EXAMINATION OF A NEW VIRULENCE FACTOR IN A SELECT STRAIN OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS

A Dissertation

Submitted to the Graduate School of the University of Notre Dame in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

By

Trevor L. Kane

________________________________________________________________________

Shaun W. Lee, Director

Graduate Program in Biological Sciences

Notre Dame, Indiana

May 2017
EXAMINATION OF A NEW VIRULENCE FACTOR IN A SELECT STRAIN OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS

Abstract

By

Trevor L. Kane

In this dissertation I address the topic of a newly discovered virulence factor in a select strain of methicillin resistant Staphylococcus aureus (MRSA) JKD6159. S. aureus has been able to acquire resistance to a wide variety of antibiotics including methicillin and vancomycin. While this antibiotic resistance is a growing concern, S. aureus also encodes an assortment of virulence factors. In this dissertation, we examine the emergence of a streptolysin S like gene cluster (sag) gene cluster that has appeared in a select strain of MRSA. The sag (Streptolysin S-associated gene) cluster is a major virulence factor in Streptococcus pyogenes, as it is responsible for the biosynthesis of the historical hemolysin toxin known as Streptolysin S. Using bioinformatic analysis, we have identified the presence of the sag gene cluster in the MRSA strain JKD6159, a recent MRSA isolate from Australia. To examine the function of the Sag-associated gene
cluster in the MRSA JKD6159 isolate, we took several experimental approaches to gain a,
deeper understanding of the mechanistic role of this gene cluster in the JKD6159 isolate.
We generated an isogenic mutant in which one of the genes responsible for the
biosynthesis of the putative SLS-like toxin was inactivated (JKD::ΔsagB) in order to more
precisely determine the role of the presence of this gene cluster in the MRSA JKD6159
strain. Disruption of the sag gene cluster in JKD6159 did not result in significant
differences in hemolysis. Bacterial growth and biofilm formation was also not found to
be affected between wt and the ΔsagB mutant. Conditioned media from the wt and
ΔsagB strain do not result in any growth inhibition of S. pyogenes, E. coli, or S.
epidermidis. During co-culture experiments with JDK6159 and E. coli, differential
morphology of the E. coli can be observed between the wt JKD6159 and the ΔsagB
strain. Eukaryotic cell infections with wt and ΔsagB JDK6159 did not result in differential
cytotoxicity or differential cytokine expression. In vivo mice infections revealed no
significant difference in either lesion size or recovered CFUs between the wt, ΔsagB or
ΔsagB+sagB strains. Analysis of in vitro generated toxin revealed no evidence of the
hypothesized post translational modifications. 2D proteomic studies were also
undertaken to examine if the sag cluster played a role in the regulation of expressed
proteins in JKD6159. Metabolic protein differences were observed between the wt and
ΔsagB strains including an increase in carbamate kinase and threonine synthase
expression and a decrease in the catabolite control protein A and MraW in wt vs. SagB
mutants. Although we have obtained evidence 1. that E. coli during co-culture exhibits
differential morphology between wt and ΔsagB 2. that the Sag-like gene associates with
the production of different *S. aureus* proteins during *in vitro* growth, the exact role of
the Sag-like gene cluster in the MRSA strain JKD6159 remains to be determined.
CONTENTS

Figures ........................................................................................................................................ iv

Tables ......................................................................................................................................... vii

Acknowledgments .................................................................................................................... viii

Chapter 1: Introduction .............................................................................................................. 1
  1.1 Disease impact of *Staphylococcus aureus* ............................................................... 1
  1.2 Virulence factors in *Staphylococcus aureus* ............................................................... 3
    1.2.1 *agr* operon ................................................................................................. 4
    1.2.2 α-toxin ........................................................................................................ 6
    1.2.3 Phenol-soluble modulins ............................................................................ 7
    1.2.4 Protein A ....................................................................................................... 9
    1.2.5 Panton-Valentine leukocidin ....................................................................... 10
    1.2.6 Staphylococcal enterotoxins ....................................................................... 12
    1.2.7 Two-component systems ............................................................................ 13
    1.2.8 Staphyloxanthin .......................................................................................... 14
  1.3 Conclusion ...................................................................................................................... 16

Chapter 2: Materials and methods ......................................................................................... 17
  2.1 Bacterial strain information ...................................................................................... 17
  2.2 Preparation of competent bacteria ........................................................................... 17
  2.3 Genetic manipulation of *S. aureus* strain JKD6159 ............................................. 18
  2.4 Sheep blood hemolysis assay .................................................................................. 26
  2.5 Antibacterial assays ................................................................................................. 26
  2.6 Biofilm formation assay ............................................................................................ 27
  2.7 2D proteomics .......................................................................................................... 28
  2.8 Cytotoxicity and host cell signaling ......................................................................... 30
  2.9 *In vivo* mice infections .......................................................................................... 33
  2.10 Generation of *in vitro* active toxin ........................................................................ 34
    2.10.1 Cloning of genes into pET28B-MBP ............................................................. 34
    2.10.2 Expression and purification of MBP linked proteins ................................... 37
    2.10.3 *In vitro* toxin generation .......................................................................... 38
    2.10.4 Digestion and mass spectrometry of *in vitro* generated toxins ............... 38

Chapter 3: Examination of the sag cluster in *S. aureus* JKD6159 .......................................... 40
  3.1 Abstract ....................................................................................................................... 40
  3.2 Research aims ............................................................................................................. 40
  3.3 Introduction .................................................................................................................. 41
  3.4 Streptolysin associated gene cluster (sag) in a select strain of *Staphylococcus aureus* ................................................................. 42
3.5 Bioinformatic discovery of a sag-like cluster in community acquired methicillin resistant S. aureus strain JKD6159 ........................................ 45
3.6 RT-PCR and gene deletion in S. aureus strain JKD6159 ...................... 49
3.7 Functional analysis of the JKD6159 wt and ΔsagB .................................. 54
  3.7.1 Sheep blood hemolysis assay .......................................................... 54
  3.7.2 Cytotoxicity of HaCat epithelial cells ............................................. 56
  3.7.3 Antibacterial assays against Escherichia coli, Staphylococcus pyogenes, and Staphylococcus epidermidis ........................................... 59
  3.7.4 Biofilm formation by wt JKD6159 and ΔsagB JKD6159 .................. 62
  3.7.5 Eukaryotic cell signaling changes .................................................. 64
  3.7.6 In vivo mice infections with wt, ΔsagB, and ΔsagB + sagB strains .. 70
3.8 Discussion .......................................................................................... 72

Chapter 4: Proteomic studies of the sag cluster in JKD6159 ........................... 75
4.1 Abstract .......................................................................................... 75
4.2 Introduction ...................................................................................... 76
4.3 2D proteomics .................................................................................. 76
4.4 In vitro toxin reconstitution .............................................................. 83
4.5 Discussion ........................................................................................ 85

Chapter 5: Conclusions and future directions ............................................. 88
5.1 Summary of conclusions .................................................................... 88
5.2 Future directions .............................................................................. 90
5.3 Closing perspectives .......................................................................... 92

Appendix A: Full list of proteins from Kinexus antibody screen ...................... 93
Appendix B: Metabolic maps of proteins identified from 2D proteomic studies .... 96
Bibliography ............................................................................................. 107
FIGURES

Figure 1.1: MRSA prevalence worldwide. In some areas up to 90% of all *S. aureus* infections are MRSA. Adapted from Lowy et al 1998 ............................................. 2

Figure 2.1: Diagram of steps to genetically modify JKD6159 ........................................... 23

Figure 3.1: Clustalω protein alignments of SagB, SagC, and SagD of JKD6159 and *Streptococcus pyogenes* (GAS). SagB from JKD6159 is 31% identical to GAS, SagC is 21% identical, SagD is 28% identical. Identical percent determined using BLAST. ......................................................................................................... 46

Figure 3.2: Cartoon alignment of *sag* cluster as found in JKD6159 compared to *sag* cluster in *Streptococcus pyogenes*. ............................................................. 47

Figure 3.3: GC analysis of the *sag* like cluster in JKD6159 and bovine isolate RF122. USA300 TCH1516 was used as the outgroup as it lacks the *sag* cluster. GC profiles are very similar between the RD122 strain and the JKD6159 strain. Image courtesy of YunJuan Bao. ...................................................................................... 48

Figure 3.4: Reverse transcriptase PCR on *sagB*, *sagC*, and *sagD* genes in JKD6159. –RT lanes indicate non-reverse transcriptase treated controls to ensure no gDNA present. ................................................................................................................. 50

Figure 3.5: PCR confirming gene deletion of *sagB* in JKD6159. Using primers outside the *sagB* gene, wildtype JKD6159 will yield ~2500 bp amplicon while Δ*sagB* will yield ~1700 bp amplicon. ........................................................................................................ 52

Figure 3.6: Growth curve of wildtype JKD6159, Δ*sagB*, and Δ*sagB+sagB* JKD6159 in TSB broth. Representative curve of biological triplicates. .......................................... 53

Figure 3.7: Hemolysis of sheep RBC when exposed to 25% conditioned media from wt or Δ*sagB* strains of *S. aureus*. One of three biological replicates. .................. 55

Figure 3.8: Growth curve of JKD6159 wt and Δ*sagB* in cell culture media used for cytotoxicity and cell signaling assays. One of three biological replicates. ........... 57
Figure 3.9: HaCat cytotoxicity from transwell infection of wt JKD6159 and ΔsagB strains. HaCats were infected at MOI50 for 8 hours and cytotoxicity was assessed using ethidium homodimer. One of three biological replicates. ............................... 58

Figure 3.10: Inhibition of *E. coli*, *S. epidermidis*, and *S. pyogenes*. A) No significant growth inhibition was observed when 25% conditioned media from JKD6159 wt, ΔsagB or ΔsagB + sagB was tested against *E. coli*. B) No inhibition of *S. epidermidis* was observed. C) No inhibition of *S. pyogenes* observed. Representative curves of triplicate biological replicates. .............................................................................. 60

Figure 3.11: Live imaging co-culture with GFP *E. coli* and JKD6159 wt or ΔsagB strain. Select time points of *E. coli* are shown. .............................................................................. 61

Figure 3.12: Biofilm formation of the JKD6159 wt, ΔsagB, and ΔsagB+sagB strains. Biofilm formation was measured at the indicated time points as measured by crystal violet. Representative of three biological replicates. ............................... 63

Figure 3.13: Quantitation of western blots to targets identified by cytokine array. Biological triplicate infections are shown, protein levels were normalized to a Sypro stained gel, and wt is set to 100%. .............................................................................. 69

Figure 3.14: Results from the *in vivo* mice infections. A) weight loss during the course of infection (error bars omitted, no significance). B) 5 day lesion size as quantified by ImageJ. C) CFU/mL recovered from the lesion ................................................. 71

Figure 4.1: Overlay of wt and ΔsagB protein gels. wt=green, ΔsagB=red markers indicate spots that were repeatable between technical and biological duplicates. Image generated using Delta2D software. .............................................................................. 78

Figure 4.2: Image of spot 1 from wt gel (left) compared to same location on ΔsagB gel (right). This spot was consistently identified as present significantly more in the wt proteome than in the ΔsagB strain. ................................................. 80

Figure 4.3: Image of spot from ΔsagB gel (left) compared to same region on wt gel (right). This spot was consistently identified as present significantly more in the ΔsagB proteome than in the wt strain. ................................................. 81

Figure 4.4: Purified proteins linked to MBP tag. SagB-MBP=75.5 kda, SagC-MBP=78.5 kda, SagD-MBP=93 kda, SagA1-MBP=53 kda, SagA2-MBP=49kda. ................................. 84
Figure 5.1: Summary of approaches taken to elucidate the role of the $sag$-like cluster in $S.\ aureus$ JKD6159................................................................. 89

Figure B.1: Threonine synthase role in Vitamin B6 metabolism from spot wt 1. .......... 96

Figure B.2: Role of 2-oxoisovalerate E1 subunit alpha in Valine, Leucine, and Isoleucine degradation from wt spot 1................................................................. 97

Figure B.3: Role of carbamate kinase 2 in arginine biosynthesis from wt spot 1. ........ 98

Figure B.4: Role of succinate CoA ligase ADP forming alpha subunit in carbon fixation from wt spot 1......................................................................................... 99

Figure B.5: Role of thioredoxin reductase in pyrimidine metabolism from wt spot 1... 100

Figure B.6: Role of putative glucokinase ROK family in amino sugar and nucleotide sugar metabolism from wt spot 1. ................................................................. 101

Figure B.7: Role of D-isomer specific 2-hydroxyacid dehydrogenase in pyruvate metabolism from wt spot 1. ................................................................. 102

Figure B.8: Role of uroporphyrinogen decarboxylase (4.1.1.37) in porphyrin and chlorophyll metabolism from $\Delta sagB$ spot 4 ........................................ 103

Figure B.9: Role of FolD (3.5.4.9) in one carbon pool by folate from $\Delta sagB$ spot 4..... 104

Figure B.10: Role of hydroxymethylbilane synthase (2.5.1.61) in porphyrin and chlorophyll metabolism from $\Delta sagB$ spot 4. ........................................ 105

Figure B.11: Role of FAD dependent pyridine nucleotide disulphide oxidoreductase in pyrimidine metabolism from $\Delta sagB$ spot 4. ................................. 106
TABLES

Table 1.1: List of virulence factors discussed ................................................................. 15
Table 2.1: Primer sets used for gene knockouts and in vitro toxin generation .......... 24
Table 2.2: Primer sets for in vitro toxin generation ....................................................... 36
Table 3.1: Top hits from kinexus antibody array ......................................................... 65
Table 3.2: Results from cytokine array between wt and ΔsagB infections ............... 68
Table 4.1: Proteins identified from 2d proteomic experiements ............................. 82
Table A.1: list of identified proteins ............................................................................. 93
ACKNOWLEDGMENTS

Thanks to everyone who has supported me throughout this Ph.D. especially my boss Dr. Shaun Lee, and my committee members Drs. Miguel Morales, Jeff Schorey, and Josh Shrout. Thanks to all the members of the lab who aided my research. My work was supported through TA ships, a CBBI grant, and two Eck fellowships.

Most of all thanks to my family, my wife Rebecca and my son Robert for keeping me sane.
CHAPTER 1:
INTRODUCTION

1.1 Disease impact of *Staphylococcus aureus*

*Staphylococcus aureus* is a ubiquitous, opportunistic pathogen whose disease in humans can range from mild symptoms to severe, including sepsis, necrotizing fasciitis and hospital-acquired bloodstream infections. Especially relevant are antibiotic and multi-antibiotic resistant strains, termed MRSA (methicillin-resistant *S. aureus*). MRSA is a global problem, and in some areas of the world, as many as 90% of all *S. aureus* infections present as methicillin resistant (WHO, Figure 1.1). *S. aureus* was able to acquire resistance to antibiotics very early, with the first incidence of penicillin resistance evolving in 1941 [1]. This evolution has continued with resistance to methicillin emerging in 1961, and vancomycin resistance emerging in 1996 [2,3]. Resistance to methicillin/β-lactam drugs arises through two primary methods, the acquisition of the *mecA* gene which encodes a penicillin binding protein with lower affinity β-lactams, and the expression of β-lactamase, which can cleave the β-lactam ring on drugs [4,5]. The primary method of vancomycin resistance is from the acquisition of the *vanA* gene cluster from *Enterococcus sp.* [6].
Figure 1.1: MRSA prevalence worldwide. In some areas up to 90% of all S. aureus infections are MRSA. Adapted from Lowy et al 1998
MRSA comes in two designations depending on the location of acquisition, hospital acquired MRSA (HA-MRSA), or community acquired MRSA (CA-MRSA). HA-MRSA strains tend to have higher level antibiotic resistance and are resistant to more forms of antibiotics than the CA-MRSA counterparts, however CA-MRSA strains tend to be more virulent [7,8]. This increased virulence is clearly shown by the ability of CA-MRSA to cause severe infections in otherwise healthy patients, whereas HA-MRSA strains infect patients that are in the hospital for other reasons and tend to have lower immunity [9]. MRSA encodes a wide variety of virulence factors which will be discussed in the following sections, but some of the increased virulence that CA-MRSA strains exhibit could be due to specific virulence factors such as Panton-Valentine Leukocidin which have been primarily been observed in community acquired strains of MRSA [10]. Regardless of the source of the infection, *S. aureus* has become a severe threat to public health aided by the numerous virulence factors it encodes.

