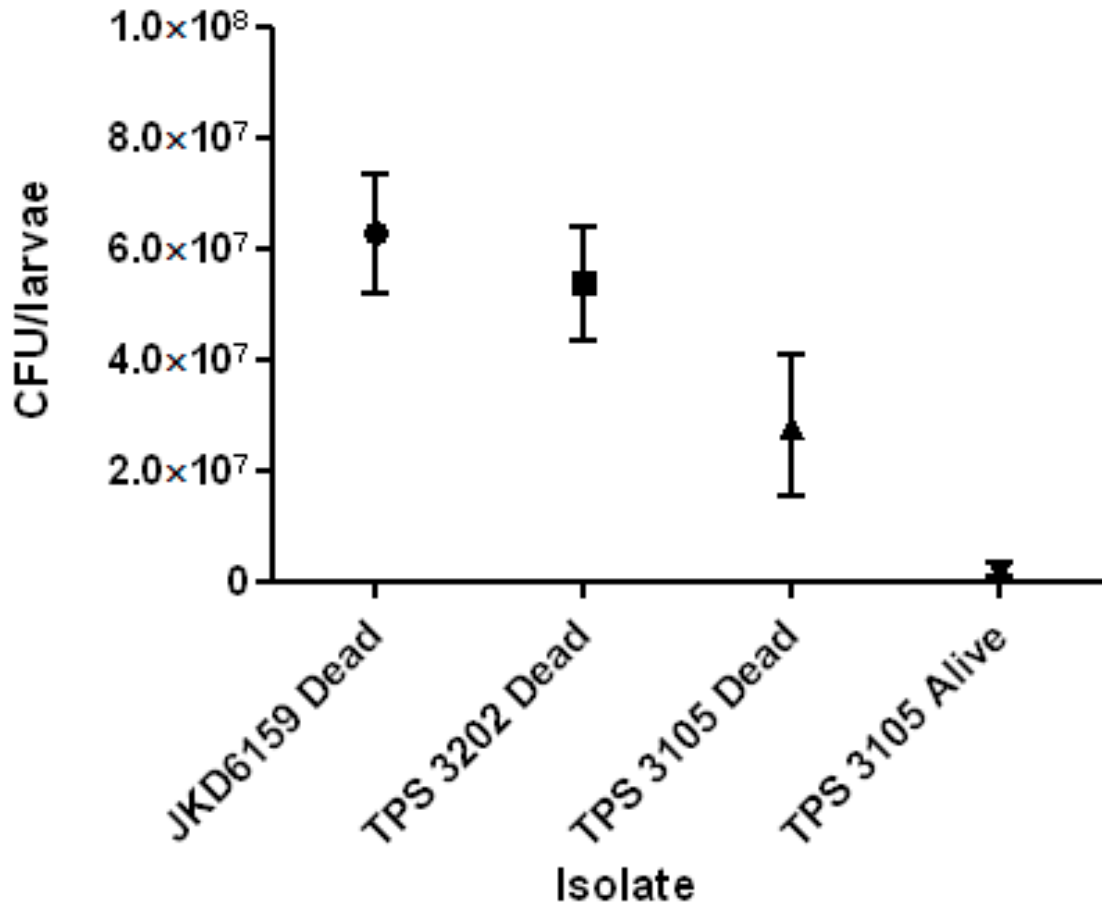
Figure 3.3. *G. mellonella* larvae *in vivo* bacterial loads. *G. mellonella* were injected with live *S. aureus* cells of isolates JKD6159, TPS3105 and TPS3202 and CFU/larvae determined. CFU/larvae counts were performed at the day of death, day one or two, for all larvae of isolates JKD6159 and TPS3105. For isolate TPS3105 CFU/larvae were either performed at day of death; days one, two, three and five, or when the larvae was alive; days two and six. Data shown are mean CFU/larvae and SEM.

3.3 Discussion

In this chapter it was confirmed by using the *G. mellonella* killing assay that isolates belonging to ST93 in this study can differ significantly in virulence profiles. Out of 58 clinical and colonisation isolates 28 were avirulent and 30 were virulent when compared to JKD6159. It was assumed that JKD6159 represents a virulent ST93 isolate as it was isolated from a patient with severe, disseminated *S. aureus* disease. The laboratory isolate, TPS3202, was the only isolate significantly more virulent than JKD6159. This was the first study to show that ST93 isolates can significantly differ in virulence *in vivo*. Significant differences in virulence of *S. aureus* isolates belonging to the same ST (not ST93) have previously been observed in murine models [139].

All ST93 isolates had similar *in vitro* growth kinetics and there was no significant difference between avirulent and virulent isolates. As previously stated this may not necessarily reflect the *in vivo* growth rates as host factors of the *G. mellonella* such as immune response to infection and the chemical environment of the larvae may have have an effect on growth rate. A previous study using CC8 isolates has also observed virtually the same *in vitro* growth rates yet some of the isolates differing significantly in virulence [206].

With the injection of cell-free filtered culture supernatant from an avirulent, virulent and hypervirulent isolate having no significant effect on the survival of *G. mellonella,* the results of a previous study were confirmed [188]. It had previously been observed that there was no significant effect on larval survival after injection of *G. mellonella* with culture filtrate from *S. aureus* Newman cells harvested in mid- to late- exponential phase [188]. However, this does not completely disprove the hypothesis that secreted factors contribute to the death of *G. mellonella* as live cells injected into *G. mellonella* may secrete more toxins than the quantity injected from the culture supernatants. To confirm the hypothesis that secreted toxins and peptides are not responsible for virulence in the *G. mellonella* model, experiments should be performed using gene deletion mutants and, where relevant, complemented strains. Deletion of genes encoding important CA-MRSA virulence determinants including α-hemolysin, PSMα3, and PVL would all be worth testing. For a different invertebrate model, the silk worm model, this has been performed with deletion mutants and parental strains [207]. Results of this study showed that disruption of the α-hemolysin, β-hemolysin, and PSMα operon did not affect the virulence on

silkworms, therefore secreted virulence factors did not have a role in the death of the silk worm. The data here supports the hypothesis that this would also be the case in the *G. mellonella* assay.

In this study *S. aureus* divided within the larvae, as has previously been observed [189]. Results from this study therefore indicate that bacterial burden contributed to larval death, contrary to a previous study where attenuation of virulence was determined not due to bacterial burden as infected larva all had equivalent bacterial burden 48 hours post infection [189]. This previous study however did not count the bacterial burden at any other time point.

The ability of *S. aureus* not only to divide but also to attach to cells within *G. mellonella* may be a major contributing factor for the virulence of isolates. Deletion mutants of genes that encode cell-wall-anchored proteins have attenuated virulence in the silk worm model [207], leading to the hypothesis that expression of cell surface proteins allow the bacterial cells to attach and divide in the *G. mellonella*. To confirm this hypothesis, deletion mutants of *S. aureus* surface binding proteins should be created and virulence assessed using the *G. mellonella* assay. Measurement of the bacterial burden in the larva from a variety of other ST93 isolates, and isolates of other MLSTs, also need to be performed to confirm and identify the threshold level of bacterial load that leads to death. This would be important to establish because it may be found that different isolates and STs kill the organism at different levels of bacterial burden

To further verify the *G. mellonella* killing assay control *S. aureus* strains could be used in future experiments as standards. Previous isolates known to kill *G. mellonella*, a positive control, and avirulent isolates in the *G. mellonella* model. Isolates ATCC 29213 and A8090 could have been used as positive controls and A8094 and A6226 as avirulent isolates.[186]. Well studied mutant *S. aureus* isolates with or without known virulence factors could also be used.

In conclusion, *in vivo* virulence analysis using the *G. mellonella* model demonstrated significant variability within the ST93 isolates in this study. Further experimental work to understand the major determinants of *S. aureus* virulence in this model, and to define the genetic mediators of altered virulence in this isolate collection is warranted.

Chapter Four: Genome Comparisons of Avirulent and Virulent Isolates to Identify Mutations That Potentially Cause the Avirulent Phenotype

4.1 Introduction

Understanding of the *S. aureus* genome including antibiotic resistance determinants, pathogenic mechanisms and relatedness of isolates has increased at a rapid rate with high-throughput DNA sequencing technologies. There are currently hundreds of publically available draft and completed genomes of *S. aureus*.

In this study 58 *S. aureus* ST93 isolates underwent whole-genomes shotgun sequencing and a read mapping approach was used to align the sequence reads from each isolate to the *S. aureus* complete ST93-MRSA-IV JKD6159 genome. SNPs and indels up to ~10 bp were identified and a tally of putative differences at each nucleotide position constructed. These differences were used to construct a ML phylogenetic tree for the collection of strains and determine the SNPs and indels in the population. These were then scrutinized to uncover SNPs or indels that may have accounted for the attenuated virulence of some ST93 isolates in the *G. mellonella* killing assays.