1.2 Virulence factors in *Staphylococcus aureus*

The following is adapted from Kane et al 2016. *Staphylococcus aureus* (*S. aureus*) is a gram positive bacterium that can colonize up to 30% of the human population[11–13]. Prior to the development of antibiotics, *S. aureus* infections caused up to 80% mortality in severe bacteremia cases [14]. While antibiotics have helped to reduce the rates of mortality dramatically, *S. aureus* has also shown a remarkable ability to acquire resistance to antibiotics, with the first isolate of penicillin resistant *S. aureus* being isolated just one year after the widespread use of penicillin [1]. *S. aureus* encodes a
wide range of virulence factors including the accessory gene regulator system (agr), α-toxin, phenol-soluble modulins (PSM), protein A (SpA), Panton-Valentine Leukocidin (PVL), staphylococcal enterotoxins (SE), two component signaling systems (TCS), and staphyloxanthin. This myriad of virulence factors and antibiotic resistance has made MRSA become classified as a serious bacterial threat by the United States Centers for Disease Control where there are over 80,000 infections with MRSA annually and nearly 11,500 deaths (CDC 2013). This dissertation describes the first identification and examination of a newly emerging virulence factor in a select strain of *S. aureus*. This virulence factor, identified through bioinformatics analysis, displays considerable homology to a potent toxin known as Streptolysin S produced by the related human pathogen *Streptococcus pyogenes*. The Streptolysin S associated gene (sag) cluster is a group on nine genes responsible for the biosynthesis of Streptolysin S. We have identified a highly similar gene cluster in the MRSA isolate JKD6159, and we have undergone several approaches to determine the role of the sag-like cluster on the overall virulence of the CA-MRSA strain JKD6159.

1.2.1 *agr* operon

The accessory gene regulator system (*agr*) operon was first discovered in 1986 by Recsei et al using a transposon insertion [15]. They observed a large decrease in alpha, beta, and delta hemolysins, but an increase in the protein A production, and they termed this gene cluster *agr* for accessory gene regulator [15]. Decreases in hemolysins alpha, beta, and delta were determined by growth on blood agar plates, while protein A
production was verified by western blot [15]. The *agr* cluster encodes two RNAs, RNAII and RNAIII [16,17]. RNAII codes for the AgrD precursor which is eventually converted to AgrD, the extracellular signaling peptide [16]. The operon also encodes AgrB, the maturation and export protein for AgrD, as well as a two-component regulatory system consisting of AgrC, a sensor histidine kinase, and AgrA, the response regulator. AgrA is activated when a quorum sensing molecule binds to AgrC. AgrA then binds to promoter regions in the *agr* operon, resulting in a positive feedback loop [16]. RNAIII serves as a transcription factor responsible for the change in gene expression [18]. One of the primary functions upon the transcription of RNAIII is the binding of RNAIII to the mRNA of the repressor of toxins (Rot) mRNA [19]. Antisense binding of the RNAIII to the mRNA of Rot results in the cleavage of Rot mRNA by RNase III as shown by northern blot [19,20]. Specific roles of *agr* during the course of infection are complicated due to varying infections, disease models, and patient populations [21]. In acute mice infection models including intracranial abscesses and necrotizing pneumonia, there is positive evidence that *agr* is important for the virulence [22–25]. In humans, there has been a link between persistent colonization in nosocomial infections with *S. aureus* and *agr* dysfunction [26–29]. Other studies have found no link between *agr* deficiency and poor clinical outcomes [30]. In a case study of 75 patients with MRSA pneumonia, increased death was associated with dysfunctional *agr*, however, these data lacked statistical significance due to low sampling [30]. Further studies are needed to establish a clear link between the *agr* operon and specific pathogenic outcomes, as current studies have shown varied conclusions, which may be the result of strain-specific differences. There
are at least four alleles of agr among the various strains of S. aureus, and each one of the groups is inhibitory to the other three agr [31–33]. Despite the differences in host pathologies implicated with agr regulation, the agr operon has been shown to be responsible for upregulating a variety of known virulence genes including SEs, hemolysins, capsular polysaccharides, and proteases [15,17]. Given the known virulence factors that agr has been shown to regulate, strategies to target agr may serve as an attractive target for antivirulence approaches. Experiments where agr has been knocked out in select S. aureus strains have been shown to exhibit decreased virulence in murine models of S. aureus pneumonia and dermonecrosis [25]. This decreased virulence was shown to coincide with reduced transcription of Panton-Valentine leukocidin and α-toxin [25]. These findings demonstrate that at least in the context of an acute infection mouse model, toxins such as hemolysins or PVL, that are regulated by agr during this type of infection, could play an important role in the progression of disease [15,25].

1.2.2 α-toxin

α-toxin is a secreted 33 kDa protein that is able to lyse cells by forming pores on the membrane of target host cells via interaction with the specific host component ADAM10 [34,35]. ADAM10 is a cellular metalloprotease responsible for a variety of functions including E-cadherin shedding and endothelial permeability [36,37]. α-toxin is repressed by ROT (Repressor of Toxins) and stimulated by the agr system [15,38].

A study by Kennedy et al was able to show that immunization to α-toxin using antisera or a non-toxic form of α-toxin (HlaH35L) resulted in a reduction of skin lesions
and dermonecrosis in a murine model of *S. aureus* infection [39]. HlaH35L was demonstrated to reduce α-toxin oligomerization as shown by liposomal-membrane binding study [40,41]. The Nagy group was able to develop a cross-reactive antibody that was capable of binding conserved conformational epitopes of α-toxin, γ-hemolysin, and Panton-Valentine leucocidin [42]. They were able to use this antibody to increase survival in murine challenges both intranasally and intravenously delivered MRSA strain USA300 [42]. Targeting multiple virulence factors using a single antibody may be a very effective and promising means of treatment with further development. Currently, there is at least one antibody targeting α-toxin in current phase two clinical trials, AstraZeneca compound MEDI4893 [42,43].

1.2.3 Phenol-soluble modulins

Phenol-soluble modulins (PSMs) comprise a family of amphipathic, α-helical peptides with diverse roles in staphylococcal pathogenesis [44]. The peptides were named for their ability to be soluble in the phenol layer rather than the aqueous layer during hot phenol extraction, a characteristic attributed to the presence of the amphipathic α-helix, and was originally isolated from the concentrated supernatant of *Staphylococcus epidermidis*. Three forms of PSMs were identified during the course of the *S. epidermidis* study: PSMα, PSMβ, and PSMγ. PSMα and PSMβ did not exhibit strong homology to known staphylococcal toxins, but PSMγ was identified as the delta toxin of *S. epidermidis* [45]. PSMs were identified in *S. aureus* using a combination of reversed-phase HPLC/electrospray mass spectrometry and N-terminal peptide
sequencing, which resulted in a total of seven PSMs: PSM\(\alpha\)-1-4, PSM\(\beta\)-1-2, and \textit{S. aureus} \(\delta\)-toxin, related to PSM\(\alpha\) [46]. PSM\(\alpha\) and PSM\(\beta\) are grouped by size and charge; \(\alpha\)-PSMs are shorter, 20-25 amino acids long and primarily have a net neutral or positive charge, while the \(\beta\)-PSMs are larger, with 43-45 amino acids, and primarily have a negative charge [44]. Unlike many of the virulence factors produced by \textit{S. aureus} which are encoded by mobile genetic elements, PSMs are encoded in the core genome and thus are present in nearly all strains of \textit{S. aureus} [44]. One exception is the recently characterized PSM-mec, encoded on the mobile genetic element (MGE) staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}). This MGE also encodes resistance to methicillin, and may contribute to horizontal gene transfer of both virulence factors and antibiotic resistance in \textit{S. aureus} strains [47]. Nevertheless, the ubiquitous nature of PSM\(\alpha\) and PSM\(\beta\) in \textit{S. aureus} strains makes them suitable potential targets for anti-virulence therapy.

In addition to different size and charge, the two general categories of PSMs have differing roles in both \textit{S. aureus} pathogenicity and initial colonization of human epithelial surfaces. PSM\(\alpha\) peptides are cytolytic, with the ability to lyse a variety of human cells, including leukocytes and erythrocytes. Interestingly, while PSM\(\alpha\)s have been show to lyse neutrophils, they can also trigger inflammatory responses through recruitment and activation of neutrophils prior to lysis [46]. This highlights the importance of stringent PSM regulation by bacteria to maintain a balance between immune evasion and immune cell recruitment. PSM\(\beta\) peptides have been implicated in the spread of biofilms but have less cytolytic activity [46,48,49]. This alternative role of
PSMs has been more thoroughly studied in *S. epidermidis*, where PSMβs were found at higher levels relative to other PSM peptides in biofilms compared to planktonic growth [50]. In *S. aureus*, all PSM classes have been implicated in both biofilm formation and detachment, including structuring of channels and dissemination from biofilms in an *in vivo* infection model [51]. With respect to overall virulence, it has been proposed that the increased virulence exhibited by CA-MRSA can be attributed at least in part to higher PSM expression levels in CA-MRSA strains compared to HA-MRSA strains [46,52].

1.2.4 Protein A

Protein A (SpA) is a cell wall anchored protein with a mass of approximately 42kDa, released during bacterial growth and encoded by the *spa* gene [53–56]. As with many *S. aureus* virulence factors, protein A is regulated by the *agr* system, and expression of protein A is specifically repressed by RNAIII. The 3′ end of RNAIII binds to the complementary portion of the 5′ end of *spa* mRNA. This binding both blocks the mRNA from translational machinery and allows for recruitment of double strand specific RNase III to degrade the mRNA [57]. Protein A is composed of two regions with clear structural and functional differences: Region X and the immunoglobulin binding domains. Region X contains two parts: the repetitive region (*Xr*) consisting of repeating octapeptide units, and the C-terminal domain (*Xc*) with a unique amino acid sequence. Region X is responsible for the cell wall attachment function of protein A [54]. The N-terminal region of protein A contains five immunoglobulin-binding domains (E, D, A, B, C) that can bind the Fc of IgG antibodies and the Fab of Variable Heavy 3 (VH3) idiotype
B-cell receptors [58–60]. In fact, protein A binds to human IgG at these five sites so effectively that it is used as a column substrate to purify antibodies [61–63]. Production of protein A contributes to S. aureus pathogenesis by evasion and suppression of host immune system components. IgG binding activity protects the pathogen from opsonophagocytic killing [64,65], and the protein acts as a superantigen against B-cells, inducing rapid activation and expansion followed by apoptotic death [66].

1.2.5 Panton-Valentine leukocidin

Panton-Valentine Leukocidin was first described by Van deVelde in 1894 when he noticed its ability to lyse leukocytes [67,68]. Later work on this toxin was done by Panton and Valentine who were able to associate the toxin with skin and tissue infections, and show that the hemolytic ability was distinct and not caused by the S. aureus leukocidin [67,69]. Panton and Valentine were able to show that these phenotypes were distinct by isolating the toxin from S. aureus, then subjecting the purified toxin to rabbit blood or leukocytes isolated from human blood [69,70]. The toxin consists of two distinct subunits that are both needed for its function, named LukS-PV and LukF-PV, with sizes of 38kDa and 32 kDa respectively [71,72]. Upon column separation of the two subunits, macrophage lysis was found to be abolished, but lytic activity was restored upon combination of the two subunit fractions [71,72]. Binding of the LukS subunit of the toxin to the neutrophil occurs first, and thus allows the binding of the LukF subunit to form the fully active toxin [73]. Binding order was determined by using $^{125}$I-labeled PVL subunits which were then incubated with PMNs for a given time,
then washed and counted in a gamma counter [73]. Binding of LukF was only observed when PMNs were pretreated with LukS [73]. LukS binds to the human complement receptors C5aR and C5L2 which are abundant on human neutrophils, and protection is conferred using the C5aR inhibitor CHIPS [74]. Specific binding was determined by allowing the proteins to bind to neutrophils, then western blotting for the C5aR receptor [74]. An octamer of four LukF and four LukS subunits then forms the open pore in the cell [75]. In low concentrations, PVL localizes to the mitochondrial membrane and induces apoptosis [76]. Upon incubation of isolated mitochondria with rPVL, release of apoptotic proteins cytochrome c and Smac/DIABLO can be observed via western blot [76]. Genes encoding PVL are nearly universally found in strains of CA-MRSA, and thus there is a strong link between severe infection outcomes and the PVL genes in S. aureus strains [67,77].

Despite this close correlation between severe disease outcomes and the presence of PVL genes, establishing the role that PVL plays during the course of infection remains controversial, with some studies stressing the importance of PVL, and others refuting the notion that PVL is important [78–80]. Some of the dispute regarding the importance of PVL is likely due to the differing susceptibilities of various eukaryotic cell types to PVL toxin. PVL is active against human leukocytes and rabbit leukocytes [81,82] but not against mouse leukocytes [74,81,83].
1.2.6 Staphylococcal enterotoxins

Staphylococcal enterotoxins (SE) are also dubbed ‘superantigens’ for their ability to bind to human T-cells and induce hyperstimulation [84]. SEs specifically bind to MHC class II T-cell receptors [85,86]. X-ray crystallography was used to image T-cell receptors coupled to SE and confirm its direct binding mechanism [85]. Staphylococcal enterotoxins are the primary cause of staphylococcal food poisoning [87,88]. SEs are very robust and are resistant to heating, and proteolysis [89,90]. Biological activity of SE has been shown to be recalcitrant to heating to 60°C for up to 16 hours, or to digestion with trypsin, chymotrypsin, rennin, or papain [89]. Although SEs are a member of the streptococcal superantigen family, only toxins that cause emesis in primate models are formally designated SEs [91]. Currently there are twenty-one known SEs, designated SE-A to SE-U [90,92,93]. Due to the expense of testing emesis in primates and the limited number of labs capable of testing in primates, toxins similar to SEs but unable to be validated in primate models are designated as staphylococcal enterotoxin-like (SEls) [87,91]. As little as 10 µg of SE-B administered intragastrically has been shown to be sufficient to cause disease symptoms in primate models [94]. There are limited data regarding human exposure to various purities of SEB as a result of lab accidents, and symptoms such as ocular swelling and discharge can be observed upon exposure to as little as 50 µg of toxin [95]. Due to the small quantity of protein needed for symptoms, the ease of spread, and the robust nature of SEs, they are listed as a Category B select agent by the CDC (CDC, [90]. Strains of *S. aureus* produce vastly different amounts of SE toxin, ranging from more than 1 µg/mL culture to less than 10 ng/mL [96]. SEs do not
appear to be fully regulated using the agr system, and higher production of SE toxins appears to be linked with prophage induction [96,97]. Transposon insertions in the agr allele was not found to affect overall levels of SE in S. aureus strains [97]. In strains of S. aureus that naturally produce high levels of SE-A (>1000 ng/mL measured via ELISA), induction of prophages using mitomycin C resulted in further increases of up to 10 fold [96].