4.2 Overview of SNPs Found within the Genomes

Analysis of the 58 ST93 isolates sequenced and aligned against reference ST93 isolate JKD6159 found a total of 518 SNPs, only ~0.002% of the entire 2,811,435bp genome. Two hundred and nineteen SNPs were non-synonymous, 195 SNPs lay within intergenic regions and the remaining 104 SNPs were synonymous mutations. The numbers of SNPs found in isolates in relation to reference isolate JKD6159 ranged from 11 in the avirulent isolate TPS3139, to 55 in the avirulent isolate TPS3180. Isolate JKD6159 contained six SNPs not present in any other isolate

(Table 4.1). Three SNPs were non-synonymous, one SNP was synonymous and two SNPs lay within intergenic regions of the genome.

Table 4.1. Unique sequence variation of JKD6159 when compared to other sequenced ST93 isolates.

Mutation (JKD6159 chromosome position)	Locus tag (JKD6159)	Protein product	Amino acid change
C to T (365526)	SAA6159_00311	Glycerol-3-phosphate transporter	D46N
T to A (641715)	Intergenic		
C to T (1182955)	SAA6159_01072	Protein phosphatase 2C domain-containing protein	Synonymous
T to G (1978462)	SAA6159_01814	Putative membrane protein	V368G
T to G (2567440)	Intergenic		
T to C (2798673)	SAA6159_02594	HdeD putative membrane protein	I160V

Forty-nine out of the 58 isolates contained at least one SNP that was only present in the chromosome of that isolate. Out of the remaining nine isolates four of them were avirulent, isolates TPS3136, TPS3139, TPS3142 and TPS3163. The total number of genes that contained a minimum of one non-synonymous SNP was 179. Eleven out of those 179 genes contained a non-sense mutation and one gene contained a non-synonymous mutation that changed a stop codon to an encoding amino acid in isolate TPS3181.

4.2.1 Isolate Correlation Based on Maximum Likelihood Phylogenetic Analyses

An unrooted NJ tree was constructed and six clades were identified (Figure 4.1). These same clades and tree topology were also resolved by maximum likelihood (ML) (Figure 4.2). The ML tree was visually examined to assess if there was any correlation with isolate *SCCmec* element carriage, virulence status, origin of isolation and specimen type. The largest clade contained 20 isolates and the smallest clade contained two isolates. Twelve MSSA isolates were present across three clades with ten of the twelve present in one clade and the two remaining MSSA isolates were in two separate clades.

The virulent and avirulent isolates were distributed throughout the tree, with no clear clonal association with either phenotype. One cluster contained only virulent isolates; TPS3132,

TPS3134 and TPS137. The largest number of avriulent isolates that grouped together was four, in two separate clades. The largest number of clustered virulent isolates was three in three clades including the cluster with only three virulent isolates. These observations show that the virulence profile of an isolate cannot be predicted based on the position within the tree.

Isolates from Western Australia were present in five of the six clades. The only clade that did not contain a Western Australian isolate contained three isolates with one each one from Victoria, South Australia and New South Wales. Isolates not from Western Australia were present in a maximum of three clades.

Eight out of the twelve MSSA isolates represented on the phylogeny were collected from Western Australia, two were collected from the Northern Territory and the remaining two from Queensland and Victoria. All MSSA isolates from Western Australia and the Northern Territory belonged to one clade with no other isolates in the clade. The MSSA isolate from Queensland was in a clade with only one other isolate, TPS3106, an MRSA isolate from Western Australia. TPS3106 was the only MRSA isolate to contain SCC*mec* element V. The MSSA isolate from Victoria was in a clade with 12 MRSA isolates.

The specimen type was also investigated in relation to where they were positioned on the tree. There were a total of 23 specimen types listed therefore known specimens were broadly classified into five categories; skin, blood, respiratory, lip and eye. The specimen type of five isolates was unknown. Sixty-seven percent (38/57) of specimens were obtained from the skin. Six isolates were blood specimens and present in three of six clades with no isolates grouping together. From this analysis no correlation between specimen type and position on the tree could be determined.
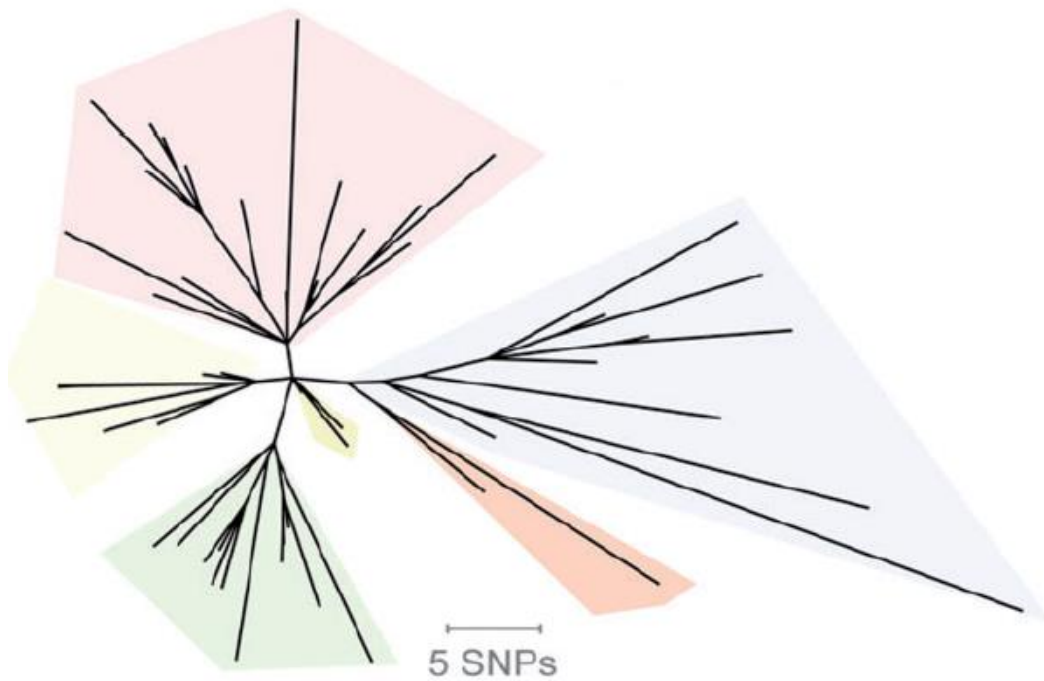
Figure 4.1. Unrooted neighbour-joining tree of ST93 isolates. Six clades are present (shaded). This was constructed by alignment of 519 SNPs using uncorrected *P* distances (SplitsTree4 v 4.13.1) [200]. Figure reproduced from [55].
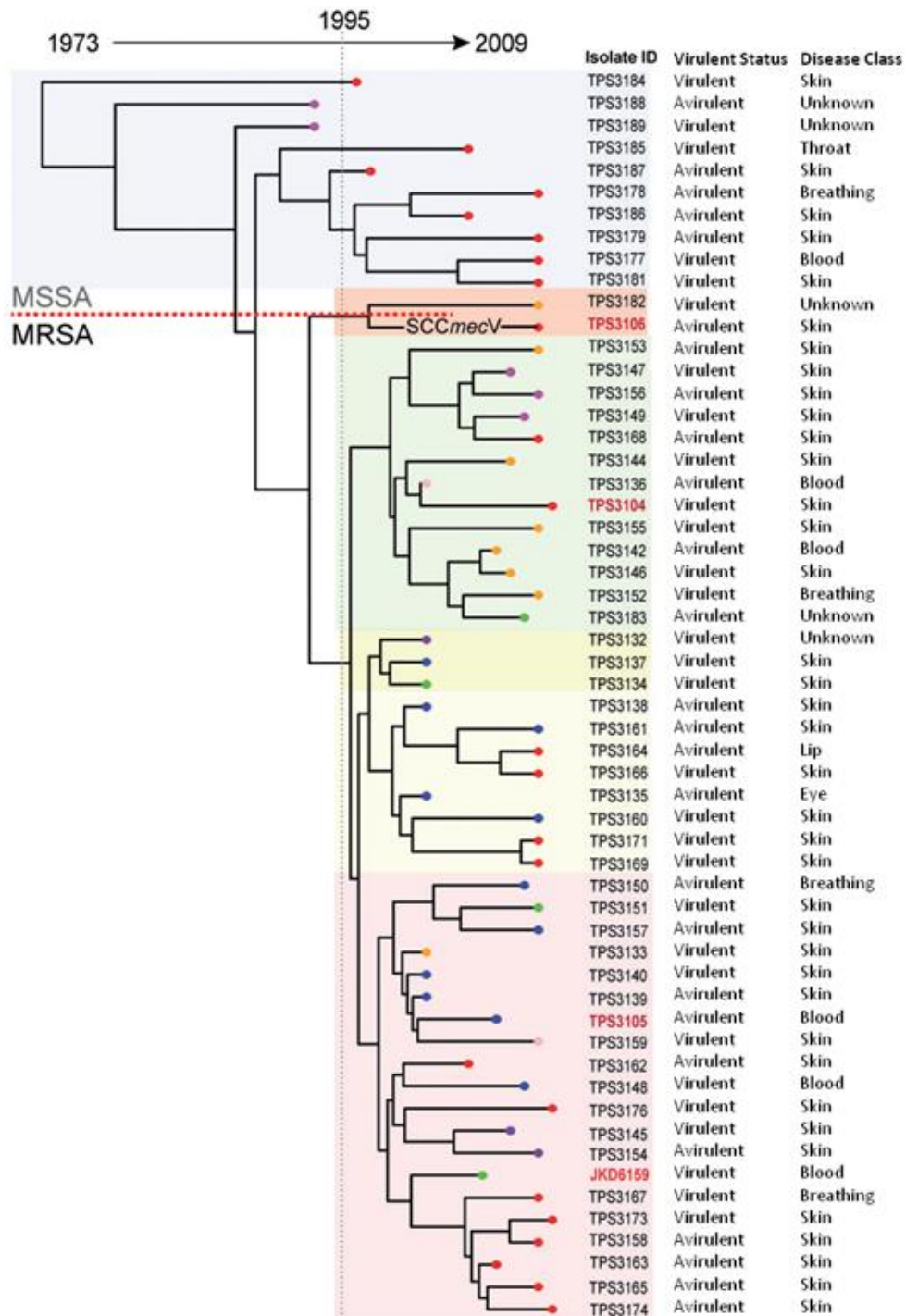
Figure 4.2. Maximum likelihood phylogeny tree of *S. aureus* ST93 isolates. Constructed based on 519 SNPs with ancestral state reconstruction and rooted using *Staphylococcus epidermidis* as an outlier. Tips are colour coded to match the state of origin. Figure reproduced from [55].

4.2.2 Phenol Soluble Modulin and δ-Toxin Detection

The concentration of secreted δ-toxin N-formylated ranged from 0µg/100µL from isolates TPS3105 and TPS3106 to 18.9µg/100µL in isolate TPS3150 (Table 4.2). Isolate TPS3105 also did not secrete PSMα3 N-formylated. Isolates TPS3139 and TPS3151 were two other isolates that did not secrete PSMα3 N-formylated. The highest value for secretion of this peptide was 3.83µg/100µL from isolate TPS3146. Twelve isolates did not secrete PSMα3 N-deformylated including isolates TPS3139 and TPS3151. Isolate TPS3133 secreted the highest concentration of PSMα3 N-deformylated at 6.4µg/100µL. All isolates secreted at least one of the peptides tested. The vast majority of isolates secreted more of the PSMα3 N-deformylated form than the N-formylated form. Only six isolates secreted more of the N-formylated form.

Table 4.2. Mean toxin expression levels of ST93 isolates.

Isolate (TPS)	δ-toxin N-Formylated (µg/100µL, Mean ± SD)	PSMα3 N-Formylated (µg/100µL, Mean ± SD)	PSMα3 N-Deformylated (µg/100µL Mean ± SD)	Percentage PSMα3 N-Deformylated
3104	11.57 ± 0.79	2.62 ± 0.26	5.52 ± 0.25	67.82
3105	0.00 ± 0.00	0.00 ± 0.00	0.76 ± 0.18	100.00
3106	0.00 ± 0.00	3.55 ± 0.26	0.61 ± 0.02	14.75
3132	13.78 ± 0.11	1.55 ± 0.06	5.75 ± 0.09	78.75
3133	13.61 ± 0.58	2.79 ± 0.39	6.38 ± 1.16	69.61
3134	15.81 ± 0.08	2.80 ± 0.17	2.58 ± 0.22	47.92
3135	14.04 ± 0.92	2.80 ± 0.21	3.20 ± 0.35	53.33
3136	14.08 ± 0.57	1.60 ± 0.23	6.04 ± 0.79	79.10
3137	16.85 ±0.60	2.95 ± 0.24	2.89 ± 0.34	49.47
3138	17.50 ± 0.67	3.31 ± 0.16	2.37 ± 0.36	41.70
3139	10.21 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	0.00
3140	12.67 ± 0.41	2.44 ± 0.12	5.41 ± 0.63	68.95
3141	13.24 ± 1.29	2.50 ± 0.32	4.33 ± 0.79	63.43
3142	12.81 ± 1.97	1.84 ± 0.19	5.37 ± 0.93	74.50
3144	14.15 ± 0.23	2.64 ± 0.07	3.94 ± 0.19	59.91
3145	7.21 ± 1.12	1.40 ± 0.21	4.21 ± 0.38	75.08
3146	10.81 ± 0.59	3.83 ± 0.41	4.20 ± 0.53	52.30
3147	10.78 ± 0.30	0.33 ± 0.09	0.00 ± 0.00	0.00
3148	8.65 ± 0.72	0.00 ± 0.00	0.00 ± 0.00	0.00
3149	14.02 ± 0.13	1.76 ± 0.22	5.33 ± 0.66	75.15
3150	18.88 ± 0.97	0.45 ± 0.01	0.00 ± 0.00	0.00
3151	5.81 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00

3152	13.31 ± 0.83	2.98 ± 0.49	4.27 ± 0.79	58.91
3153	11.91 ± 1.63	0.22 ± 0.28	0.00 ± 0.00	0.00
3154	11.77 ± 1.14	2.28 ± 0.46	2.38 ± 0.07	51.09
3155	7.61 ± 0.97	0.94 ± 0.21	0.00 ± 0.00	0.00
3156	13.36 ± 0.66	2.54 ± 0.20	3.87 ± 0.30	60.41
3157	15.51 ± 0.21	1.47 ± 0.01	3.50 ± 0.07	70.38
3158	6.84 ± 0.16	1.44 ± 0.08	4.38 ± 0.10	75.24
3159	13.82 ± 0.45	1.55 ± 0.02	3.77 ± 0.12	70.90
3160	11.17 ± 0.77	2.59 ± 0.15	3.60 ± 0.09	58.20
3161	4.62 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00
3162	6.46 ± 0.28	1.56 ± 0.08	4.18 ± 0.07	72.87
3163	8.53 ± 0.43	1.57 ± 0.07	3.41 ± 0.07	68.49
3164	16.58 ± 0.70	3.00 ± 0.40	4.60 ± 0.53	60.51
3165	8.63 ± 0.20	0.26 ± 0.05	0.00 ± 0.00	0.00
3166	16.92 ± 0.39	3.49 ± 0.22	3.05 ± 0.35	46.59
3167	13.08 ± 0.71	0.88 ± 0.04	0.00 ± 0.00	0.00
3168	12.17 ± 0.34	2.59 ± 0.03	4.11 ± 0.24	61.40
3169	16.08 ± 0.32	0.99 ± 0.05	1.26 ± 0.11	55.93
3170	11.50 ± 1.99	0.05 ± 0.14	0.43 ± 0.40	89.30
3171	9.71 ± 1.67	0.00 ± 0.00	0.00 ± 0.00	0.00
3173	8.81 ± 0.75	0.74 ± 0.07	2.11 ± 0.42	74.05
3174	5.39 ± 0.21	0.73 ± 0.01	3.96 ± 0.01	84.38
3176	8.11 ± 2.30	1.10 ± 0.11	4.27 ± 0.16	79.55
3177	10.91 ± 0.43	2.42 ± 0.24	4.65 ± 0.35	65.73
3178	12.99 ± 0.63	2.03 ± 0.10	1.28 ± 0.06	38.72
3179	8.54 ± 0.65	1.75 ± 0.23	5.56 ± 0.71	76.06
3180	3.99 ± 0.08	2.18 ± 0.05	3.26 ± 0.34	59.90
3181	7.97 ± 0.31	2.44 ± 0.33	5.50 ± 0.99	69.23
3182	13.50 ± 1.35	1.63 ± 0.12	4.53 ± 0.10	73.59
3183	5.17 ± 0.77	0.37 ± 0.04	0.00 ± 0.00	0.00
3184	13.64 ± 0.26	1.98 ± 0.18	0.00 ± 0.00	0.00
3185	10.50 ± 0.59	1.61 ± 0.11	0.00 ± 0.00	0.00
3186	10.70 ± 0.89	2.32 ± 0.25	4.41 ± 0.27	65.55
3187	11.89 ± 0.29	1.45 ± 0.01	4.27 ± -.14	74.61
3188	10.65 ± 0.89	2.49 ± 0.35	4.72 ± 0.66	65.45
3189	13.67 ± 0.36	3.64 ± 0.72	4.67 ± 1.05	56.20
JKD6159	8.25 ± 1.05	0.78 ± 0.14	3.84 ±0.58	83.09
Average	11.03	1.72	2.93	63.07