1.2.7 Two-component systems

Bacterial two-component systems (TCS) are one of the primary mechanisms bacteria use to sense their environment [98,99]. As the name suggests, TCSs consist of two proteins, a sensor kinase which responds to the signal and a response regulator that binds to DNA [98,99]. In S. aureus, these TCS can affect the virulence of the bacterium. agr is an important regulator of virulence and is also a TCS consisting of the response regulator AgrA and the kinase AgrC. The SaeR/S TCS in essential to the evasion of the innate immune system by reducing reactive oxygen species, aiding survival in the face of host defense peptides, reducing expression of IL-8, and increasing the expression of leukocidins [100–104]. SaeRS can also regulate the expression of virulence genes including hla [105].
1.2.8 Staphyloxanthin

Staphyloxanthin is the pigment that *S. aureus* produces that is primarily responsible for the characteristic golden color that some strains of *S. aureus* exhibit [106]. This compound was originally isolated from *S. aureus* by Marshall and Wilmoth in 1981. Using a warm methanol extraction method followed by chromatography separation, they were able to obtain four fractions, and fraction three contained the staphyloxanthin [106]. Subsequent work using gene deletions in the staphyloxanthin biosynthesis pathway confirmed that staphyloxanthin functions as a virulence factor by serving as an antioxidant protecting the bacterium from neutrophil oxidative burst [107,108].
**TABLE 1.1:**

**LIST OF VIRULENCE FACTORS DISCUSSED**

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>agr</strong></td>
<td><em>agr</em> is a quorum sensing system that is dependent on cell density. AgrD binds to AgrC, which results in activation of AgrA the transcription factor. AgrA is then able to increase transcription of SE, hemolysins, proteases, and capsular polysaccharides.</td>
</tr>
<tr>
<td><strong>α-toxin</strong></td>
<td>Binds to ADAM10 on eukaryotic cells and oligomerizes, forming pores and lysing the cell.</td>
</tr>
<tr>
<td><strong>Phenol Soluble Modulins (PSM)</strong></td>
<td>Family of 7 α helical peptides, split into PSMα and PSMβ. PSMα are able to lyse a variety of cells including leukocytes and erythrocytes. PSMβ are implicated in the spread of biofilms.</td>
</tr>
<tr>
<td><strong>Protein A</strong></td>
<td>Cell wall anchored protein that binds to the FC region of IgG antibodies and to the Fab of Variable Heavy 3 (VH3) idiotype B-cell receptors.</td>
</tr>
<tr>
<td><strong>Panton-Valentine Leukocidin (PVL)</strong></td>
<td>PVL consists of two subunits, LukS and LukF. LukS binds to human complement receptors C5aR and C5L2 and allows docking of LukF. The Luk subunits oligomerizes and form a pore lysing the cell.</td>
</tr>
<tr>
<td><strong>Staphylococcal Enterotoxins (SE)</strong></td>
<td>There are 21 known SEs. SEs bind MHC class II receptors on T-cells and hyperstimulate the T-cells.</td>
</tr>
<tr>
<td><strong>Two-Component System (TCS)</strong></td>
<td>Consist of a sensor kinase which senses the environmental signal and the response regulator which is activated and is able to bind DNA to affect transcription.</td>
</tr>
<tr>
<td><strong>Staphyloxanthin</strong></td>
<td>Pigment responsible for some strains of <em>S. aureus</em> golden color. Protects the bacterium from oxidative stress.</td>
</tr>
</tbody>
</table>
1.3 Conclusion

*Staphylococcus aureus* is able to encode a vast range of virulence factors in addition to its ability to acquire antibiotic resistance. While the mechanisms of virulence vary, from killing immune cells to lysing red blood cells to blocking antibodies, the end result is a bacterium that encodes a variety of proteins that are able to damage the host. One of the virulence factors, Panton-Valentine Leukocidin is primarily found in CA-MRSA strains. As discussed earlier, there is some evidence that the presence of these genes is at least partially responsible for the increased virulence that is characteristic of CA-MRSA strains. To date the *sag* like cluster has only appeared in a CA-MRSA strain and a bovine isolate of *S. aureus* (Kane, T. unpublished). This dissertation describes approaches aimed at identifying the role that a potential new virulence factor with homology to Streptolysin S plays in a highly virulent CA-MRSA strain, JKD6159. As the function of this gene cluster in *S. aureus* is unknown, we examined a wide range of potential phenotypes including, hemolysis, antibacterial assays, biofilm formation, 2D proteomics, *in vitro* toxin generation, cytotoxicity, host cell signaling changes, and *in vivo* mice infections. This range of experiments will help us to elucidate the role of the *sag* cluster in *S. aureus* JKD6159.
2.1 Bacterial strain information

*S. aureus* strain JKD6159 was the generous gift of Dr. Timothy Stinear. Vector pIMAY and pCL55 was generous gift of Dr. Ian Monk and Dr. Taeok Bae. *E. coli* strain IM93B was generous gift of Dr. Ian Monk and BCCM. Unless otherwise mentioned all strains were grown at 37°C.

2.2 Preparation of competent bacteria

*E. coli* strain IM93B was prepared according to protocols from [109,110]. Summary of method follows.

IM93B was grown in LB (Fisher Scientific) broth overnight shaking at 225 RPM, and diluted the following morning 1:1000 in fresh LB media. The culture was allowed to grow shaking until an OD550nm was reached of 0.7 as measured on Eppendorf BioSpectrometer Basic. Bacteria were then centrifuged at 4°C at 3500 RPM in an Eppendorf 5810R centrifuge. The pellet was resuspended in 45 mL ice-cold 10% glycerol (Fisher Scientific) water, centrifuged as above, and resuspended again in 45 mL ice-cold 10% glycerol water. Cells were then resuspended in 10 mL ice-cold glycerol water, centrifuged, and resuspended a final time in 2 mL/L starting volume ice-cold 10%
glycerol water. Cells were separated into 100 µL aliquots, and snap frozen in an ethanol dry ice bath, and stored at -80°C.

JKD6159 was made competent using a protocol courtesy of Dr. Taeok Bae’s laboratory. 3 mL overnight culture in tryptic soy broth (TSB, Acumedia) was diluted in 300 mL TSB. Cultures were allowed to grow until an OD600 nm of 0.5 was reached according to Eppendorf BioSpectrometer Basic. JKD6159 was then centrifuged 4°C at 3500 RPM on an Eppendorf 5810R centrifuge. The bacteria were washed once using 60 mL ice-cold 0.5 M sucrose (Fisher Scientific) water, then washed three more times in 9 mL 0.5M ice-cold sucrose. Cells were then resuspended in 3 mL 0.5M ice-cold sucrose, aliquoted into 100 µL, and snap frozen in an ethanol dry ice bath and stored at -80°C.

2.3 Genetic manipulation of S. aureus strain JKD6159

Isogenic gene deletions were obtained following the protocols in [109] and [111], and is described below. ~800 bp regions of the chromosome upstream and downstream of the sagB gene to be deleted was amplified using sagB KO downF, sagB KO downR, sagB KO upF, and sagB KO upR as found in Table 2.1. PCR reactions were visualized on a 1% agarose gel (Sigma-Aldrich) containing a 1:10,000 dilution of GelRed (Biotium). pIMAY and the upstream region were digested with EcoR1 and Not1 (New England BioLabs) according to manufacturer instructions. Digested DNA was then purified using QIAquick PCR purification kit from Qiagen according to manufacturer instructions. The digested upstream region and pIMAY were ligated together using T4 DNA Ligase (New England BioLabs) according to manufacturer instructions. 1 µL ligation
reaction was then transformed into *E. coli* strain One Shot ®Top10 (Invitrogen) according to manufacturer instructions and plated on LB containing 10 µg chloramphenicol (Cm, Sigma-Aldrich) for selection. *E. coli* clones able to grow on Cm 10 were then PCR amplified for the presence of the upstream region using primer set T7F and T7term.

Phusion High-Fidelity DNA polymerase from New England BioLabs was used according to manufacturer instructions and cycled at 98°C 2 min initial denature, 96°C 30s repeat denature, 55°C 30s primer anneal, 72°C 30s extension time, 10°C hold. Denature, anneal, and extension cycles were repeated 30 times total. This was the standard PCR conditions used throughout the procedure unless otherwise noted. Clones that contained the insert as shown on an agarose gel stained with GelRed (Biotium) were then grown up in 10 mL LB (Fisher Scientific) Cm 10 and subjected to Zippy plasmid Miniprep Kit (Zymo Research) according to manufacturer recommendations. Purified plasmid was then quantified on a NanoDrop 2000 (Thermo Scientific). 300 ng of purified plasmid was then sequenced using T7F and T7term on an Applied Biosystems 96-capillary 3730xl DNA Analyzer. Sequencing results were the analyzed using Sequencher (Gene Codes). Plasmids containing the correct insert were then digested along with the downstream region using Kpn1 and EcoR1 (New England BioLabs). Digested DNA was then purified with QIAquick kit (Qiagen) according to manufacturer recommendations. DNA was then ligated using T4 DNA ligase (New England BioLabs) and 1 µL ligation reaction was transformed into *E. coli* strain One Shot ®Top10 (Invitrogen). Clones were selected for on LB (Fisher Scientific) Cm 10 µg agar plates, and individual colonies were checked with PCR for bands corresponding to ~1600 base pairs indicating that the
vector contained both the upstream and downstream region. PCR was repeated as above with the only change being the extension time was increased to 1 minute from 30 seconds. Positive clones were grown up; miniprep kit was performed to isolate plasmids, and sequenced as above. Plasmids that contained the correct upstream and downstream region as shown by Sanger sequencing was then transformed into *E. coli* strain IM93B. Competent IM93B was thawed on ice, and then added to chilled 0.1 cm electroporation cuvettes (BioRad). 100 ng of pIMAY containing the upstream and downstream regions to *sagB* was added to the competent IM93B and electroporation was carried out at 100 Ω, 1.8 kV, 25 μF using a BioRad Gene Pulser Xcell. 500 μL SOC media (Invitrogen) was added, and the bacteria were allowed to recover shaking at 37°C for 45 minutes. Cells were then plated on LB Cm 10 µg and allowed to grow overnight. Colonies were checked for presence of pIMAY with correct inserts using PCR, miniprep, and sequencing as above to ensure that no additional mutations had occurred. Clones containing the correct insert were then grown up and plasmid isolated using a Qiagen QIA Filter Plasmid Maxi kit according to instructions. Competent JKD6159 was thawed on ice for 15 minutes then spun at 8000 g for 3 minutes in an Eppendorf 5415R centrifuge. Bacteria were then resuspended in 100 μL 10% glycerol containing 0.5M sucrose (Fisher Scientific). 2 μg plasmid passed from IM93B was added to the competent JKD6159. Plasmids were introduced into the JKD6159 bacteria via electroporation in a 0.1 cm electroporation cuvette (BioRad) at 100 Ω, 2.5 kV, 25 μF. After electroporation the JKD6159 was allowed to recover in 1 mL Brain Heart Infusion (BHI, Fluka) for one hour at 37°C. Cells were centrifuged at 8000 g for 3 minutes using the Eppendorf 5415R
and resuspended in 100 µL BHI and plated on BHI Cm 10 µg/mL agar plates and incubated at 28°C for 2 days. Colonies that grew under selective pressure at 28°C were homogenized in 200 µL TSB, diluted 100 fold, and 100 µL were plated on BHI Cm 10 µg/mL at 37°C to force the first integration of the pIMAY plasmid. Colonies that grew were grown in 1 mL BHI Cm 10 µg/mL and genome DNA recovered using MasterPure™ Gram Positive DNA Purification Kit from Epicenter. Integration of the plasmid was verified using pIMAYF and BR KO chromosome. Clones that successfully integrated the vector were grown overnight in BHI at 28°C. Dilutions of 10^-6 and 10^-7 were plated on BHI agar containing 1 µg/mL anhydrotetracycline (ATC, Sigma-Aldrich) at 28°C for 24-48 hours. Large colonies were patched onto both BHI ATC 1µg/mL and BHI Cm 10 µg/mL and allowed to grow overnight at 37°C. Colonies that were able to grow on BHI ATC but not on BHI Cm were selected as potential positive candidates for the gene deletion. Cm sensitive colonies were grown in BHI overnight at 37°C overnight and genome DNA purified using the MasterPure™ Gram Positive DNA Purification Kit. Chromosomal primers BF KO chromosome and BR KO chromosome were used to verify the loss of the sagB gene. PCR for the verification of gene deletion is the same as above however the extension time was increased to two minutes. 50 mL of overnight culture of the ΔsagB were grown in BHI, centrifuged at 2500 g for 10 minutes, resuspended in 1 mL BHI with 30% glycerol (Fisher Scientific) and stored at -80°C. Summary of the steps to successfully genetically manipulate JKD6159 found in Figure 2.1.

ΔsagB was complemented using the pCL55 plasmid [112]. sagB was amplified from wt genomic DNA using the sagB compF and sagB compR primer set, digested with
BamH1 and Kpn1, and ligated as before into pCL55. The vector was then transformed into IM93Bs, selected with ampicillin 25 µg/mL, and colonies were screened for the \textit{sagB} insert using pCL55 insertF and pCL insertR primers. Colonies containing the correct size band were grown up and subject to a miniprep kit to isolate the plasmid, and sequenced using the pCL55 sequence F and pCL55 sequence R primers in addition to the pCL55 insertF and pCL55R primers. The correct plasmid was then inserted into competent Δ\textit{sagB} JKD6159 and allowed to grow under selection of chloramphenicol 5 µg/mL at 37°C for 2 days until colonies appeared. Individual colonies were genome DNA isolated as above and PCR amplified using the \textit{sagB} compF and \textit{sagB} compR primer set. The PCR product was then purified using QIAquick PCR Purification Kit according to manufacturer instructions, and submitted to sequencing using the same primer pair to ensure no mutations in \textit{sagB} had occurred.
Figure 2.1: Diagram of steps to genetically modify JKD6159

1. Transform into IM93B *E. coli* and reisolate
2. pIMAY now has correct methylation pattern
3. Transform into JKD6159

---

JKD genomic DNA

28°C, CM 10

---

37°C CM10

---

Integrated pIMAY
### TABLE 2.1:

**PRIMER SETS USED FOR GENE KNOCKOUTS AND IN VITRO TOXIN GENERATION**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Restriction Enzyme Site (Red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sagB KO downF</td>
<td>ATA <strong>GGTACC</strong> TGA GAC GCT GAA GCG ATT AAA ATA TT</td>
<td>Kpn1</td>
</tr>
<tr>
<td>sagB KO downR</td>
<td>ATA <strong>GAATTC</strong> TCA TTG CTT TTT ACC TCT TTT GTT AGT</td>
<td>EcoR1</td>
</tr>
<tr>
<td>sagB KO upF</td>
<td>ATA <strong>GAATTC</strong> TTG AGC AAT GAA AAC GTT TAT TAA ACC</td>
<td>EcoR1</td>
</tr>
<tr>
<td>sagB KO upR</td>
<td>ATA <strong>GCGGCCGC</strong> ATG TGG GTA AAT AAA AAT GAA GTA CAT TCC</td>
<td>Not1</td>
</tr>
<tr>
<td>BF KO chromosome</td>
<td>CCA ATT GCT TTT ATT AGT TTT CCA ATT TG</td>
<td></td>
</tr>
<tr>
<td>BR KO chromosome</td>
<td>ACA TGT CGA ACA AGT AGA CTG TC</td>
<td></td>
</tr>
<tr>
<td>sagBF</td>
<td>GCC <strong>GGATCC</strong> ATG ATT AAT TAT ACA GTT G</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagBR</td>
<td>ATA <strong>GCGGCCGCG</strong> GTT TTC ATT</td>
<td>Not1</td>
</tr>
<tr>
<td>sagCF</td>
<td>GGT <strong>GGATCC</strong> ATG AAG TTT ATT AAA C</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagCR</td>
<td>TAT <strong>GCGGCCGCG</strong> TTA AAG GTT ATC</td>
<td>Not1</td>
</tr>
<tr>
<td>sagDF</td>
<td>ATA <strong>GCGGCCGCG</strong> ATG AAT TAT C</td>
<td>Not1</td>
</tr>
<tr>
<td>sagDR</td>
<td>ATAT <strong>GGATCC</strong> CTA TGC CAA AGG ATG</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagA1F</td>
<td>GAG <strong>GGATCC</strong> ATG AGG AGA ATA ATG ATG TTG AAG AGA</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagA1R</td>
<td>ACA <strong>GCGGCCGCG</strong> TCA TTG CTT TTT ACC TCT TTT</td>
<td>Not1</td>
</tr>
<tr>
<td>sagA2F</td>
<td>GAG <strong>GGATCC</strong> ATG GAT ATT CAG ACA TTA GTA GTT C</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagA2R</td>
<td>ACA <strong>GCGGCCGCG</strong> TCA CCT ATC AAA TTT GGA AAA</td>
<td>Not1</td>
</tr>
<tr>
<td>T7F</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
<td></td>
</tr>
<tr>
<td>T7term</td>
<td>GCT AGT TAT TGC TCA GCG G</td>
<td></td>
</tr>
<tr>
<td>pIMAYF</td>
<td>GCT TTG GCA GTT TAT TCT TGA CAT GTA</td>
<td></td>
</tr>
<tr>
<td>sagB comp F</td>
<td>ATA <strong>GGATCC</strong> GAA TTG CTT GTA GCA ACT AAC AAA AGA</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagB comp R</td>
<td>ATA <strong>GGTACC</strong> CTA TTC CTT CCC ACA AAT ATT CGT</td>
<td>Kpn1</td>
</tr>
<tr>
<td>pCL55 insertF</td>
<td>CTT ATT TTT AAA TTT TTC AAA CCA CAT TTT</td>
<td></td>
</tr>
<tr>
<td>Primer Name</td>
<td>Primer Sequence</td>
<td>Restriction Enzyme Site (Red)</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>pCL55 insertR</td>
<td>CTT TCG TCT TCA AGA ATT CGA GC</td>
<td></td>
</tr>
<tr>
<td>pCL55 sequence F</td>
<td>GAC AAC ACT TAC ACG TTT CCA TTT</td>
<td></td>
</tr>
<tr>
<td>pCL55 sequence R</td>
<td>CCA CCT GAC GTC TAA GAA ACC</td>
<td></td>
</tr>
</tbody>
</table>
2.4 Sheep blood hemolysis assay

1 mL defibrinated sheep blood (ThermoFisher Scientific) was washed in 40 mL ice cold PBS to remove the free heme from the lysed red blood cells (RBC). RBCs were then centrifuged at 4°C 1200 RPM for 10 minutes in Eppendorf 5810R centrifuge. RBCs were determined to be free of lysed cells when the supernatant was mostly clear, usually two wash cycles. Intact RBCs were resuspended in ice cold PBS 40 mL, and aliquoted into 1.5 mL snap top tubes. Sterile conditioned media was generated from the wt and ΔsagB JKD6159 strains by growing the bacteria overnight in TSB media, centrifuging the bacteria at 3500 RPM in an Eppendorf 5810R centrifuge and sterile filtering the supernatant using Millipore 0.22 μM luer lok syringe filters. This conditioned media was then added to the washed RBCs to a concentration of 25%. The tubes were then incubated at 37°C for the designated time points. To read hemolysis, the tubes were centrifuged at 1200 RPM in an Eppendorf 5415R centrifuge for 10 minutes. 100 μL of the supernatant was transferred in Eppendorf UVette cuvettes before being read at O.D. 450 nm on an Eppendorf Biospectrometer Basic. Hemolytic potential was calculated by normalizing the lysis to 0.01% Trition X-100 positive control and TSB negative control. Experiments were conducted in triplicate.

2.5 Antibacterial assays

Overnight cultures of *E. coli, S. pyogenes* and *S. epidermidis* in LB media (Fisher BioReagents) were diluted in LB (*E. coli*, Fisher Scientific), TH (*S. pyogenes*, Acumedia), or Nutrient Broth (*S. epidermidis*, Sigma) to an OD$_{600}$ of ~0.1. Conditioned media from the
JKD6159 wt and ΔsagB strains were prepared by overnight growth in TSB media followed by centrifugation at 3500 RPM and sterile filtration through Millipore 0.22 µm luer lok syringe filter. Conditioned media was added to the diluted overnight cultures of *S. epidermidis*, *S. pyogenes* and *E. coli* at 25% total volume ratio in Eppendorf 96 well plates. The plates were read on a Synergy H1 microplate reader by BioTek. The plate reader was heated to 37°C, set to continual shaking, and read OD600 every 10 minutes for 16-24 hours. Experiments were conducted in biological and technical triplicate.

Co-culture experiments were also carried out using GFP *E. coli* pND1308 (pCR-Fcr/GFP) TOP10 (Gift of Zhong Liang). Experiments were carried out by Claudia Park. *E. coli* and JKD6159 were grown overnight before being diluted to OD600 0.1 (*E. coli*). Wt and ΔsagB JKD6159 was diluted 1000 fold less than *E. coli* before being added to glass bottom petri dish (MatTek) containing 1 mL of 1:1 LB:TSB. Live imaging was acquired on Nikon Eclipse Ti-E Inverted Microscope on a 60X oil immersion NA1.40 objective. Images were captured using EMCCD camera (Andor Ixon Ultra 88 ECCD Oxford Instruments) with a 10 ms exposure time. Petri dish was placed in an environmental chamber at 37°C for 12 hours and imaged continuously for 12 hours in DIC and FITC channels.

2.6 Biofilm formation assay

Overnight cultures of wt, ΔsagB, and ΔsagB+sagB were incubated in TSB. Cultures were then diluted 1:100 in TSB in Eppendorf 24 well plates and placed in a humidified 37°C, 5% CO₂ incubator for 24, 48, or 72 hours. Following the time point, wells were washed 2x H₂O, and 1 mL crystal violet (Remel) was added and incubated a
room temperature for 10 minutes. Crystal violet was then aspirated, and wells were washed 2x H$_2$O. Wells were allowed to air dry, and remained at room temperature until solubilization with 33% acetic acid in water (Sigma) and reading at OD570 on a Synergy H1 microplate reader by BioTek. Experiments were conducted in technical triplicate and biological triplicate.

2.7 2D proteomics

The procedure below was adapted from [113] and [114]. wt JKD6159 and $\Delta$sagB JKD6159 were grown in 10 mL TSB at 37°C for 48 hours. Bacteria were centrifuged at 4°C at 2500 g for 10 minutes. The pellet was then washed twice in 500 µL PBS containing SigmaFast protease inhibitor tablet (Sigma Aldrich). Cells were resuspended in 500 µL PBS with protease inhibitor and added to ~200 µL 0.1mm glass beads (Biospec) before being placed in Mini Bead Beater (Biospec) for 4 cycles of 30 seconds each, with one minute on ice between each cycle. Upon conclusion of mechanical disruption, 500 µL of 2D protein Extraction Buffer V (GE Healthcare), 6 µL Destreak reagent (GE Healthcare), and 2.5 µL IPG buffer 3-10 pH NL (GE Healthcare) was added to samples, and samples were allowed to incubate at room temperature for one hour with vortexing every 10 minutes for 10 seconds. Lysates were then centrifuged at 4°C at 16,000 g for 30 minutes. Supernatants were then centrifuged at 50,000 g for 2x1 hour to remove any additional insoluble material. Supernatant containing the soluble proteins was quantified using Comassie Plus Assay Kit (Thermo Scientific) according to manufacturer instructions. 300 µg total protein was prepared for 2D proteomics using the 2D Clean Up
Kit (GE Healthcare) according to manufacture instructions. Proteins were then resuspended in 250 µL Destreak Rehydration Solution (GE Healthcare), 6 µL of DTT 1M, and 6 µL of IPG 3-10 pH NL (GE Healthcare). Samples were vortexed for 15 minutes, and any remaining insoluble material was removed via centrifugation at 16000 g for 1 minute. Samples were then allowed to adsorb into Immobiline DryStrip 3-10 pH NL 13 cm (GE Healthcare) overnight. The first dimension separation was achieved using an Ettan IPGphore 3 (GE Healthcare) following suggested protocols. Prior to second dimension separation, the strips were equilibrated by incubation at room temperature in 5 mL SDS equilibration buffer (50mM Tris-HCl, 6M urea, 30% glycerol, 2% SDS, 0.01% bromophenol blue) supplemented with 650 µL of 1M DTT for 15 minutes, followed by 5 mL SDS equilibration buffer supplemented with 1350 µL of 1M iodoacetamide. Second dimension separation was made on a 12.5% polyacrylamide gel using the SE600 Ruby system (GE Healthcare). Mark 12 unstained standard was used for molecular weight estimations. The DryStrip was covered with sealing agarose (Bio-Rad). The gel was then run at 600 V, 30 mA, 100 W for 15 mins, then 600 V, 60 mA, 100 W for 4-6 hours. mA was set as the limit for the power supply. Gels were then stained using Sypro Ruby according to manufacturer instructions and imaged using a Typhoon imager FLA9500 (GE Healthcare). Data was analyzed using Delta 2D (Decodon). Spots that were determined to be significantly different were manually excised and subjected to mass spectrometry.

Gel spots were excised from the gel, destained in 25mM ammonium bicarbonate (ABC) in 50% water 50% acetonitrile. The spots were then placed in an Eppendorf Speed
Vac at 35°C until spots were completely dried. 25 µL of 10 mM DTT in 25 mM ABC was added to dried spots and incubated at 56°C for 1 hour. The supernatant was then removed and spots were washed 2x by adding 100 µL 25 mM ABC and vortexed for 10 minutes at room temperature. Gels were dehydrated using 25 mM ABC in 50% acetonitrile 50% water and vortexed for 5 minutes. Solution was then removed and new 25 mM ABC in 50% acetonitrile 50% water was added again and vortexed an additional 5 minutes. Gel spots were again placed in an Eppendorf Speed Vac at 35°C until completely dried. 500 ng of Trypsin Gold (Promega) in 25mM ABC was added to gel spots and allowed to rehydrate on ice for 30 minutes. If needed, 25 mM ABC was added to cover the gel spots after the rehydration. Trypsin was allowed to digest at 37°C overnight. Supernatant was reserved and the spot was covered in extraction buffer (45% water, 50% acetonitrile, 5% formic acid (Fisher Optima)) and vortexed for 30 minutes. Extraction of peptides from spot was repeated once and all supernatants were pooled and concentrated using a Speed Vac to 10µL total volume. Peptides were then run on a Thermo Fisher Q-Exactive mass spectrometer. The 2D proteomic studies were carried out biological and technical duplicate.

2.8 Cytotoxicity and host cell signaling

HaCat keratinocyte cells were used for all cytotoxicity and cell signaling assays. Unless otherwise indicated, cells were kept in a humidified, 37°C, 5% CO₂ environment in DMEM containing 10% fetal bovine serum (Biowest) in T75 flasks. In cytotoxicity assays, HaCat cells were grown in DMEM (Gibco) lacking FBS in 24 well plates. HaCats
were allowed to grow to 80-90% confluency. Prior to infection, cells were washed twice with sterile PBS (Gibco) and then fresh DMEM (Gibco) media was added to the wells. wt and ΔsagB JKD6159 bacteria were added at an MOI of 50 bacteria/1 host cell in a Millipore Transwell 0.4 µm. Cells and bacteria were allowed to incubate for 8 hours at 37°C, 5% CO₂. Upon completion of the time points, the transwells were removed, and HaCats rinsed with PBS. Ethidium homodimer (Sigma Aldrich) at a concentration of 4 µM was added, and the cells were allowed to incubate at room temperature in the dark for 30 minutes. The plate was then read on a BioTek Synergy H1 at excitation 528 nm, and emission 617 nm to obtain a treated level of fluorescence. Saponin (Sigma) was added at a concentration of 0.1% w/v and allowed to rock at room temperature in the dark for 20 minutes before being read as above. The total fluorescence from the saponin treatment was then divided by the initial treated fluorescence to obtain percent cytotoxicity.

Cell signaling data was obtained by using a Kinexus antibody array. HaCats were treated as above for the cytotoxicity assay, but upon completion of the 8 hour time point, cells were washed twice in ice cold PBS, before being lysed using Kinexus lysis buffer plus protease inhibitor. 8 wells of the 24 well plate were lysed for each of the wt and ΔsagB conditions. The cells were sonicated for 4x10 seconds with 30 seconds on ice in between each pulse. The lysates were then spun at 16,000 g at 4°C for 30 minutes to remove cell debris. Lysates were then stored at -80°C until they could be sent to Kinexus for antibody array analysis.
Cytokine antibody array analysis was also performed using the Abcam Human Cytokine array kit with 80 targets. Prior to transwell infection as outlined above, the HaCat cells were serum starved for 16 hours before being infected by the wt and ΔsagB mutant strain for eight hours. Infections were carried out in triplicate wells and 400 µL from each well was collected and pooled before being frozen at -20°C until the array analysis was performed. The antibody array was performed as instructed by the manufacturer, with the membranes being incubated overnight at 4°C with the supernatants collected from the HaCats, and overnight incubation at 4°C with the primary antibodies. Membranes were imaged using an Azure Biosystems c600 imager, and images were then quantified using ImageJ.

Cytokine hits IL-10, I-309, osteopontin, and MIP-1δ were verified via western blots. IL-10 antibody was from Abcam number ab34843, osteopontin was Abcam number ab8448, MIP-1δ was R&D systems AF628, and I-309 was from Abcam ab109788. Supernatants were collected as done for the cytokine array, and all of the gels that were probed using antibodies were compared to a control gel that was stained with Sypro Ruby (Invitrogen). The Sypro stained gel served as a loading control for normalization of the western blots. Proteins were transferred to a nitrocellulose membrane at 20 V for 2 hours followed by 70 v for an additional hour. The membrane was blocked using 10% milk in TBST rocking at room temperature for 1 hour. The membrane was then incubated with the primary antibody according to manufacturer recommended dilutions at 4°C rocking overnight. Following the primary antibody incubation, the membrane was washed in TBST every 5 minutes for 45 minutes. For the IL-10, osteopontin, and I-
309 anti-rabbit secondary antibody at 1:5000 dilution (Santa Cruz Biotechnology sc-2357) was used. For MIP-16, anti-goat HRP 1:5000 dilution (Santa Cruz Biotechnology sc-2354) was used. In all cases, the secondary antibody was in 10% milk TBST and was allowed to rock at room temperature for 1 hour. Membranes were then washed for 1 hour with TBST changed every 5 minutes. ECL chemiluminescence reagent (KPL) was then added to membrane according to manufacturer recommendations and imaged on Azure Biosystems c600. Following imaging, the western blots were normalized to the Sypro gel to account for any protein loading differences, and quantified in ImageJ.