There was no significant difference between the concentrations of δ-toxin, PSMα3 N-formylated and PSMα3 N-deformylated secreted by avirulent and virulent isolates (P>0.05) (Figure 4.3). Reference ST93 isolate, JKD6159 only produced a moderate concentration of δ-toxin, demonstrating that virulence cannot be predicted by the concentration of δ-toxin produced.
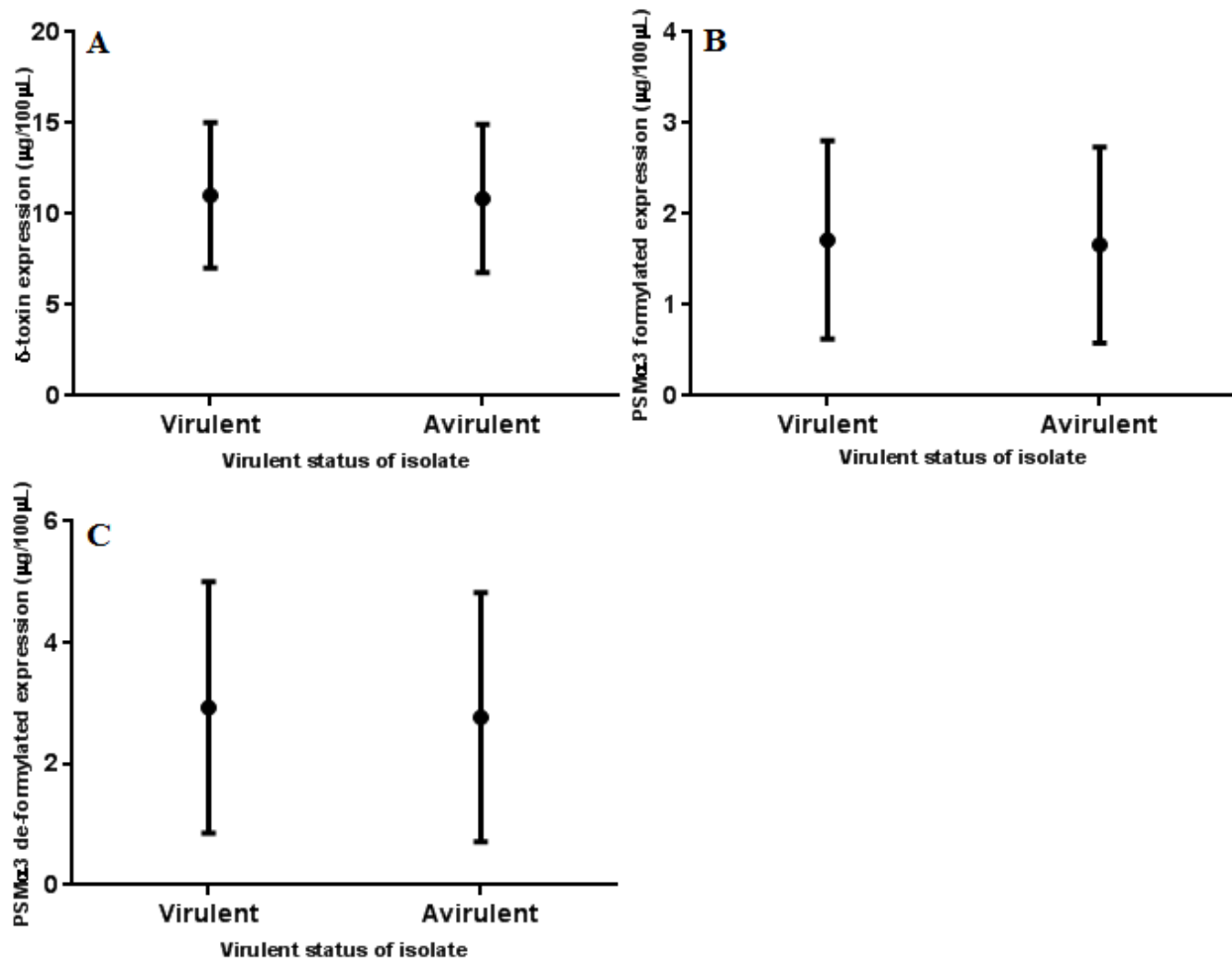
Figure 4.3. Comparison of Toxin Expression Levels Between Avirulent and Virulent Isolates. (A) δ-toxin (B) PSMα3 N-formylated (C) PSMα3 N-deformylated. Data shown are mean toxin expression +/- SEM.

4.2.3 Expression of δ-toxin Over Time

The change in exotoxin expression during the evolution of ST93 isolates was investigated. There was a weak negative correlation between the mean concentration of δ-toxin expressed by isolates and the ML phylogeny root-to-tip branch length (Figure 4.4). This result suggests that the ST93 clone is under selective pressure to reduce virulence gene expression.
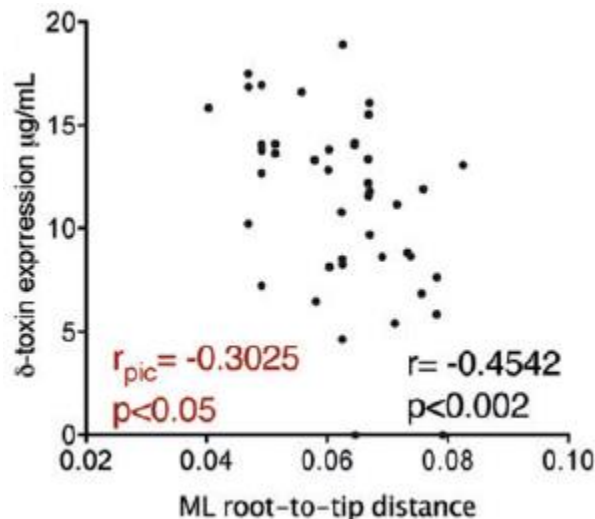
Figure 4.4. Correlation of ML root-to-tip distance with δ-toxin expression. Shown is Spearman's rank correlation coefficient (*r*) and significance indicated with a two-sided *P* value. Assessment of the significance of this relationship was also measured using Felsenstein's PIC method [202]. The correlation coefficient of this statistic is indicated by r_{pic}. Figure reproduced from [55].

4.3.1 Mutations in the *agr* locus

As previously described (Section 1.3.1.2) the two-component *agr* quorum-sensing system in *S. aureus* plays an integral role in the co-ordination of population density and the expression of genes. Many of these genes controlled by the *agr* system are involved in the virulence of this organism. Upon activation of the *agr* system genes encoding virulence factors are up-regulated and genes involved with cell attachment to host cells such as surface proteins are down-regulated. Mutations within the *agr* locus could reduce or completely disrupt the activity of this TCRS.

4.3.2 Confirmation of Deletion of *hld* Region in Isolate TPS3106

The intracellular effector molecule of the *agr* system is a 514 nt transcript RNA molecule, RNAIII [208]. This molecule is a mRNA encoding a 26 amino acid protein, δ-toxin [209]. Visualisation of mapped sequence data analysis of the TPS3106 genome aligned to the genome of reference isolate JKD6159 uncovered an apparent deletion of the *hld* gene ~350bp in length. To confirm this observation PCR amplification of the region spanning the *hld* gene was performed with gDNA from JKD6159 and TPS3106 (Figure 4.5).The PCR amplification product of JKD6159 was ~350bp longer than that of the PCR amplification product of TPS3106. This initial result confirmed that there was a deletion in the *hld* gene region of isolate TPS3106.

The PCR amplification product of isolate TPS3106 was then Sanger sequenced and aligned against the genome of JKD6159 to determine the exact length and location of the deleted sequence in relation to the JKD6159 genome. There was a 356bp deletion that spanned the entire *hld* gene, from position 2097732 to 2093728 in the chromosome of TPS3106, which was intact in JKD6159. These findings confirm that isolate TPS3106 had a deleted region in the *hld*.

Isolate TPS3106 was previously found to produce no formylated δ-toxin (Table 4.2) and the deletion of the *hld* gene is the likely explanation for this. While inactive *agr* loci have been frequently reported due to mutations within the locus, this isolate appears to be the first reported clinical isolate to contain a deletion of the *hld* gene.