2.9 In vivo mice infections

Mice infections were based off of Chua et al 2011 [115], conducted with the generous assistance of Dr. WonSik Yeo and Dr. Taeok Bae at Indiana University School of Medicine Gary, IN, and a summary of the experiment follows. 5 week old female Balb/Cj mice were obtained from the Jackson Laboratory. When the mice had aged to 6 weeks the right flank hair was removed, sprayed with 70% ethanol, and $10^8$ CFU of wt, ΔsagB, ΔsagB + sagB or PBS in 50 µL sterile PBS were injected into 11 mice/treatment condition. Mice were monitored for 5 days post infection and lesion size was measured by taking pictures with the lesion next to the lesion and later quantified in ImageJ (NIH). Mice were also weighed daily. During the course of infection, 2 mice from wt, 1 mouse from ΔsagB, and 3 mice from ΔsagB + sagB infection groups died. Following the 5 day time course mice were sacrificed and the lesion was excised to allow for enumeration of CFUs. 2 outlier mice from the ΔsagB + sagB group are omitted from data presented.
2.10 Generation of in-vitro active toxin

Generation of the active toxin took place in several steps detailed below.

2.10.1 Cloning of genes into pET28B-MBP

Protocol was derived from previous studies [116,117]. In order to create the active toxin in vitro, the sagA, sagB, sagC, and sagD genes were cloned from JKD6159 genomic DNA (Epicenter MasterPure Gram Positive Genomic DNA kit according to manufacturer instructions). Primer sets for the appropriate genes are listed in Table 2.2. Following amplification, the size of the PCR product was verified on a 1% agarose gel (Sigma), and digested with Bam H1 and Not1 restriction enzymes (New England Biolabs). pET28B-MBP was also digested with the same enzymes. Vector and insert were then ligated using T4 DNA ligase (New England Biolabs) according to protocol. Following overnight ligation, 1 µL of ligation reaction was transformed into Top10 E. coli (Thermo Scientific) according to manufacturer protocol, and plated on LB plates containing 30 µg/mL kanamycin. Colonies were allowed to grow overnight and subjected to colony PCR to verify correct integration of the gene insert. Colonies containing the insert were grown overnight in 10 mL LB containing 30 µg/mL kanamycin. The following morning, plasmid DNA was collected using a Zippy miniprep kit according to instructions. Plasmids were then subjected to Sanger DNA sequencing using the appropriate primers listed in Table 2.2 and M13F and M13R. Sequencing results were then analyzed using Sequencher software by Genecodes. Vectors that did not contain any mutations were then transformed into BL21 E. coli optimized for protein production (Thermo Scientific).
and plated on LB plates containing 30 µg/mL kanamycin. Colonies that grew were again
tested for insert gene DNA using PCR, and positive colonies were grown overnight in 10
mL LB containing 30 µg/mL kanamycin. Miniprep, sequencing, and analysis were
performed as above. Clones containing the correct $sagA$, $sagB$, $sagC$, and $sagD$ genes
were stored at -80 °C in LB 30% glycerol.
### TABLE 2.2:
PRIMER SETS FOR IN VITRO TOXIN GENERATION

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Restriction Enzyme Site (Red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sagBF</td>
<td>GCC GGATCC ATG ATT AAT TAT ACA GTT G</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagBR</td>
<td>ATA GCGGCCGG GTT TTC ATT</td>
<td>Not1</td>
</tr>
<tr>
<td>sagCF</td>
<td>GGT GGATCC ATG AAA ACG TTT ATT AAA C</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagCR</td>
<td>TAT GCGGCCGC TTA AAG GTT ATC</td>
<td>Not1</td>
</tr>
<tr>
<td>sagDF</td>
<td>ATA GCGGCCGC ATG AAT TAT C</td>
<td>Not1</td>
</tr>
<tr>
<td>sagDR</td>
<td>ATAT GGATCC CTA TGC CAA AGG ATG</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagA1F</td>
<td>GAG GGATCC ATG AGG AGA ATA ATG ATG TTT AAG AGA</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagA1R</td>
<td>ACA GCGGCCGC TCA TTG CTT TTT ACC TCT TTT</td>
<td>Not1</td>
</tr>
<tr>
<td>sagA2F</td>
<td>GAG GGATCC ATG GAT ATT CAG ACA TTA GTA GTT C</td>
<td>BamH1</td>
</tr>
<tr>
<td>sagA2R</td>
<td>ACA GCGGCCGC TCA CCT ATC AAA TTT GGA AAA GC</td>
<td>Not1</td>
</tr>
<tr>
<td>M13F</td>
<td>GTA AAA CGA CGG CCA GT</td>
<td></td>
</tr>
<tr>
<td>M13R</td>
<td>CAC ACA GGA AAC AGC TAT GAC CAT</td>
<td></td>
</tr>
</tbody>
</table>
2.10.2 Expression and purification of MBP linked proteins

Expression and purification of MBP linked SagB, SagC, SagD, SagA1, and SagA2 proteins were carried out as described previously [116,117], and description follows.

Autoinduction of proteins was carried out according to [118]. 10 mL of LB + Kan 30 µg/mL of the BL21 strain containing the sagB, sagC, sagD, sagA1, or sagA2 was grown overnight at 37°C. The following morning, the culture was added to 500 mL autoinduction media (460 mL ZY broth, 500 µL 1M MgSO₄, 500 µL 1000× trace metals (Teknova), 25 mL 20x NPS, 10 mL 50x 5052). 2L baffled flasks were incubated at 37°C shaking at 300 rpm for 5 hours, then the temperature was reduced to 26°C for overnight incubation. The cultures were then centrifuged in Eppendorf 5810R at 4000 rpm for 30 minutes. Bacterial pellets were resuspended in 25 mL MBP binding buffer containing EDTA free protease inhibitor tablet (Roche) and 0.1% Triton X-100 (Sigma-Aldrich). Bacteria were then lysed in a French Pressure Cell at 10,000 psi. Lysed bacteria were allowed to incubate with 0.5 mg/mL DNase 1 (Sigma) rocking at room temperature for 45 minutes. Cellular debris was centrifuged at 15,000 g for 30 minutes in Eppendorf 5810R. Supernatant was sterile filtered with SteriFlip (Millipore) and was ready for purification using an AKTA Pure system.

AKTA was run as according to manufacturer recommendations. A 5 mL MBPtrap (GE Life Sciences) was equilibrated with 5 column volumes (CV) MBP binding buffer at a flow rate of 2 mL/min. Sample was applied at 1 mL/min using a sample pump until air was sensed. The column was then washed with 10 CV binding buffer at 2 mL/min, and sample was eluted in 5 CV elution buffer at a flow rate of 1 mL/min and collected in 1 CV
fractions. Fractions containing protein as shown by UV 280 trace on the AKTA was then pooled and concentrated using MacroSep Advanced Centrifugal Device (Pall) with a 10 kda MWCO membrane. Concentrated protein was quantitated using Pierce Coomassie Plus according to manufacturer directions and run out on an SDS page gel to check purity. Protein was then frozen at -80°C until needed.

2.10.3 *In vitro* toxin generation

*In vitro* toxin reactions were carried out as previously described [116,117]. 10x reaction buffer consisting of 250 mM Tris pH7.5, 625 mM NaCl, 100 mM MgCl2, 10 mM ATP, 50mM DTT was added to 1 µM SagB, SagC, SagD, and 10 µM SagA1 or SagA2. Small amounts of TEV protease (0.5 µL) and trace Triton X-100 (0.005%) was also added to reaction and allowed to incubate overnight at 37°C. Active toxin was then subject to mass spectrometry to examine if any of the hypothesized post translational modifications had taken place.

2.10.4 Digestion and mass spectrometry of *in vitro* generated toxins

*In vitro* generated toxins were prepped for mass spectrometry as follows. 25 µL 2,2,2, trifluoroethanol (Sigma) and 25 µL 50 mM ammonium bicarbonate (ABC, Sigma) was added to sample and vortexed. Dithiothreitol (DTT, Sigma) dissolved in 50 mM ABC was added to samples to a final concentration of 20 mM DTT and samples were incubated at 60°C for one hour. Iodacetamide (Sigma) dissolved in 50 mM ABC was added to a final concentration of 40 mM and allowed to incubate in the dark at room temperature for 20 minutes. Sample was placed in an Eppendorf Speed Vac for 15
minutes to remove trifluoroethanol, and then 500 ng Trypsin Gold (Promega) was added and allowed to incubate at 37°C overnight. Samples were then quenched with 10% formic acid, vortexed and concentrated using a Speed Vac before being desalted using ZipTip C18 tips (Millipore) according to manufacture instructions. The samples were then run on a Thermo Fisher Q-Exactive mass spectrometer. Spectra gathered from the Q-Exactive were then searched using Protein Pilot (Paragon) and MaxQuant (Andromeda) to examine if any heterocycle formation had taken place.
3.1 Abstract

*Staphylococcus aureus* is a major human pathogen that encodes a wide range of virulence factors as well as resistance to numerous antibiotics. We have discovered a streptolysin-S like gene cluster (*sag*) in a highly virulent strain of community-acquired methicillin resistant *S. aureus*, JKD6159. In *Streptococcus pyogenes*, the *sag* cluster encodes a potent cytolysin, and this work examines the role it plays in the JKD6159 strain. This work attempts to take a comprehensive approach to elucidating the role of the *sag*-like cluster in JKD6159, including examining cytotoxicity, hemolysis, biofilm formation, host cell signaling, antibacterial assays, and *in vivo* mice infections. No significant differences were observed in any of the parameters studied, however during co-culture with *E. coli* and wt or Δ*sagB* JKD6159, morphological differences in *E. coli* can be observed.

3.2 Research aims

My dissertation research aims to elucidate the role that the *sag*-like cluster plays in the JKD6159 strain of MRSA. We anticipate that this cluster will begin to appear in additional strains of MRSA as the *sag*-like family of bacteriocin gene cluster is already
present in a wide range of other bacterial organisms [116]. This research will help to show if the sag-like cluster is functioning as a virulence factor, regulatory protein, or antimicrobial peptide. If this protein does indeed serve as a virulence factor, further research can examine ways to mitigate the damage caused by the peptide. The primary way this research will be achieved is through generation of an isogenic gene deletion in the sagB found in JKD6159 as prior work has shown that disruption of any of the genes in the sag cluster led to loss of functionality of the toxin [119]. Mutants can then be assessed for changes in cytotoxicity, hemolysis, antibacterial activity, and changes in host cell signaling. 2D proteomics were also carried out to determine if disruption of the sag cluster has any additional effects on the S. aureus proteome. As the sag cluster is a member of the TOMM family, part of my dissertation research has also focused on creating the active toxin in vitro then subjecting the hypothetically modified toxin to mass spectrometry to verify the heterocyclic conversions of residues on the active form of the peptide.

3.3 Introduction

*Staphylococcus aureus* (*S. aureus*) is a common human commensal that is also capable of causing severe disease including lesions and sepsis [120–122]. As discussed in Chapter 1, *S. aureus* is encodes a wide range of virulence factors including protein A, Panton-Valentine Leukocidin, and α-toxin among others. *S. aureus* commonly presents as methicillin resistant in roughly half of all infections in the United States [123]. Methicillin resistant *S. aureus* (MRSA) comes in two distinct designations based on
where the infection is acquired, community acquired MRSA (CA-MRSA) or hospital acquired MRSA (HA-MRSA). CA-MRSA tends to be highly virulent, and can encode toxins that are exclusive to CA-MRSA such as Panton-Valentine Leukocidin. This work examines a highly virulent strain of CA-MRSA, JKD6159 from Australia. The JKD6159 strain has become the dominant strain of MRSA found in Australia despite the initial isolation of the strain only occurring ~15 years ago [124,125]. This strain encodes a sag-like cluster that in a related pathogen, Streptococcus pyogenes is a potent virulence factor [116,119]. This chapter will examine how disruption of the sag cluster affects hemolysis, cytotoxicity, antibacterial effects on E. coli, S. pyogenes, and S. epidermidis, biofilm formation, epithelial cell signaling changes, and in vivo mice infections.

3.4 Streptolysin associated gene cluster (sag) in a select strain of Staphylococcus aureus

The streptolysin associated gene cluster was identified using transposon mutagenesis in Group A Streptococcus pyogenes (GAS) [119]. One of the hallmarks of GAS is the beta-hemolysis it exhibits when plated on blood agar plates which is caused by the oxygen stable toxin Streptolysin S (SLS) [116,119]. The identification of the operon responsible for SLS production was found in 2000 and was found to consist of 9 genes that when disrupted resulted in the loss of the beta-hemolysis phenotype [119]. The genes in the operon were dubbed sag for SLS-associated gene [119]. SLS fits into a family of post-translationally modified peptides dubbed TOMMs short for thiazole/oxazole modified microcins [126,127]. These peptides are ribosomally synthesized then post-translationally modified via the instillation of heterocycles on
cysteine, serine, or threonine residues [127]. Peptides in the TOMM family can have a range of functions, ranging from toxins such as SLS to antimicrobial peptides such as microcin b17 [116,119,128]. With regards to the specific functions of the proteins in the sag cluster, SagA functions as the protoxin, SagB as a dehydrogenase, SagC as a cyclodehydrogenase, SagD as a scaffold, 2-3 genes serving as ABC type transporters, and an immunity like gene [116,117,129]. Work by our laboratory has shown that in Streptococcus pyogenes, we can recreate the active toxin in vitro, demonstrate that disruption of the sag cluster can affect host cell signaling, and that the product of the sag cluster in Streptococcus pyogenes SLS dependent hemolysis requires the band 3 protein on erythrocytes. This prior work served as a template for the research that was conducted and described in this dissertation.

Work by Dr. Shaun Lee in 2008 was able to demonstrate that by expressing the SagA, SagB, SagC, and SagD proteins from Streptococcus pyogenes in E. coli expression vectors, purifying the proteins, and allowing them to incubate together, the activity of the toxin could be reconstituted [116]. Functionality of the toxin was confirmed by hemolysis and cytotoxic activities with the with fully active toxin SagA converted by SagB,C,D, or conditions were SagA or one of the modifying proteins SagB,C or D were omitted. Hemolysis and cytotoxicity was only observed when the four proteins SagA-D were utilized [116]. This approach was attempted with the proteins from the JKD6159 strain of S. aureus, and is discussed in Chapter 4.

Dr. Rebecca Flaherty was able to examine the signaling changes that occurred from HaCat epithelial cell infections with Streptococcus pyogenes wt and ΔsagA strains
Using an antibody array the authors were able to find and verify several changes in eukaryotic cell signaling that occurs in treatment with wt of ΔsagA S. pyogenes. Specifically, the authors found that activation of p38 MAPK and NF-κB were caused by SLS, and that Akt was down regulated. This approach was carried out and discussed in detail in Chapter 3.

Dustin Higashi carried out experiments to identify the protein target on erythrocytes that SLS binds to in order to lyse the blood cell [131]. Using SLS-preparations from wt, ΔsagA, and ΔsagA +sagA, the authors were able to show that the blood cells took in large quantities of water prior to the lysis, and this influx of water was a response to influx of Cl- into the cell. Band 3 is the primary anion transporter found on erythrocytes, and blocking the anion channel with DIDS resulted in loss of hemolysis.

Using bioinformatic searches to the SagB protein from S. pyogenes, we were able to identify a strain of methicillin resistant S. aureus that encoded a sag-like cluster (Figures 3.1, 3.2). This strain was identified as JKD6159, an Australian clone of community acquired MRSA (CA-MRSA). The JKD6159 is a member of the ST93-IV or Queensland clone strain of CA-MRSA [115]. JKD6159 was initially isolated roughly 15 years ago from a young male IV drug user who was presenting with severe sepsis [132]. We were able to obtain this strain from Dr. Timothy Stinear in order to investigate the role that the sag-like cluster may be playing in the virulence of this strain.
3.5 Bioinformatic discovery of a sag-like cluster in community acquired methicillin resistant *S. aureus* strain JKD6159

Using the gene and protein sequence from *Streptococcus pyogenes* strain M1 *sagB* was searched against the non-redundant gene or protein entry using NCBI BLAST bioinformatics tool. We observed a match on the *S. aureus* strain JKD6159 with high levels of homology (Figure 3.1). The gene cluster observed in the *S. aureus* strain contained all of the components of the *sag* cluster as found in *S. pyogenes* including the *sagB*, *sagC*, *sagD*, and ABC transporters (Figure 3.2). Analysis of the GC content in the gene cluster indicates it was likely horizontally acquired and integrated between the *gnd* gene and the *gloxalase* gene (Figure 3.3). We also observed several small open reading frames in the vicinity of the modifying genes that could possibly serve as the protoxin gene. This strain of community acquired MRSA was originally isolated from a male IV drug user and had been sequenced by Dr. Timothy Stinear [115].
Figure 3.1: ClustalW protein alignments of SagB, SagC, and SagD of JKD6159 and *Streptococcus pyogenes* (GAS). SagB from JKD6159 is 31% identical to GAS, SagC is 21% identical, SagD is 28% identical. Identical percent determined using BLAST.
Figure 3.2: Cartoon alignment of sag cluster as found in JKD6159 compared to sag cluster in *Streptococcus pyogenes*
Figure 3.3: GC analysis of the sag-like cluster in JKD6159 and bovine isolate RF122. USA300 TCH1516 was used as the outgroup as it lacks the sag cluster. GC profiles are very similar between the RD122 strain and the JKD6159 strain. Image courtesy of YunJuan Bao
3.6 RT-PCR and gene deletion in *S. aureus* strain JKD6159

Upon receiving the JKD6159 strain, we first wanted to ensure that the genes of
the *sag*-like cluster were transcribed. To do this we performed reverse transcriptase PCR
whereby RNA was isolated from the bacterium, converted to cDNA, and PCR amplified.
Expression of the *sagB*, *sagC*, and *sagD* genes were confirmed (Figure 3.4).
Figure 3.4: Reverse transcriptase PCR on *sagB*, *sagC*, and *sagD* genes in JKD6159. –RT lanes indicate non-reverse transcriptase treated controls to ensure no gDNA present
Upon confirmation that the sag cluster genes were transcribed and based on the knowledge that disruption of any of the 9 genes found in the sag cluster results in loss of function[119], we endeavored to create a deletion in the sagB gene. JKD6159 encodes a robust restriction enzyme system, and to bypass the nuclease system we utilized the IM93B strain of E. coli obtained from Dr. Ian Monk [109,111]. IM93B was engineered to produce the methyltransferases from JKD6159, and thus plasmids passed through the IM93B would have the proper methylation system and not be digested by the nucleases endogenous to JKD6159. Using this E. coli intermediary, we were able to successfully generate a knockout of the sagB gene in JKD6159 (Figure 3.5). This gene deletion and complement strain did not exhibit any growth defect in standard bacterial TSB media (Figure 3.6).
Figure 3.5: PCR confirming gene deletion of $sagB$ in JKD6159. Using primers outside the $sagB$ gene, wildtype JKD6159 will yield ~2500 bp amplicon while $\Delta sagB$ will yield ~1700 bp amplicon.
Figure 3.6: Growth curve of wildtype JKD6159, ΔsagB, and ΔsagB+sagB JKD6159 in TSB broth. Representative curve of biological triplicates.
3.7 Functional analysis of the JKD6159 wt and ΔsagB

Functional assays were undertaken to examine the role that the sag-like cluster may be playing in JKD6159. The following experiments were undertaken 1: Hemolysis, 2: Cytotoxicity, 3: Antibacterial Assays, 4: Biofilm Formation, and 5: Cell Signaling Changes.

3.7.1 Sheep blood hemolysis assay

Hemolysis is one of the classic readouts of the SLS toxin found in *S. pyogenes* [116,119], and as such was a logical starting point to explore our hypothesis that the sag-like cluster is serving as a toxin. Defibrinated sheep blood was used to examine the hemolytic potential of the conditioned media from the wt and ΔsagB JKD6159 strain. 25% conditioned media from overnight growth was sterilized and added to sheep blood and incubated at 37°C for the indicated time points (Figure 3.7). Hemolysis levels were normalized to a TSB negative control and a 0.1% Triton X-100 control. No significant difference in hemolysis was observed between the wt and ΔsagB strains (Figure 3.7).
Figure 3.7: Hemolysis of sheep RBC when exposed to 25% conditioned media from wt or ΔsagB strains of *S. aureus*. One of three biological replicates.
3.7.2 Cytotoxicity of HaCat epithelial cells

The SLS toxin in *S. pyogenes* is a secreted factor and thus, the cytotoxicity of the wt and ΔsagB strains were assessed using transwells at a multiplicity of infection of 50 bacteria/1 host cell. Prior to assays using host cells, strains were grown in cell culture media to ensure no growth differential between strains (Figure 3.8). No significant differences in cytotoxicity were observed (Figure 3.9).
Figure 3.8: Growth curve of JKD6159 wt and ΔsagB in cell culture media used for cytotoxicity and cell signaling assays. One of three biological replicates.
Figure 3.9: HaCat cytotoxicity from transwell infection of wt JKD6159 and ΔsagB strains. HaCats were infected at MOI50 for 8 hours and cytotoxicity was assessed using ethidium homodimer. One of three biological replicates.
3.7.3 Antibacterial assays against *Escherichia coli*, *Streptococcus pyogenes*, and *Staphylococcus epidermidis*.

In strains of *E. coli*, the microcin B17 peptide is post-translationally modified into a DNA gyrase inhibitor [128]. Additionally, antimicrobial TOMMs have been discovered in both *Listeria monocytogenes* and *Bacillus amyloliquefaciens* [133,134]. These TOMMs target *Staphylococcus aureus* and related *Bacillus* species respectively [133,134]. Based on these reports we examined the wt, Δ*sagB* and Δ*sagB+sagB* strain effects on the growth of *E. coli*, the common skin commensal *S. epidermidis*, and the pathogen *S. pyogenes*. Conditioned media from overnight growth of the *S. aureus* wt, Δ*sagB*, and Δ*sagB+sagB* strains were sterilized and added to early growth *E. coli*, *S. epidermidis*, and *S. pyogenes*. Results indicate no inhibition of any tested bacterial strain (Figure 3.10). Co-culture experiments were carried out to observe morphological differences. Using GFP *E. coli* and either wt or Δ*sagB* JKD6159, different *E. coli* morphologies were observed (Figure 3.11). In particular, *E. coli* cultures grown in the presence of the wt JKD6159 strain were found to have diffuse GFP staining and less pronounced membrane density suggesting that the *E. coli* in under increased stress when cultured with wt JKD6159 as compared to Δ*sagB*. It may be possible that while *E. coli* growth is not significantly inhibited by wt JKD6159 the production of the hypothetical toxin may influence *E. coli* membrane morphology as observed by high-resolution microscopy. This avenue of research will be explored in future studies.
Figure 3.10: Inhibition of *E. coli*, *S. epidermidis*, and *S. pyogenes*.

A) No significant growth inhibition was observed when 25% conditioned media from JKD6159 wt, ΔsagB or ΔsagB + sagB was tested against *E. coli*.  
B) No inhibition of *S. epidermidis* was observed.  
C) No inhibition of *S. pyogenes* observed.  
Representative curves of triplicate biological replicates.
Figure 3.11: Live imaging co-culture with GFP *E. coli* and JKD6159 wt or ΔsagB strain. Select time points of *E. coli* are shown.
3.7.4 Biofilm formation by wt JKD6159 and ΔsagB JKD6159

*S. aureus* biofilms can play an important role in infections, with the ability to create biofilms associated with virulence [135–137]. Bacterial biofilms are associated with a reduction in antibiotic availability, slower clearing infections, and persister cells [136,138]. To this end we assessed the ability of the wt and ΔsagB and ΔsagB + sagB strains to form biofilms (Figure 3.12). Overnight cultures were diluted in fresh TSB in 24 well tissue culture plates and allowed to incubate in a humidified environment for 24, 48, or 72 hours. No significant differences between the strains were observed as measured by crystal violet biofilm assay.
Figure 3.12: Biofilm formation of the JKD6159 wt, ΔsagB, and ΔsagB+sagB strains. Biofilm formation was measured at the indicated time points as measured by crystal violet. Representative of three biological replicates.
3.7.5 Eukaryotic cell signaling changes

Despite the lack of differences observed in eukaryotic cytotoxicity, there is still a possibility that the *sag* cluster would result in altered cell signaling. Prior work by our lab has shown that the deletion of the protoxin in *S. pyogenes* can result in differential cell signaling including an increase in NF-κB [130]. To assess this, after an 8 hour transwell infection at an MOI of 50 of the wt, Δ*sag*-*B*, and Δ*sag*B+sag*B strains, HaCat cells were lysed and an antibody array was carried out. Transwells were utilized to ensure that contact dependent signaling was removed from the infection. As the toxin from the *sag* cluster in JKD6159 is hypothesized to be a soluble factor, having a membrane separating the host HaCat cells from the bacterium will limit the signaling changes observed to soluble factors. As the only difference in soluble factors between the wt and Δ*sag*-*B* strains is the *sag* produced toxin, we can attribute any signaling changes to the toxin produced by the *sag* cluster in JKD6159. Samples were submitted to Kinexus for the 900P antibody array. The array contains 878 antibodies. A full table of results can be found in Appendix 1. Proteins that were down regulated in wt as compared to Δ*sag*-*B* included cyclin-dependent protein serine kinases (CDKs) and epidermal growth factor receptor tyrosine kinase (EGFR) (Table 3.1).
### TABLE 3.1:

**TOP HITS FROM KINEXUS ANTIBODY ARRAY**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Phosphorylation site</th>
<th>% down regulation in wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>T14</td>
<td>97</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>T161</td>
<td>97</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>T15/Y15</td>
<td>55</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>Pan-specific</td>
<td>51</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 6</td>
<td>Y13</td>
<td>101</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 6</td>
<td>Y24</td>
<td>65</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 7</td>
<td>T170</td>
<td>52</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 9</td>
<td>T186</td>
<td>60</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 10</td>
<td>T196</td>
<td>64</td>
</tr>
<tr>
<td>Epidermal growth factor-tyrosine kinase</td>
<td>Y998</td>
<td>85</td>
</tr>
<tr>
<td>Epidermal growth factor-tyrosine kinase</td>
<td>T693</td>
<td>143</td>
</tr>
</tbody>
</table>
In bacterial infections by *Salmonella* and *Streptococcus pneumoniae*, changes in phosphorylation of CDKs can be observed between various wt and mutant strains but the ultimate function of these changes was not understood [139,140]. Research in cancer cells has shown that altered CDK phosphorylation can affect cell migration in cancer cell lines [141,142]. Based off this work we attempted scratch assays with HaCat cells to see if there was a difference in the rate of wound closure between the strains of JKD6159. However, the toxicity of the bacteria even after one hour of exposure resulted in non-interpretable results due to cell death (data not shown). There is a paucity of publications regarding *S. aureus* scratch assays possibly due to the high toxicity the bacterium exhibits.

EGFR can affect the cytokines that eukaryotic cells express, and the changes in cytokine expression during *S. aureus* infections can be traced through EGFR [143,144]. Using an 80 target cytokine array kit from Abcam we examined the differences in cytokine signaling between the wt and ΔsagB strains of JKD6159. Results are summarized in Table 3.2. The hits from the cytokine array were attempted to be verified via western blots for I-309, IL-10, MIP-1δ, and osteoponitin, but no significant differences were observed indicating that the cytokine array may have resulted in false positives (Figure 3.13). The root cause of the array failure is unknown. The wt and ΔsagB infections were carried out identically and identical volumes from the pooled infected wells were applied to the membrane. The membrane contained internal positive and negative controls for normalization, and imaging and normalization was done exactly as the kit instructions laid out. It is possible that one of the membranes was defective or
stored incorrectly leading to the false positives. This array should be repeated in the future.
TABLE 3.2:
RESULTS FROM CYTOKINE ARRAY BETWEEN WT AND ΔSAGB INFECTIONS

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>WT intensity (normalized)</th>
<th>KO intensity (normalized)</th>
<th>WT/KO ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-309</td>
<td>631</td>
<td>1439</td>
<td>0.43</td>
</tr>
<tr>
<td>MIP-1δ</td>
<td>1024</td>
<td>2430</td>
<td>0.42</td>
</tr>
<tr>
<td>Osteoponitin</td>
<td>13369</td>
<td>35231</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-10</td>
<td>3492</td>
<td>1417</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Figure 3.13: Quantitation of western blots to targets identified by cytokine array. Biological triplicate infections are shown, protein levels were normalized to a Sypro stained gel, and wt is set to 100%.
I-309 is a monocyte attractant [145], MIP-1δ is a chemokine for monocytes and neutrophils [146], osteoponitin is also chemotactic for neutrophils [147], and IL-10 has anti-inflammatory functions [148]. Results from the cytokine array indicate that the sag like cluster in JKD6159 could be modulating the host immune system to prevent recruitment of immune cells. Taken together, these cytokine changes indicate that the sag cluster may enhance the virulence of JKD6159 by suppressing innate immunity during the course of an infection. However, the hits from the cytokine array were attempted to be verified via western blots for the I-309, IL-10, MIP-1δ, and osteoponitin, but no significant differences were observed indicating that the cytokine array may have failed.

3.7.6 In vivo mice infections with wt, ΔsagB, and ΔsagB + sagB strains

The gold standard for assessing virulence cause by S. aureus is mice infections. We used a mice skin and soft tissue infection model using Balb/C mice based on prior experimentations utilizing the JKD6159 strain of S. aureus [115,149]. In brief, 6 week old Blab/C female mice were injected with 10⁸ CFU in the flank and lesion size and mice mass were assessed daily for 5 days before mice were sacrificed and wounds were enumerated for CFUs (Figure 3.14). Experiments were carried out in the laboratory of Dr. Taeok Bae at Indiana School of Medicine Gary.
Figure 3.14: Results from the *in vivo* mice infections. A) weight loss during the course of infection (error bars omitted, no significance). B) 5 day lesion size as quantified by ImageJ. C) CFU/mL recovered from the lesion.
Mice infections did not exhibit any significant difference in weight loss, lesion size, or recovered CFU/mL between any strains as determined by one way Anova statistics.