With the *agr* system known to play an integral role in the virulence of *S. aureus*, the presence of mutations in the *agr* locus in all isolates in this study was then investigated in the SNP data analysis.
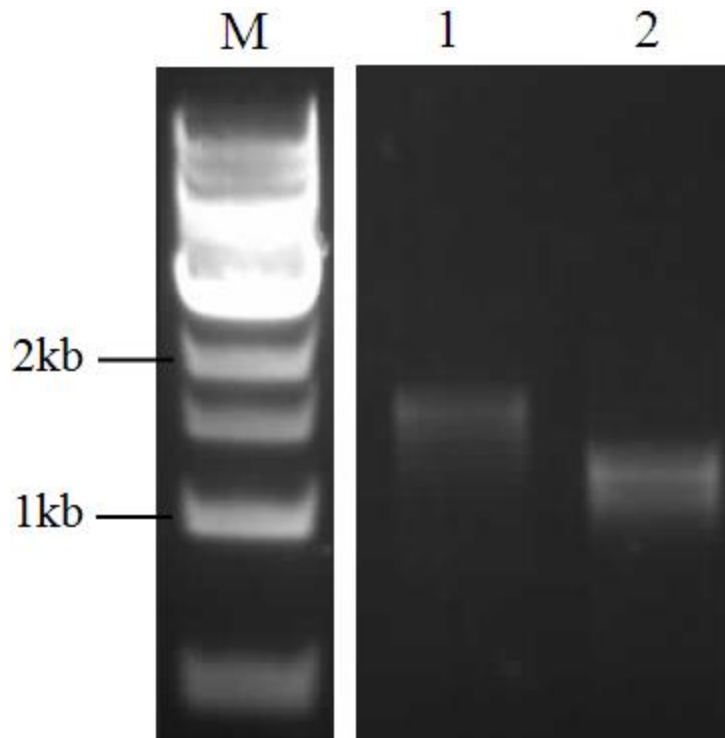
Figure 4.5. *hld* gene deletion confirmation of isolate TPS3106. Polymerase chain reaction (PCR) amplification shows PCR amplified fragment of DNA from isolate JKD6159 (lane 1) running ~350bp larger than isolate TPS3106 (lane 2), region deleted.

4.3.3 Isolates that Contain Mutations in the *agr* Locus

Seven of the sequenced ST93 isolates contained a mutation in the *agr* locus; five isolates containing a non-synonymous SNP in *agrC*, one isolate containing a frame-shift mutation in *agrA* and one isolate with a 356bp deletion region spanning the *hld* gene, as discussed above (Table 4.3). Only two isolates produced no δ-toxin (agr-), TPS3105 and TPS3106; isolate TPS3105 was agr- due to a frameshift mutation in the *agrA* gene and TPS3106 contained a 356bp deletion spanning the *hld* gene. Both isolates were avirulent in the *G. mellonella* animal model.

Five isolates contained a non-synonymous SNP in the *agrC* gene and all secreted δ-toxin (Table 4.4). All five non-synonymous SNPs were non-conservative. These results show that isolates can contain SNPs in the *agr* locus and remain functional. Three of the isolates containing a mutation in the *agr* locus were virulent with the invertebrate animal model used (Table 3.1).

The isolates containing a SNP in the *agrC* gene were distributed throughout the phylogenetic tree.

Table 4.3. Characteristics of isolates that contain a mutation in the *agr* locus.

Isolate (TPS)	Virulence Profile	Agr Locus Mutation	Domain affected
3105	Avirulent	CDS frame-shift base 533 codon 178 of codons 156..239 *agr*A	-
3106	Avirulent	CDS SAA6159_02612 hld	-
3148	Virulent	*agr*C $R_{235}C$	HK Domain, DHp
3151	Virulent	*agr*C $R_{240}N$	HK Domain, DHp
3155	Virulent	*agr*C $Y_{71}H$	Sensor Domain, Intracellular
3161	Avirulent	*agr*C $G_{284}D$	HK Domain, CA
3165	Avirulent	*agr*C $F_{162}S$	Sensor Domain, Transmembrane
3167	Virulent	*agr*C $M_{20}I$	Sensor Domain, Transmembrane

All SNPs in the *agrC* gene were non-synonymous and the predicted amino acid changes were non-conservative. These mutations appeared to have an impact on exotoxin expression. TPS3183 was the only isolate that expressed a lower concentration of each peptide measured than at least one isolate with a mutation in *agrC* (Table 4.4). The expression of three exotoxins of isolates with a mutation in *agrC* was extremely low compared to other ST93 isolates (Table 4.2). Expression of PSMα3 N-deforymlated was not detected in any of these isolates and only isolate, TPS3167 contained a detectable level of PSMα1 N-formylated at a concentration of 0.15µg/100µL. The concentration of PSMα3 N-formylated was <1.00µg/100µL for all isolates. The δ-toxin N-formylated concentrations of isolates with a mutation in *agrC* however were similar to all other ST93 isolates used in this study with three isolates, TPS3148, TPS3165 and TPS3167 expressing a higher concentration than reference isolate JKD6159 (Table 4.2). Therefore expression of δ-toxin N-formylated and the exotoxins measured in this study are not directly linked.

Table 4.4. Exotoxin expression concentrations of all isolates containing a SNP in *agrC* and isolate TPS3183, the only isolate that expressed a lower concentration of each peptide measured than at least one isolate with a mutation in *agrC*.

Isolate (TPS)	δ-toxin N-Formylated (µg/100µL, Mean ± SD)	PSMα3 N-Formylated (µg/100µL, Mean ± SD)	PSMα3 N-Deformylated (µg/100µL Mean ± SD)	PSMα1 N-Formylated (µg/100µL, Mean ± SD)
3148*	8.65 ± 0.72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3151*	5.81 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3155*	7.61 ± 0.97	0.94 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
3161*	4.62 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3165*	8.63 ± 0.20	0.26 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
3167*	13.08 ± 0.71	0.88 ± 0.04	0.00 ± 0.00	0.15 ± 0.01
3183	5.17 ± 0.77	0.37 ± 0.04	0.00 ± 0.00	0.00 ± 0.00

* Contains a SNP in *agrC*

4.4 The ABC Superfamily and Isolates That Contain Mutations Within These Genes

Twelve of the 58 isolates in this study contained a SNP in at least one ABC transporter, and isolates TPS3178 and TPS3182 contained mutations in two ABC transporters (Table 4.5). Six of the eleven ABC SNPs were in isolates that were avirulent. Two of the ABC SNPs were hypothesised to account for the avirulent phenotype, a SNP in gene *SAA6159_02264* of isolate TPS3157 and a SNP in gene *SAA6159_00640* of isolates TPS3178 and TPS3186.

The SNP in isolate TPS3157 resulted in a non-sense mutation in gene *SAA6159_02264* the hemin ABC superfamily ATP binding cassette transporter permease component, at codon 30. This SNP was hypothesised to account for the avirulence of this isolate as the full length protein, if expressed, is 351 amino acids long. Therefore, the truncated 30 amino acid protein would be dysfunctional. The SNP in isolates TPS3178 and TPS3186 encoded a non-synonymous mutation of gene *SAA6159_00640* an ABC superfamily ATP binding cassette transporter; ABC protein at codon 335. As two avirulent isolates contained this non-synonymous SNP this SNP was hypothesised to account for the avirulent phenotype of both isolates. Isolate TPS3178 also contained a SNP in gene *SAA6159_01864* but was not hypothesised to account for avirulence as the SNP in gene *SAA6159_00640* was also found in another avirulent isolate.

Table 4.5. Isolates containing a non-synonymous mutation in an ABC superfamily protein.

Isolate	Virulent Status	Mutation (JKD6159 chromosome position)	Locus tag (JKD6159)	Protein Product	Effect of mutation
3105	Avirulent	G to A (1378362)	SAA6159_01253	PstB	P128S amino acid change
3157	Avirulent	A to T (2426993)	SAA6159_02264	hemin ABC superfamily ATP binding cassette transporter; permease component	L30* amino acid change
3169/3171	Virulent	T to A (1585898)	SAA6159_01445	ABC transporter ATP-binding protein	I27L amino acid change
3177/3181	Virulent	T to C (142219)	SAA6159_00126	PhnD	D197G amino acid change
3178	Avirulent	T to C (2030675)	SAA6159_01864	ABC superfamily ATP binding cassette transporter; ABC protein	T269A amino acid change
3178/3186	Avirulent	G to A (718299)	SAA6159_00640	ABC superfamily ATP binding cassette transporter; ABC protein	A335T amino acid change
3182	Virulent	T to C (1305497)	SAA6159_01186	ABC transporter ATP-binding protein	L227S amino acid change
3182	Virulent	G to A (2086330)	SAA6159_01942	TrkG	V408I amino acid change
3184	Virulent	T to G (757639)	SAA6159_00677	ABC-type proline/glycine betaine transport systems; permease component	S187A amino acid change
3187	Avirulent	C to A (1967991)	SAA6159_01802	ABC-type polysaccharide/polyol phosphate transport system ATPase component-like protein	D382Y amino acid change
3188	Avirulent	C to T (1946158)	SAA6159_01787	ABC transporter protein	G329E amino acid change

After investigation of the mutations in the *agr* locus and the ABC superfamily genes, analysis of the remaining non-synonymous SNPs in avirulent isolates was conducted to determine what SNPs were the cause of the avirulent phenotype in the *G. mellonella* animal model.

4.5 SNPs that Might Explain Avirulent Phenotype

It was reasoned that avirulent isolates may have accumulated loss-of-function mutations in key virulence genes. To test this SNPs and indels in genomes of avirulent isolates were investigated. The first step in determining the SNP(s) that contributed to the avirulent profile of ST93 isolates was to investigate if there was a SNP(s) only present in a minimum of two avirulent isolates. This would indicate that the gene with a SNP may play a key role in the virulence of *S. aureus* ST93 isolates. A total of four SNPs in four genes were found in a minimum of two avirulent isolates (Table 4.6). Each of the four SNPs had a pair of avirulent isolates with isolates TPS3153 and TPS3170 representing three of the four pairs and avirulent isolates TPS3178 and TPS3186 the remaining pair. The most closely related isolate to isolate TPS3153 on the ML phylogenetic tree was TPS3170. This was also the case for isolates TPS3178 and TPS3186.

One clade contained five avirulent isolates, TPS3178, TPS3179, TPS3180, TPS3186 and TPS3187, clustered together with no virulent isolate separating these five isolates. Twenty SNPs were common to all five isolates but none were common to either only these isolates or other avirulent isolates.

The least number of SNPs separating two isolates was two, virulent isolates TPS3169 and TPS3171. Four pairs of isolates differed in sequence variation by three point mutations, two of these pairs differing in virulence. These pairs were; virulent isolate TPS3142 and avirulent isolate TPS3146 and avirulent isolate TPS3164 and virulent isolate TPS3166.

Table 4.6. Proteins that contain a predicted mutation only in avirulent isolates.

Strains (TPS)	Mutation (JKD6159 chromosome position)	Locus tag (JKD6159)	Protein product	Amino acid change
3153 and 3170	789230	SAA6159_00707	YfiA	A51V
3153 and 3170	1889809	SAA6159_01732	Conserved hypothetical membrane protein	E46*
3153 and 3170	2449473	SAA6159_02283	AraC	Synonymous
3178 and 3186	718229	SAA6159_00640	ABC superfamily ATP binding cassette transporter; ABC protein	A335T

Of the four SNPs from this analysis the SNP in the *araC* gene was disregarded as a mutation that may cause the avirulent phenotype in ST93 isolates as this mutation was synonymous. The non-synonymous SNP in the *yfiA* gene encoded for a conservative amino acid substitution and therefore the SNP was determined to be unlikely to change the virulence phenotype.

Avirulent isolates TPS3153 and TPS3170 both contained a non-synonymous SNP encoding a stop codon replacing that of the amino acid glutamic acid in the hypothetical membrane protein SAA6159_1732. The full length SAA6159_01732 protein is 119 amino acids in length and the length of the truncated form encoded on the chromosome of isolates TPS3153 and TPS3170 is less than half that at 45 amino acids. Protein SAA6159_01732 is a hypothetical membrane protein and may have a significant role in virulence of *S. aureus* if expressed on the cell surface interacting with host cells and therefore hypothesised to account for the avirulent phenotype of isolates TPS3153 and TPS3170. However this SNP is unlikely to be homoplastic as these two isolates are clustered together on the ML tree, therefore this SNP may not be the cause of avirulence.

The remaining non-synonymous SNP only common to avirulent isolates was a non-conservative mutation in gene SAA6159_00640. As was the case for the SNP in SAA6159_01732 this SNP is also unlikely to be homoplastic with the two avirulent isolates containing this SNP, TPS3178 and TPS3186, clustering together on the ML tree. With only four SNPs common to more than one

avirulent isolate further investigation was necessary to determine what non-synonymous SNPs may cause the avirulence of isolates.

The next step to determine SNPs that cause the avirulence of isolates was to compare genomes of closely related isolates that differed in virulence. There were three SNPs difference between the core genomes of avirulent isolate TPS3142 and virulent isolate TPS3146. This was also the case for avirulent isolate TPS3164 and virulent isolate TPS3166.

For isolates TPS3142 and TPS3146 all three SNP differences were in the intergenic region of the genome. Isolate TPS3142 contained one of the SNPs and isolate TPS3146 the remaining two. The SNP in the genome of TPS3142, position 1739874 A to a T, was therefore hypothesised to account for the avirulence of this isolate.

The three SNPs that differed between TPS3164 and TPS3166 were all in isolate TPS3164. Two of the SNPs were in intergenic regions and one SNP was a synonymous SNP in JKD6159 locus tag SAA6159_01298, a putatative integral membrane protein. Therefore either of the SNPs in the intergenic regions or both of the SNPs together were hypothesised to have caused the avirulent phenotype of isolate TPS3164.

After an extensive analysis of SNPs of 58 ST93 isolates aligned to the reference ST93 genome, JKD6159, at least one non-synonymous mutation in 20 genes were hypothesised to account for the avirulence of isolates (Table 4.7). All of the SNPs presented in the table were non-synonymous non-conservative mutations.

Table 4.7. Avirulent isolates and potential point mutations hypothesised to account for avirulence of isolates.

Strains (TPS)	Mutation (JKD6159 chromosome position)	Locus tag (JKD6159)	Protein Product	Effect of mutation
3105	Frame-shift 2096569	SAA6159_01952	AgrA	Frame-shift mutation
3106	Deletion 2093372 to 2093728	SAA6159_02612	Hld	Deletion
3161	G to A (2095576)	SAA6159_01951	AgrC	G284D amino acid change
3165	C to T	SAA6159_01951	AgrC	F162S amino acid change
3153 and 3170	C to A (1889809)	SAA6159_01732	Conserved hypothetical membrane protein	E46* amino acid change
3178 and 3186	G to A (718229)	SAA6159_00640	ABC superfamily ATP binding cassette transporter; ABC protein	A335T
3170	C to G (2074391), T to A (2074306), A to T (2074245) and C to T (2074247), G to T (2074218) and A to T (2074219), A to C (2074205)	SAA6159_01927	Conserved hypothetical membrane protein	E2Q, Y30K, D50F, I59K, Y64D amino acid changes
3170	A to T (2077507)	SAA6159_01934	Conserved hypothetical membrane protein	M61L amino acid change
3150	T to G (316822)	SAA6159_00267	EssB	F227C amino acid change
3162	C to T (1156675)	SAA6159_01049	IspA	H50Y amino acid change
3138	A to T (2763617)	SAA6159_02558	Cap1C	W254R amino acid change
3157	A to T (2426993)	SAA6159_02264	Hemin ABC superfamily ATP binding cassette transporter; permease component	L30* amino acid change

3158	T to G (2669325)	SAA6159_02486	PyrD	H341G amino acid change
3158	A to T (989773)	SAA6159_00891	Conserved hypothetical membrane protein	K33* amino acid change
3180	T to C (443469), A to G (443499) and G to A (443500), C to T (433509)	SAA6159_00391	Lpl2	F175L, S185D, T188I amino acid changes
3168	T to C (1094506)	SAA6159_00989	SrtB	I240T amino acid change
3188	T to C (1386867)	SAA6159_01260	Asd	L21S amino acid change
3188	T to A (176274)	SAA6159_00157	Binding-protein-dependent transport systems inner membrane component	L209* amino acid change
3170	A to C (2769773)	SAA6159_02565	IcaC	K31N amino acid change
3187	C to T (1003169)	SAA6159_00905	Glutamyl endopeptidase	A14T amino acid change
3180	A to T (445084), A to G (445094)	SAA6159_00393	Conserved hypothetical membrane protein	K146N, K150E amino acid changes

Avirulence of isolates TPS3105 and TPS 3106 were determined to be due to mutations in the *agr* locus. TPS3105 contained a frame-shift mutation in *agrA* and TPS3106 contained a 356bp deletion spanning the *hld* gene.

The *agrC* SNPs in six isolates TPS3148, TPS3151, TPS3155, TPS3161, TPS3165 and TPS3167 were assumed to account for the reduction in exotoxin expression in this study however exotoxin expression does not have a role in *G. mellonella* killing. Not every SNP in the *agrC* gene accounts for avirulence of isolates as three of the five isolates with an *agrC* were virulent. The SNPs in the *agrC* gene of avirulent isolates TPS3161 and TPS3165 were hypothesised to account for the avirulence of these isolates in the *G. mellonella* killing model.

A non-sense mutation in a gene encoding a hemin ABC superfamily ATP binding cassette transporter, permease component (SAA6159_02264) was hypothesised to account for the

avirulent phenotype of isolate TPS3157. Mutations in iron ABC transporters of *Streptococcus pyogenes* and *Yersinia pestis* have previously been shown to have a significant reduction in virulence in subcutaneous murine models [210-211]. A significant reduction in virulence of other bacteria with mutations in iron ABC transporters has also been observed in a range of animal models [174, 212-216].