3.8 Discussion

In this chapter we have utilized numerous approaches to determine the function of the sag like gene cluster found the CA-MRSA strain JKD6159. We engineered a sagB deletion in the cluster as the protoxin gene was not readily apparent, and prior work has shown that deletion of any of the 9 genes found in the cluster led to loss of function of the active toxin [119]. The peptides that are in the TOMM family are responsible for a range of phenotypes, everything from antibiotic activity to hemolysis [116,128,130]. Based on the range of activities that the sag cluster in JKD6159 could potentially be responsible for, we examined a wide range of activities. We were able to show that the sag cluster has no effect on either hemolysis or cytotoxicity. No effect on the growth of E. coli, S. epidermidis, or S. pyogenes was observed. While no growth differences were observed, the co-culture experiments showed that the E. coli when cultured with wt JKD6159 exhibits different morphology than when E. coli is cultured in the presence of the ΔsagB strain. The E. coli rods appear more diffuse in the wt conditions than the ΔsagB conditions. This could point to the E. coli undergoing some additional stress that is not present in the ΔsagB conditions. Co-culture experiments should be carried out using S. epidermidis and S. pyogenes as well. Biofilm formation was unaffected by the deletion of the sagB gene.
The results from the cytokine array initially pointed to the modulation of the innate immune system. If the JKD6159 strain is able to reduce the inflammation response during the initial stages of infection, this could aid the bacteria in establishing an initial foothold in the patient and thus increase the likelihood of causing an infection. Additionally, the innate immune system being blinded to the bacteria by the reduction in I-309, MIP-1δ, and osteoponitn, this could result in a more severe infection and/or a longer lasting infection as the body is slower to clear the infection. This could also increase the total bacterial burden which would result in a longer infection. The data from the cytokine array could not be verified by western blots, so the cytokine array will need to be repeated and if any additional cytokines are expressed differently, those results will need to be verified via western blotting as well.

In vivo mice infections show no significant differences in any of the parameters measured, weight loss, lesion size, or recovered CFUs. Mice are the most commonly used model animal for *S. aureus* infections, and the infections are commonly subdermal as we have done or intraperitoneal (IP) injections. These experiments should be repeated using IP injections and the time of death monitored to see if any strain specific differences are observed. Additionally, it is possible that the mice do not respond to the peptide produced by the *sag* cluster as is observed with the Panton-Valentin Leukocidin [74,81–83]. As with PVL, for effects of the gene deletion to be observed, a different model organism such as rabbits may be utilized.

We have been able to show that the *sag* cluster in JKD6159 has no effect on a variety of processes, including cytotoxicity of epithelial cells, hemolysis, inhibition of *E.*
coli, S. pyogenes, or S. epidermidis, biofilm formation, and mice subcutaneous infections.
4.1 Abstract

The SLS toxin produced by the sag cluster in *Streptococcus pyogenes* is hypothesized to undergo extensive posttranslational modification via the addition of heterocycles, and we predict that that is the case with the protein product from the sag-like cluster found in *Staphylococcus aureus*. Using MBP tagged recombinant protein produced in *E. coli*, we were able to generate the toxin and examine it via mass spectrometry for the posttranslational modifications. No heterocycle formation was observed. Additionally we assessed the proteomic profiles of the wt and ΔsagB JKD6159 using 2D proteomics to see if the disruption of the sag cluster resulted in differences in the proteomic profile of the bacteria. We were able to observe several differentially regulated metabolic proteins between the wt and ΔsagB JKD6159 strains including the wt strain exhibiting an increase in threonine synthase and carbamate kinase, and in ΔsagB, an increase in catabolite control protein A and FolD. Catabolite control protein A (CcpA) is linked to virulence in *S. pyogenes* and *S. pneumoniae*, but the current functionality of the increase in CcpA in the ΔsagB strain is currently unknown.
4.2 Introduction

To assess the effect that the *sag* cluster may have on the proteome of JKD6159 we carried out comprehensive total proteomic studies using 2D gel electrophoresis. This method provided a visual way to assess the changes in the proteome upon the deletion of the *sagB* gene. The studies were carried out in biological and technical duplicate, and spots that were consistently differentially expressed were excised from the gel and subjected to LC/MS/MS for protein identification.

Prior work has shown that the active toxin from the *sag* cluster found in *S. pyogenes* and *C. botulinum* can be created *in vitro* by expressing the SagA-D proteins in *E. coli*, purifying them, and combining them in reaction buffer overnight [116,117]. Based on this work we created maltose binding protein (MBP) linked SagA-D protein for expression in BL-21 *E. coli*. As there was not a distinct phenotype that we were able to determine based on the functional assays we carried out in Chapter 3, the *in vitro* generated toxin was only subjected to mass spectrometry to determine the nature of the hypothesized posttranslational modifications. If a distinct phenotype can be attributed to the *sag* cluster in JKD6159 in the future, the *in vitro* toxin could be used to recapitulate the phenotype observed.

4.3 2D proteomics

In order to determine if disruption of the *sag* cluster in the JKD6159 strain of *S. aureus* affects the expression of proteins outside of the gene cluster we undertook a 2D proteomic approach to visualize the proteome. Proteins were isolated and separated on
a pH 3-10 non-linear strip to disperse the proteins by isoelectric point before being run on a 12.5% polyacrylamide gel to separate based on size. The gels were then stained with Sypro Ruby stain, scanned using a Typhoon Imager, and the images were overlain using Delta2D software (Figure 4.1).
Figure 4.1: Overlay of wt and ΔsagB protein gels. wt=green, ΔsagB=red markers indicate spots that were repeatable between technical and biological duplicates. Image generated using Delta2D software
Analysis of biological duplicate and technical duplicates revealed one spot in each the wt and ΔsagB strains that was consistently differentially expressed (Figures 4.2, 4.3). These spots were excised, de-stained, digested and subjected to LC/MS/MS. Proteins discovered were searched on the MASCOT database for identification, and results are shown in Table 4.1. These protein hits were then mapped to their metabolic pathways using Blast2Go (Appendix B).
Figure 4.2: Image of spot 1 from wt gel (left) compared to same location on ΔsagB gel (right). This spot was consistently identified as present significantly more in the wt proteome than in the ΔsagB strain.
Figure 4.3: Image of spot from ΔsagB gel (left) compared to same region on wt gel (right). This spot was consistently identified as present significantly more in the ΔsagB proteome than in the wt strain.
TABLE 4.1:
PROTEINS IDENTIFIED FROM 2D PROTEOMIC EXPERIEMENTS

<table>
<thead>
<tr>
<th>Spot origin</th>
<th>Protein Name</th>
<th>% coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt 1</td>
<td>Threonine synthase</td>
<td>13.60</td>
</tr>
<tr>
<td></td>
<td>2 oxoisovalerate dehydrogenase subunit alpha</td>
<td>7.58</td>
</tr>
<tr>
<td></td>
<td>Carbamate kinase</td>
<td>26.20</td>
</tr>
<tr>
<td></td>
<td>NH3 dependent NAD synthetase</td>
<td>49.08</td>
</tr>
<tr>
<td></td>
<td>Succinate CoA ligase ADP forming alpha subunit</td>
<td>45.03</td>
</tr>
<tr>
<td></td>
<td>Thioredoxin disulfide reductase</td>
<td>36.98</td>
</tr>
<tr>
<td></td>
<td>Putative glucokinase ROK family</td>
<td>10.98</td>
</tr>
<tr>
<td></td>
<td>D isomer specific 2 hydroxyacid dehydrogenase family protein</td>
<td>26.90</td>
</tr>
<tr>
<td>ΔsagB 4</td>
<td>Uroporphyrinogen decarboxylase</td>
<td>12.17</td>
</tr>
<tr>
<td></td>
<td>FolD</td>
<td>38.47</td>
</tr>
<tr>
<td></td>
<td>Global transcriptional regulator catabolite control protein A</td>
<td>46.50</td>
</tr>
<tr>
<td></td>
<td>Hydroxymethylbilane synthase</td>
<td>11.36</td>
</tr>
<tr>
<td></td>
<td>FAD dependent pyridine nucleotide disulphide oxidoreductase</td>
<td>9.45</td>
</tr>
<tr>
<td></td>
<td>S adenosyl methyltransferase MraW</td>
<td>15.11</td>
</tr>
</tbody>
</table>
4.4 *In vitro* toxin reconstitution

The protein products in the TOMM family of which the *sag* cluster is a member of, undergo extensive posttranslational modification [127,150]. These modifications typically consist of dehydration of the cysteine, threonine, or serine residues [127,150,151]. Prior work has shown that in order to generate the active toxins from the *sag* gene cluster in *S. pyogenes* or *C. botulinum*, the SagA-D protein are necessary and sufficient for the reaction to occur *in vitro* [116,117]. Based on this data we created maltose binding protein (MBP) linked SagA, B, C, and D recombinant proteins generated by *E. coli* (Figure 4.4). Upon completion of the synthetase reaction, the proteins were trypsin digested and prepared for mass spectrometry. The peptides were run on a Q-Exactive mass spectrometry and results were searched using Protein Pilot and MaxQuant for the presence of the hypothesized heterocycle formation. No heterocycle formation was observed.
Figure 4.4: Purified proteins linked to MBP tag. SagB-MBP=75.5 kda, SagC-MBP=78.5 kda, SagD-MBP=93 kda, SagA1-MBP=53 kda, SagA2-MBP=49 kda
4.5 Discussion

We were able to consistently see some differentially regulated metabolic proteins between the wt and ΔsagB JKD6159. Proteins that were higher in the wt strain compared to the ΔsagB strain included threonine synthase, 2 oxoisovalerate dehydrogenase subunit alpha, carbamate kinase, NH3 dependent NAD synthetase, succinate CoA ligase ADP forming alpha subunit, thioredoxin disulfide reductase, Putative glucokinase ROK family, and D isomer specific 2 hydroxyacid dehydrogenase family protein. Proteins that were more strongly expressed in the ΔsagB strain included uroporphyrinogen decarboxylase, FolD, Global transcriptional regulator catabolite control protein A, hydroxymethylbilane synthase, FAD dependent pyridine nucleotide disulphide oxidoreductase, and S adenosyl methyltransferase MraW.

Threonine synthase is responsible for the conversion of L-phosphohomoserine to L-threonine via the addition of water [152]. 2 oxoisovalerate dehydrogenase subunit alpha is part of a large protein complex in the TCA cycle, and is responsible for the conversion of alpha-keto acids to acetyl-CoA [153]. Carbamate kinase is responsible for transferring a phosphate from carbamoyl phosphate to ADP thereby generating ATP [154]. NH3 dependent NAD synthetase is responsible for the final step in generating NAD+ from deamindo-NAD+ [155]. Succinate CoA ligase ADP forming alpha subunit is involved in the substrate level phosphorylation of ADP in the TCA cycle [156]. Thioredoxin disulfide reductase catalyzes the reaction of NADP+ to NADPH [157]. Putative glucokinase ROK family plays a role in the glucose metabolism pathway [158]. D isomer specific 2 hydroxyacid dehydrogenase family protein is a member of the
carbohydrate metabolism pathway and it converts keto acids into chiral hydroxy acids [159].

The function of proteins that are highly expressed in the ΔsagB strain of JKD6159 follows: Uroporphyrinogen decarboxylase catalyzes the first step of the tetrapyrrole pathway [160]. FolD is a bifunctional enzyme that generates NADPH and 10-formyltetrahdrofolate [161]. Global transcriptional regulator catabolite control protein A (CcpA) is a master regulator involved in many catabolic pathways [162]. Hydroxymethylbilane synthase is involved in polymerization of porphobilinogen into 1-hydroxymethylbilane [163]. FAD dependent pyridine nucleotide disulphide oxidoreductase is responsible for catalyzing disulfide bond formation [164]. S adenosyl methyltransferase MraW is a methyltransferase that methylates 16s rRNA [165].

While the impact of many of these pathways is not understood in regards to the sagB deletion in JKD6159, CcpA has been linked to virulence in both S. pyogenes and S. pneumoniae [162,166]. In both cases, deletion of ccpA results in lessened virulence. Deletion of ccpA in S. pneumoniae results in decreased colonization of mice lungs [162]. For S. pyogenes, the loss of the regulatory protein resulted in reduced infectivity in mice models of infection, reduced colonization, and interestingly, purified CcpA binds to the promoter region of sagA, increasing the transcription rate [166]. It is possible that the increase in CcpA that the ΔsagB strain exhibits is the result of the bacterium attempting to compensate for the deficient sag cluster. Further elucidating the interplay between the sag cluster and CcpA could shed light on the role of the sag cluster in JKD6159.
While no posttranslational modifications were seen in the *in vitro* toxin generation, there are several avenues to attempt the experiment again. The proteins were single step purified on a MBP column, but that resulted in high levels of contamination. The constructs also contain a 6x His tag, so a two-step purification would help to reduce the protein contamination we observed. Additionally, in the generation of the clostridium toxin, the SagC protein was never active causing to the authors to use a SagC from a different species [117]. It may be necessary to substitute an active SagC from a different species to observe the posttranslational modifications.
5.1 Summary of conclusions

We were successful in testing a range of hypotheses regarding the impact that the sag cluster in JKD6159 could be having on virulence, and as a result we can state that the sag cluster has no impact on the following: Cytotoxicity, Hemolysis, In Vivo Mice Infections, Biofilm Formation, IL-10, I-309, MIP-1δ, and Osteoponitn expression, and inhibition of *E. coli*, *S. epidermidis*, and *S. pyogenes*. While no growth defects were observed, morphological differences can be observed in *E. coli* during co-culture of the wt and ΔsagB strains. We did see differential expression of several metabolic proteins between the two strains of JKD6159, with the potentially interesting increase in catabolic control protein A in the ΔsagB strain. Figure 5.1 shows the experiments carried out.
Figure 5.1: Summary of approaches taken to elucidate the role of the *sag*-like cluster in *S. aureus* JKD6159

- Genetic analysis
  - Generate JKD6159 mutants
  - RT-PCR to check for transcripts of *sagA-D*-like genes

- Proteomics
  - MS spot analysis from 2D gel electrophoresis
  - Posttranslational modifications on in vitro generated toxin

- Functional analysis
  - Hemolytic assays
  - Cytotoxicity assays
  - Antibacterial assays
  - PCR and clone the components of the *sag*-like cluster
  - Eukaryotic cell signaling changes
5.2 Future directions

Co-culture experiments revealed that *E. coli* may be under increased stress when cultured with wt JKD6159 as compared to the Δ*sagB* strain. While this difference was not reflected in the growth curves, the more sensitive microscopy images do appear to show additional stress on the *E. coli* during wt co-culture. Co-culture methods should be repeated using *S. epidermidis* and *S. pyogenes* in order to observe morphology changes.

The cytokine array initially had some results that could explain how the *sag* cluster in JKD6159 could modulate immune signaling via cytokine expression, but verification western blots failed to show any difference in the signaling. The array should be repeated and any further positive hits examined via western blot for verification.

While no significant difference in cytotoxicity was observed when epithelial cells were infected with the various strains of JKD6159, it is possible that other cell types would exhibit a difference. It would be worth examining if macrophage infection resulted in either a difference in cytotoxicity or a difference in cytokine signaling.