The non-synonymous SNP in the hypothetical membrane protein SAA6159_1732 of avirulent isolates TPS3153 and TPS3170 was also previously described to be likely to account for the avirulence of these two isolates. The genome of isolate TPS3170 also contained non-synonymous SNPs in three other genes that were also hypothesised to change the virulence profile of isolates from virulent to avirulent if the stop codon in the hypothetical membrane protein SAA6159_1732 did not. The non-synonymous SNPs were in genes encoding conserved hypothetical proteins SAA6159_01927, SAA6159_01934 and the *IcaC* gene. A non-synonymous SNP in the *IcaC* gene was also present in avirulent isolate 3188 however this encoded a conservative amino acid change.

The *ica* locus encodes products that synthesize a linear β-1, 6-linked glucosaminylglycan [217]. There is currrently no evidence about the impact of the predicted K31N mutation on IcaC function. A clinical isolate that was biofilm-negative was found to have an insertion element, IS256, in the *icaC* gene [218]. A *ica* null mutant has also been created and was not able to form strong biofilm compared to the wild type parent strain [102].

Non-synonymous SNP F227C in the *essB* gene of isolate 3150 was hypothesised to account for the avirulent phenotypic profile of this isolate. No isolate has previously been reported with a SNP in the *essB* gene. Two laboratory strains with disruption of *essB* have been created, an *essB* deletion mutant of USA300 and a Newman isolate with a minitransposon inserted in the chromosome at the location of the *essB* gene [100, 219]. Deletion of *essB* in strain USA300 led to the loss of EsxA secretion with EsxA remaining in the cell [219]. Secretion of EsxA and EsxB was required for establishment and virulence in a murine abscess model of infection [100, 220].

A non-synonymous SNP in the lipoprotein signal peptidase (LspA) was hypothesised to be the only mutation that caused the phenotype of isolate TPS3162 to be avirulent. LspA is involved in the lipoprotein biosynthesic pathway by the cleavage of signal peptides [221]. Three *lsp* mutants have previously been constructed in *S. aureus*; *bursa aurealis* insertion in the *lsp* gene of Newman strain and two RN6390 isolates with a Tn917 transposon insertion disrupting the *lsp* gene [83, 222-223]. Significantly attenuated virulence in murine models was observed for both *lsp* mutants [222-223].

The genome of avirulent isolate TPS3138 contained one unique non-synonymous SNP in the *cap1C* gene. This gene is one of genes in the capsule operon, suggesting that this mutation may have an impact on capsule production. A *S. aureus* strain that expressed CP1, SA1 mucoid, was significantly more virulent in mouse lethality, mouse bacteraemia and renal abscess models than Tn551 mutants, JL24 and JL25, which produced a limited and no capsule, respectively [105].

Isolate TPS3158 contained two non-synonymous SNPs that were unique to this isolate, a non-synonymous SNP in the dihydroorotate dehydrogenase gene, *PyrD* and a non-sense SNP mutation in conserved hypothetical protein SAA6159_00891. *PyrD* has been shown to be important in virulence production with a *PyrD* null mutant of *P. aeuruginosa* not producing β-hemolysin [224]. The non-sense SNP mutation of SAA6159_00891 reduced the length of the protein from 95 codons to 32 codons. This SNP may also contribute to the avirulence of this isolate.

Two mutations in genes *lpl2* and *SAA6159_00393* in isolate TPS3180 were predicted to account for the avirulence of this isolate. The *lpl2* gene encoding a staphylococcal tandem lipoprotein contained 10 SNPs. A total of eight of the 10 SNPs were non-synonymous and four of them changed the properties the amino acids encoded Staphylococcal lipoproteins are hypothesised to be exported from the cytoplasm of the cell via the Sec pathway and anchored to the membrane [225]. Lipoproteins *lpl2-9* have previously been identified as candidate virulence genes [226]. The conserved hypothetical protein, SAA6159_00393, contained three non-synonymous mutations, two of which changed the properties of the amino acids encoded.

TPS3168 contained a non-synonymous mutation in *srtB* hypothesised account for the avirulent phenotype. The *srtB* gene encoding a surface protein sortase B, required for anchoring a protein with a NPQTN motif [227]. SrtB was found to be important for persistence of infection in a murine renal abscess model [227]. A murine arthritis model of showed that Δ*srtA*, Δ*srtB* and Δ*srtA, srtB* mutants had better survival rates that the wild-type Newman strain [228]. In the same study *srtB* was found not to play a prominent role in the pathogenesis of murine arthritis but contributed to the establishment and persistence of the disease [228]. Another study found that a *srtB* mutant was able to establish infection in renal abscesses and arthritic joints equivalent to wild-type Newman strain but there was significantly reduced infective titre over the course of infection when compared to the wild-type Newman [229]. Therefore SrtB has been shown to be an important protein in the persistence of *S. aureus* disease.

Non-synonymous SNPs in the *asd* gene and a gene encoding a binding-protein-dependent transport system inner membrane component, SAA6159_00157, were hypothesised to account for the avirulence of isolate TPS3188. The SNP in the gene encoding SAA6159_00157 is non-sense mutation at codon 209 of the full length 253 amino acid protein. The *asd* gene encodes an aspartate semialdehyde dehydrogenase protein. This protein is involved in the synthesis of all four aspartate family amino acids, methionine, threonine, isoleucine and lysine and the synthesis of diaminopimelic acid (DAP), an important component of cell wall peptidoglycan [230]. The *asd* gene has been identified as a virulence gene in a murine model of bacteraemia using signature-tagged mutagenesis of *S. aureus* isolate RN6390 [231]. However a mutant *asd* strain of *S. aureus* 8325-4 revealed that there was no significant difference between the number of cells recovered from the host after 7-days of infection in three murine infection models (mouse abscess, pyelonephritis, and wound infection) [232]. Therefore the importance of the *asd* gene in the establishment and/or persistence of *S. aureus* infection is still unknown with conflicting results using different strains of *S. aureus*.

The final non-synonymous SNP hypothesised to account for the avirulence of ST93 was in the gene encoding a glutamyl endopeptidase protein. The most well characterized exoprotease expressed is the serine protease GluV8 [233]. GluV8 plays an important role in the virulence of

S. aureus by destruction of host defence mechanisms [234]. The SNP in the glutamyl endopeptidase may therefore account for the avirulence of isolate 3187.

4.6 Discussion

Overall there was restricted genetic diversity between the 58 ST93 isolates with a total of only 518 SNPs found in the whole population when compared to reference ST93 isolate JKD6159. A sequencing study has previously been conducted on USA300 isolates where the sequences of 10 USA isolates were compared to reference USA300 strain FPR3757 [235]. The study found a total of 578 unique SNPs in the core genome. Based on this the genetic divergence amongst ST93 isolates is far less than USA300 isolates, even though the isolates included in this study were collected over many years. This may mean that ST93 has recently evolved and is a newer strain of *S. aureus* than USA300. Isolation and sequence comparisons of more ST93 isolates may help to confirm this.

Out of all the isolates in this study, 13 were MSSA and the remaining were MRSA. One MRSA isolate, TPS3106, contained SCC*mec* type V element and the remaining MRSA isolates contained SCC*mec* type IV. Based on phylogenetic analysis the ST93 progenitor was likely to be an MSSA isolate from north-western Australia. The MRSA ST93 isolates have arisen on at least two occasions with the acquisition of two SCC*mec* types, IV and V. This has been previously been described with several STs containing MRSA isolates with more than one SCC*mec* element [42]. There is potential bias as to the prediction of the location of the progenitor isolates with the majority of isolates obtained from Western Australia. Therefore there may be an overrepresentation of isolates from Western Australia that may skew the tree, but this will remain unresolved until more ST93 isolates are sequenced.

In this study ~60% of PSMα3 secreted by isolates was in the N-deformylated form, as has previously been reported [236]. It is known that immune cells recognise the N-formyl methionine group of synthesized bacterial proteins therefore higher secretion of the N-deformylated will help evade the immune system [237]. There was a slight negative correlation observed between the decrease of both PSM α3 formylated and δ-toxin and branch length. A decrease of both the expression of PSMα3 formylated, a major virulence factor in murine

models, and a decrease of the activity of the *agr* system as isolates evolve indicate that isolates are under selective pressure. This attenuation of virulence may be critical for the clone to survive, and suggests that ST93 may start to emerge in the healthcare setting where inactivation or attenuation of *agr* activity is well described [238].

PSMα3 and PSMα1 expression had limited correlation with δ-toxin expression as has previously been reported [84]. Therefore other regulatory systems and/or factors must either directly regulate or dependently regulate PSM production. It has recently been reported that the *SarA* locus is required for maximum PSM production [239]. The quantity of the deformylated form of PSMα3, the form with greater cytotoxicity, was significantly higher in the majority of isolates.

It was interesting to note that avirulent and virulent sets of ST93 isolates secreted similar level of PSMα3 N-formylated and PSMα3 N-deformylated compared to USA300, a strain that has previously been shown to produce high level of PSMs and contribute to virulence (Wang 2007 and Li 2010 Chua 2014). USA300 secreted 1.91 ± 0.01 μg/100μL PSMα3 N-formylated and 3.54 ± 0.46μg/100μL PSMα3 N-deformylated [97].

It is important to note that the virulent and avirulent profiles were based on the *G. mellonella* larvae model and it was demonstrated in this study that virulence appears to be dependent on expressed factors on the surface of the cell as the supernatant had no effect on killing. A mammalian host such as a mouse or rabbit may present a different virulence profile as these models respond to toxins produced and secreted by *S. aureus* cells.

The finding that isolate TPS3106 has a deletion spanning *hld* was significant as it proves that an isolate without a functioning *agr* locus remain capable of causing human infection. Previous studies have created a total of three *hld* mutants, two *hld* gene deletions, USA300 (LAC) and USA400 (MW2), and one deletion replacement, WA400 [84, 240]. The *hld* mutants have demonstrated that *S. aureus* is viable without a functional *hld* gene, however the secretion of several exoproteins, α-toxin, serine- and metallo-protease were almost completely blocked. *hld* mutants have been used in only two studies to assess virulence; murine arthritis model and murine bacteraemia model [84, 152]. Both studies found that virulence was significantly

attenuated when compared to wild type isolates. The deletion of *hld* is therefore highly likely to account for the avirulent phenotype of isolate TPS3106.

The non-synonymous mutation in the putative secretion system component, EssB, may not be the cause of avirulence in the *G. mellonella* model as this system is known to release toxins into the extracellular environment and it has been demonstrated in this work, and previous work, that the *G. mellonella* model does not respond to exotoxins [188]. However, the Ess pathway also releases proteins on to the cell surface and is required for the establishment of disease, therefore the non-synonymous mutation in the *essB* gene is hypothesised to cause avirulence in this model.

Of the 28 avirulent isolates in this study, 14 contained at least one mutation hypothesised to account for the avirulence of isolates. Three of these 14 isolates contained mutations in multiple genes accounting for the avirulence, isolates TPS3170, TPS3180 and TPS3188. It has previously been shown that the difference between an asymptomatically carried MSSA isolate and a lethal MSSA isolate was only eight mutations, four of which were nonsense mutations [56]. Two USA300 *S. aureus* strains (18805 and 18811) that were significantly less virulent than reference USA300 strain (FPR3757) in a mouse sepsis model contained only 7 and 14 unique SNPs respectively, and 2-3 non SNP deletions in each isolate when compared to genomes of 10 virulent USA300 isolates [235]. A missense mutation in *agrA* in strain 18811 was hypothesised to account for the avirulence of the isolate, the same gene that strain TPS3105 contained a frameshift mutation in. This highlights that subtle genetic changes, including a small number of SNPs in certain loci, can significantly alter the virulence phenotype of *S. aureus* isolates.

This study also highlighted the association of the *agr* locus with the virulence of *S. aureus,* as has been previously described. The impact of a SNP in *agrA*, *agrA*$_{C123F}$, and a change in virulence gene expression has previously been demonstrated in a phage type 80/81 isolate (22251) with this isolate not producing Hla [238]. Survival of mice infected with isolate 22251 in a bacteraemia model was significantly different to phage-type 80/81 and South-west Pacific clones and similar to ERMSA-16. Isolate 22251 was also used in a mouse pneumonia model and survival was 100%. Survival of mice with two other phage-type 80/81 isolates without the *agrA* mutation was significantly reduced.

AgrC is a member of the class 10 receptor-histidine protein kinases (HPKs) comprised of a sensor domain that transverses the cell membrane and a histidine kinase (HK) domain in the intracellular domain [241]. The HK domain is further divided into two subdomains, dimerization histidine phosphotransfer (DHp) and catalytic ATP-binding (CA) subdomains. The site of phosphorylation occurs in the DHp subdomain at amino acid H239. Studies have shown that two regions in the extracellular region of the sensor domain are involved in the recognition and attachment of the AIP and the transmembrane helices transmit the AIP signal through the HK domain where phosphorylation of H239 occurs [242-243]. Two isolates, virulent isolate TPS3155 and avirulent isolate TPS3165, contained non-synonymous SNPs in the sensor domains however these non-synonymous SNPs were not in the region of the sensor domain previously shown to be crucial for AIP binding in the extracellular loop, therefore it was assumed that the AIP molecule could attach to AgrC. The other three *agrC* non-synonymous SNPs were in the HK domain.

The role of SNPs in non-coding, intergenic regions of *S. aureus* has been less well characterised. It does appear, however, that SNPs in intergenic regions may be important to the virulence profile of isolates, with this study revealing that the only genomic differences between one pair of virulent and avirulent isolates, TPS3142 and TPS3146, was three SNPs in intergenic regions. Intergenic regions may encode undiscovered RNA molecules and proteins and may also have a role in transcription and the rate of translation. Bacterial non-coding RNA molecules are known to regulate genes by regulation of transcription, RNA modification, and changes in stability and mRNA translation [244]. RNAIII, the effector molecule of the *agr* system of *S. aureus*, detected in this study is an example of virulence gene regulation by an RNA molecule [146]. Other RNA molecules that mediate virulence are Qrr1-Qrr4 and CsrB in the *Vibrio cholerae* and *Salmonella typhymurium* genomes respectively [245]. Deletion mutants of two sRNA molecules, *rli38* and *rliB*, showed attenuation in virulence of *Listeria monocytogenes* [246]. Allelic replacement of genomes and animal experiments would determine if the identified SNPs within intergenic regions do have any role in the virulence of *S. aureus*.

The avirulence of remaining isolates may be due to a combination/accumulation of SNPs, a SNP in an intergenic region or a mutation in the genome that was beyond the parameters set in the sequence analyses. One amino acid change may have little effect on protein function therefore some SNPs may have been incorrectly identified as the cause for avirulence in this study.This study has shown the difficulties in determining the phenotype based on the genotype.

Development of computational methods to predict phenotype based on genotype is crucial in the understanding of the pathogenesis and transmission of *S. aureus* and other harmful pathogens on a case by case basis. A genome-wide association study (GWAS) and machine learning approach has recently been conducted on 90 ST239 *S. aureus* isolates to predict virulence of isolates [247]. From the initial GWAS approach 121 loci were significantly associated with virulence from a total of 3060, however this method produced a high rate of false positives. An approach used to narrow this number reduced it down to only four loci. Future developments could accurately predict virulence genes for *S. aureus* from all backgrounds. This approach could be applied to the ST93 dataset in future.

Future work to be conducted for sequence analysis of this dataset includes complete genome assembly and annotation. Whole genome assembly will uncover any additional genome differences to the reference genome JKD6159 beyond the scope of this study, including any large insertions or deletions, sequence repeats, duplications, insertion sequence movements, genomic islands and also any plasmid acquisition or loss. These genetic differences may also impact virulence of the isolate or may be of interest for future study of isolates. The ~356bp deletion in the genome of isolate TPS3106 of this study that was not identified in SNP analysis demonstrates the need for this. Isolates that should be prioritised at the top of the list for further analysis are the isolates that had no unique non-synonymous mutations or non-synonymous mutations only present in other avirulent isolates. These isolates are TPS3136, TPS3139, TPS3142, TPS3163, TPS3164 and TPS3183. These isolates may not contain any other differences in the genome when further investigated and the avirulence of these isolates may be due to the accumulation of non-synonymous mutations that are also present in virulent isolates.

In addition, RNA sequencing (RNAseq) is another recently emerged tool that can be can also be used to uncover genes that are expressed in virulent isolates and not in avirulent isolates, or highly expressed in virulent isolates when compared to avirulent isolates. This method can also be used after allelic replacement experiments to determine the effect SNPs have on the whole genome transcript.

Proposed future work includes; allelic replacement experiments, animal studies and further genome sequence analyses. Allelic replacement experiments would be used to confirm that a SNP identified in this study to either be the sole reason or a major contributor to the avirulent profile of an isolate. The identified SNP in the avirulent isolate would be replaced to a wild type allele (based on JKD6159) and vice versa. The *G. mellonella* virulence assay can then be used to confirm if the SNP in the avirulent isolate is the cause of avirulence. If there is a change in the virulence profile experiments using the murine SSTI model should also be used for the repaired isolates to test the virulence. This is because *G. mellonella* model does not respond to exotoxin expression therefore the murine SSTI may correlate more closely to human disease.

In summary, this study has used genomics to reveal the close genetic relationship among a large collection of ST93 isolates. The cause of the avirulent phenotype of isolates, as determined by *G. mellonella* studies, determined by this study were a frame-shift mutation, deletion, multiple SNPs within one gene or single SNPs. Quantification of exotoxin expression was also investigated in this study with the majority of isolates expressing relatively the same concentrations. These results show that JKD6159 is a good reference candidate to represent the ST93 population. Further studies are needed to unveil the mechanisms behind the emergence and hypervirulence of CA-MRSA ST93.

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