*In vivo* mice infections failed to show any significant difference in the virulence of the JKD6159 strains tested. It is possible that mice are simply not an appropriate choice for examining the *sag* cluster, such as is the case with the *S. aureus* toxin Panton-Valentin Leukocidin. The wt, Δ*sagB*, and Δ*sagB+sagB* strain should be tested against rabbit cells *in vitro* before proceeding to rabbit infections.

While most of the metabolic changes remain a mystery and are worthy of further research, one potentially interesting area for future research is the CcpA protein that is upregulated in the Δ*sagB* JKD6169. Prior work has shown that CcpA is required
for virulence in both *S. pyogenes* and *S. pneumonia* [162,166]. It is possible that the CcpA is upregulated in the mutant strain as a mechanism to compensate for the loss of functionally of the *sag* cluster. In *S. pyogenes*, CcpA is also able to bind and enhance transcription of the *sagA* gene [166]. It would be very interesting if this was the case in the JKD6159 strain as well. Creation of a Δ*sagB::ccpA* double mutant would allow study of how the JKD6159 strain behaves without the ability of the Δ*sagB* strain to upregulate CcpA. If this is truly a codependent system, the exact role of the *sag* cluster in JKD6159 could be difficult to deduce, which is exactly the problem our research has run into. A comparison using mice models of infection utilizing the Δ*sagB*, Δ*sagB::ccpA*, and Δ*ccpA* strains could show that the double knockout is less virulent than either of the single knockouts. This could imply that the *sag* cluster is indeed encoding a virulence factor, but in our studies the exact contribution of the *sag* cluster was masked by the resulting upregulation of CcpA.

Subjecting the *in vitro* toxin reconstitution to further purification and/or substituting a different SagC protein could result in the observation of the heterocycles.

Finally it is worth examining if the *sag* cluster in JKD6159 is transient. It is possible that despite the organism harboring and expressing the genes in the cluster that it is in the process of losing the cluster if it serves no purpose to the bacterium’s survival. It would be worth passing the wt JKD6159 strain for multiple generations then subject the bacterium to PCR to see if the *sag* cluster is excised from the genome. The isolate we are using was isolated roughly 15 years ago, and since that timeframe the ST93-IV clade has become the dominate CA-MRSA strain in Australia [124,125]. To
support the possibility that the *sag* cluster in JKD6159 is a transient acquisition, it would be beneficial to obtain some more recently acquired JKD6159 examples and sequence the isolates to see if the *sag* cluster is retained in its original form. If the gene cluster is still present, it could have undergone additional mutations to either increase its contribution to virulence or be silenced.

5.3 Closing perspectives

While the effects of the deletion were not as dramatic as hoped, we were successful in showing a variety of ways that the virulence of the JKD6159 strain is unaffected by the disruption of the *sag* gene cluster. While we attempted to spread a wide net of experiments to capture the effect of the *sag* cluster in JKD6159, we were unable to point to a definitive mechanism that the cluster has on the virulence of the bacterium. Further experiments should be undertaken to see if the optimal host animal for infections is rabbits, the role that CcpA is playing in the Δ*sag* strain, or if the *sag* cluster had been removed or otherwise silenced in the more current isolates of JKD6159.
APPENDIX A:

FULL LIST OF PROTEINS FROM KINEXUS ANTIBODY SCREEN

TABLE A.1:
LIST OF IDENTIFIED PROTEINS

<table>
<thead>
<tr>
<th>Target Protein Name</th>
<th>Phospho Site (Human)</th>
<th>Full Target Protein Name</th>
<th>% ΔsagB/wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTF2F1</td>
<td>S385+T389</td>
<td>General transcription factor IIF subunit 1</td>
<td>150</td>
</tr>
<tr>
<td>VIM (Vimentin)</td>
<td>S34</td>
<td>Vimentin</td>
<td>138</td>
</tr>
<tr>
<td>Histone H3 (H3F3A)</td>
<td>S10</td>
<td>Histone H3.3</td>
<td>118</td>
</tr>
<tr>
<td>PBK</td>
<td>Y74</td>
<td>Lymphokine-activated killer T-cell-originated protein kinase</td>
<td>114</td>
</tr>
<tr>
<td>SIK3 (QSK)</td>
<td>Pan-specific</td>
<td>Serine/threonine-protein kinase SIK3</td>
<td>113</td>
</tr>
<tr>
<td>CDK6</td>
<td>Y13</td>
<td>Cyclin-dependent protein-serine kinase 6</td>
<td>101</td>
</tr>
<tr>
<td>CDK1 (CDC2)</td>
<td>T14</td>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>97</td>
</tr>
<tr>
<td>CDK1 (CDC2)</td>
<td>T161</td>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>97</td>
</tr>
<tr>
<td>Chk2 (CHEK2)</td>
<td>T68</td>
<td>Checkpoint protein-serine kinase 2</td>
<td>90</td>
</tr>
<tr>
<td>p70 S6K (RPS6KB1, p70S6Ka)</td>
<td>Pan-specific</td>
<td>Ribosomal protein S6 kinase beta-1</td>
<td>86</td>
</tr>
<tr>
<td>EGFR</td>
<td>Y998</td>
<td>Epidermal growth factor receptor-tyrosine kinase</td>
<td>85</td>
</tr>
<tr>
<td>MLK3 (MAP3K11)</td>
<td>Pan-specific</td>
<td>Mixed-lineage protein-serine kinase 3</td>
<td>79</td>
</tr>
<tr>
<td>EGFR</td>
<td>T693</td>
<td>Epidermal growth factor receptor-tyrosine kinase</td>
<td>143</td>
</tr>
<tr>
<td>GSK3b</td>
<td>Pan-specific</td>
<td>Glycogen synthase-serine kinase 3 beta</td>
<td>81</td>
</tr>
<tr>
<td>Target Protein Name</td>
<td>Phospho Site (Human)</td>
<td>Full Target Protein Name</td>
<td>% ΔsagB/wt</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------------</td>
<td>--------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Cbl</td>
<td>Y700</td>
<td>Signal transduction protein CBL</td>
<td>78</td>
</tr>
<tr>
<td>CDK11A</td>
<td>T583</td>
<td>Cell division cycle 2-like 2 protein kinase</td>
<td>74</td>
</tr>
<tr>
<td>Huntingtin</td>
<td>S421</td>
<td>Huntington's disease protein</td>
<td>73</td>
</tr>
<tr>
<td>ACK1 (TNK2)</td>
<td>Pan-specific</td>
<td>Activated CDC42 kinase 1</td>
<td>72</td>
</tr>
<tr>
<td>CDC7</td>
<td>T376</td>
<td>Cell division cycle 7-related protein kinase</td>
<td>71</td>
</tr>
<tr>
<td>SLK (STK2)</td>
<td>S189</td>
<td>STE20-like serine/threonine-protein kinase</td>
<td>71</td>
</tr>
<tr>
<td>MEK5 (MAP2K5, MKK5)</td>
<td>Pan-specific</td>
<td>MAPK/ERK protein-serine kinase 5 (MKK5)</td>
<td>70</td>
</tr>
<tr>
<td>SOX9</td>
<td>S181</td>
<td>SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)</td>
<td>69</td>
</tr>
<tr>
<td>CDK6</td>
<td>Y24</td>
<td>Cyclin-dependent protein-serine kinase 6</td>
<td>65</td>
</tr>
<tr>
<td>CDK10</td>
<td>T196</td>
<td>Cyclin-dependent protein-serine kinase 10 [PISSLRE]</td>
<td>64</td>
</tr>
<tr>
<td>CDK12</td>
<td>T893</td>
<td>Cell division protein kinase 12</td>
<td>64</td>
</tr>
<tr>
<td>CDK5</td>
<td>Y15</td>
<td>Cyclin-dependent protein-serine kinase 5</td>
<td>62</td>
</tr>
<tr>
<td>Krs-2</td>
<td>Pan-specific</td>
<td>Mammalian STE20-like protein-serine kinase 1 (KRS2)</td>
<td>61</td>
</tr>
<tr>
<td>Krs-1</td>
<td>Pan-specific</td>
<td>Protein-serine kinase suppressor of Ras 1</td>
<td>60</td>
</tr>
<tr>
<td>Chk1 (CHEK1)</td>
<td>S345</td>
<td>Checkpoint protein-serine kinase 1</td>
<td>60</td>
</tr>
<tr>
<td>CDK9</td>
<td>T186</td>
<td>Cyclin-dependent protein-serine kinase 9</td>
<td>60</td>
</tr>
<tr>
<td>JAK2</td>
<td>Pan-specific</td>
<td>Janus protein-tyrosine kinase 2</td>
<td>58</td>
</tr>
<tr>
<td>p70 S6K (RPS6KB1, p70S6Ka)</td>
<td>T252</td>
<td>Ribosomal protein S6 kinase beta-1</td>
<td>58</td>
</tr>
<tr>
<td>Csk</td>
<td>Pan-specific</td>
<td>C-terminus of Src tyrosine kinase</td>
<td>58</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>Pan-specific</td>
<td>Platelet-derived growth factor receptor kinase alpha</td>
<td>57</td>
</tr>
<tr>
<td>Target Protein Name</td>
<td>Phospho Site (Human)</td>
<td>Full Target Protein Name</td>
<td>% ΔsagB/wt</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------</td>
<td>--------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>SIK3 (QSK)</td>
<td>T163</td>
<td>Salt-inducible kinase 3</td>
<td>57</td>
</tr>
<tr>
<td>GluR1</td>
<td>S849</td>
<td>Glutamate receptor 1</td>
<td>56</td>
</tr>
<tr>
<td>MKK4 (MAP2K4, MEK4)</td>
<td>Pan-specific</td>
<td>MAPK/ERK protein-serine kinase 4 (MKK4)</td>
<td>56</td>
</tr>
<tr>
<td>MAPKAPK2 (RPS6KC1)</td>
<td>Pan-specific</td>
<td>Mitogen-activated protein kinase-activated protein kinase 2</td>
<td>56</td>
</tr>
<tr>
<td>CDK1 (CDC2)</td>
<td>T14+Y15</td>
<td>Cyclin-dependent protein-serine kinase 1/2</td>
<td>55</td>
</tr>
<tr>
<td>SIT</td>
<td>Y95</td>
<td>Signaling threshold-regulating transmembrane adapter 1</td>
<td>54</td>
</tr>
<tr>
<td>CDK7</td>
<td>T170</td>
<td>Cyclin-dependent protein-serine kinase 7</td>
<td>52</td>
</tr>
<tr>
<td>CDK1 (CDC2)</td>
<td>Pan-specific</td>
<td>Cyclin-dependent protein-serine kinase 1</td>
<td>51</td>
</tr>
<tr>
<td>TAK1 (MAP3K7)</td>
<td>S439</td>
<td>TGF-beta-activated protein-serine kinase 1</td>
<td>51</td>
</tr>
<tr>
<td>CDK12</td>
<td>S383+S385</td>
<td>Cell division protein kinase 12</td>
<td>49</td>
</tr>
<tr>
<td>STAT5B</td>
<td>Pan-specific</td>
<td>Signal transducer and activator of transcription 5B</td>
<td>49</td>
</tr>
<tr>
<td>STAT3</td>
<td>Y705</td>
<td>Signal transducer and activator of transcription 3</td>
<td>-50</td>
</tr>
<tr>
<td>FAK</td>
<td>Y576+Y577</td>
<td>Focal adhesion protein-tyrosine kinase</td>
<td>-53</td>
</tr>
<tr>
<td>MEKK2 (MAP3K2)</td>
<td>S239</td>
<td>MAPK/ERK kinase kinase 2</td>
<td>-58</td>
</tr>
</tbody>
</table>
APPENDIX B:

METABOLIC MAPS OF PROTEINS IDENTIFIED FROM 2D PROTEOMIC STUDIES

Figure B.1: Threonine synthase role in Vitamin B6 metabolism from spot wt 1.
Figure B.2: Role of 2-oxoisovalerate E1 subunit alpha in Valine, Leucine, and Isoleucine degradation from wt spot 1.
Figure B.3: Role of carbamate kinase 2 in arginine biosynthesis from wt spot 1.
Figure B.4: Role of succinate CoA ligase ADP forming alpha subunit in carbon fixation from wt spot 1.
Figure B.5: Role of thioredoxin reductase in pyrimidine metabolism from wt spot 1.
Figure B.6: Role of putative glucokinase ROK family in amino sugar and nucleotide sugar metabolism from wt spot 1.
Figure B.7: Role of D-isomer specific 2-hydroxyacid dehydrogenase in pyruvate metabolism from wt spot 1.
Figure B.8: Role of uroporphyrinogen decarboxylase (4.1.1.37) in porphyrin and chlorophyll metabolism from ΔsagB spot 4
Figure B.9: Role of FolD (3.5.4.9) in one carbon pool by folate from ΔsagB spot 4.
Figure B.10: Role of hydroxymethylbilane synthase (2.5.1.61) in porphyrin and chlorophyll metabolism from ΔsagB spot 4.
Figure B.11: Role of FAD dependent pyridine nucleotide disulphide oxidoreductase in pyrimidine metabolism from ΔsagB spot 4.
BIBLIOGRAPHY


108


Dysfunction Affects 
Staphylococcal Cassette Chromosome mec Type-Dependent Clinical Outcomes in 
Methicillin-Resistant Staphylococcus aureus Bacteremia. 
Antimicrob. Agents 
Chemother. [Internet]. 2015;59(6):3125–3132. Available from: 
http://aac.asm.org/content/59/6/3125.abstract

Prevalence of agr Dysfunction among Colonizing Staphylococcus aureus Strains. 
J. Infect. Dis. [Internet]. 2008;198(8):1171–1174. Available from: 

Mutations in agr Do Not Persist in Natural Populations of 
Methicillin-Resistant Staphylococcus aureus. J. Infect. Dis. [Internet]. 

McDanel JS, Perencevich EN, Diekema DJ, Winokur PL, Johnson JK, Herwaldt LA, 
et al. Association between microbial characteristics and poor outcomes among 
patients with methicillin-resistant Staphylococcus aureus pneumonia: a 
retrospective cohort study. Antimicrob. Resist. Infect. Control [Internet]. 
2015;4:51. Available from: 

Ji G, Beavis R, Novick RP. Bacterial interference caused by autoinducing peptide 

Exfoliatin-producing strains define a fourth agr specificity group in Staphylococcus 

Lyon GJ, Mayville P, Muir TW, Novick RP. Rational design of a global inhibitor of 
the virulence response in Staphylococcus aureus, based in part on localization of 
the site of inhibition to the receptor-histidine kinase, AgrC. Proc. Natl. Acad. Sci. 
U. S. A. 2000;97(24):13330–13335

Effectiveness of Alpha-toxin Fab Monoclonal Antibody Therapy in Limiting the 
Pathology of Staphylococcus aureus Keratitis. Ocul Immunol Inflamm [Internet]. 
2014;(April):1–7. Available from: 


entrezz&rendertype=abstract


[162] Iyer R, Baliga NS, Camilli A. Catabolite Control Protein A (CcpA) Contributes to Virulence and Regulation of Sugar Metabolism in Streptococcus pneumoniae. Catabolite Control Protein A (CcpA) Contributes to Virulence and Regulation of Sugar Metabolism in Streptococcus pneumoniae. 2005;187(24):8340–8349

