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The Role and Regulation of NsaRS: a Cell-Envelope Stress Sensing Two-Component System in Staphylococcus aureus

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The Role and Regulation of NsaRS: a Cell-Envelope Stress Sensing Two-Component System in *Staphylococcus aureus*

by

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Table of Contents

Table of Tables iv

Table of Figures v

Abstract viii

Chapter 1: Introduction 1
The Staphylococci 1
Staphylococcus aureus 2
The diseases of *S. aureus* 3
Virulence factors of *S. aureus* 5
The regulation of virulence factors in *S. aureus* 9
Antibiotic resistance of *S. aureus* 14
Hosptial-acquired and community-acquired *S. aureus* 16
Two-component systems 19
Structure and function of histidine protein kinases 23
Structure and function of response regulators 23
The occurrence of two-component systems in prokaryotes 26
Intramembrane-sensing histidine kinases 27
Subgroups of intramembrane-sensing histidine kinases 27
Two-component systems of *S. aureus* 30
The SaeRS two-component system of *S. aureus* 33
The VraRS system of *S. aureus* 33
The GraRS two-component system of *S. aureus* 34
Project aim 36

Chapter 2: The role and regulation of NsaRS: a cell-envelope stress-sensing two-component system of *S. aureus* 38
Introduction 38
Results 42

Chapter 3: The role and regulation of the ABC transporter, SACOL2525/2526 of *S. aureus* 43
Introduction 43
Materials and methods 46
Bacterial strains, plasmids and growth conditions 46
Construction of the SACOL2525/2526 mutant strain 46
Construction of the SACOL2525/2526 reporter fusion strain 48
Construction of SACOL2525/2526 complemented strain 48

Plate-based assay for determination of altered transcription from external stress....49
Real-time PCR....50
Microtitre MIC Assay for cell-wall targeting drugs....50
Survival in the free unsaturated fatty acid, oleic acid....50
Survival in toluene....51
Negative staining electron microscopy....51
Hydrophobicity assay using hexadecane....51
Cytochrome C binding assay....51
Peptidoglycan lysis kinetics assay....52
Survival in daptomycin....52
Lysostaphin treatment for removal of the cell envelope....52
Spontaneous mutation frequency....52
Results....54
In silico analysis of SACOL2525/2526....54
Transcriptional profiling of the SACOL2525/2526 ABC transporter....56
NsaRS, but not GraRS, regulates transcription of SACOL2525/2526 in response to a variety of cell-envelope-targeting compounds....63
SACOL2525/2526 aids in resistance to daptomycin....65
SACOL2525/2526 aids in the ability of *S. aureus* to spontaneously resist the action of a variety of cell-envelope targeting antibiotics....74
SACOL2525/2526 is part of the *S. aureus* resistance mechanism against toxic non-antibiotic chemicals that target the cell membrane....76
SACOL2525/2526 mutants of *S. aureus* have altered cell envelope architecture....80
SACOL2525/2526 contributes to protection against lysostaphin....87
Discussion....89

Chapter 4: The role of secreted proteases in Community Acquired-Methicillin Resistant *Staphylococcus aureus* infections....103
Introduction....103
Manuscript introduction....109
Materials and methods....113
Ethics Statement....113
Bacterial strains, plasmids and growth conditions....113
Real-time PCR....114
Whole human blood survival assay....114
Antimicrobial peptide sensitivity assay....114
Murine model of wound formation....115
Murine model of sepsis and dissemination....116
Proteomic analysis of surface and secreted proteins....116
Results....119
Verification of extracellular protease depletion in the LAC-

protease null strain 119
The protease-null strain has fitness defects during growth in peptide based media and pig serum 121
Secreted proteases play a role in resistance to antimicrobial peptides 124
Extracellular proteases are required for survival during interaction with the innate immune system 126
Secreted proteases contribute to CA-MRSA wound formation 126
Extracellular proteases play a key role during systemic CA-MRSA infections 128
Secreted proteases are primary effectors of virulence determinant stability 131
Discussion 143
Acknowledgments 150

Chapter 5: Final discussion 151
Discussion 151

References cited 169

Appendix 1 203

Table of Tables

Table 1: *S. aureus* possesses 16 two-components systems.....31

Table 2: Strains, plasmids and primers used in this study 47

Table 3: Stressor compounds used in the plate-based assay to determine the inducibility of SACOL2525/2526 expression 60

Table 4: Cell-envelope-targeting antibiotics utilized to determine the transcription of SACOL2525/2526 under cell-envelope stress 62

Table 5: Collective alterations in protein abundance for secreted proteins upon deletion of extracellular proteases in *S. aureus* USA 300 133

Table 6: Individual fraction analysis of alterations in protein stability for secreted proteins upon deletion of extracellular proteases in *S. aureus* USA 300.....135

Table 7: Collective alterations in protein abundance for surface proteins upon deletion of extracellular proteases in *S. aureus* USA 300 137

Table 8: Individual fraction analysis of alterations in protein stability for surface proteins upon deletion of extracellular proteases in *S. aureus* USA 300.....138

Table of Figures

Figure 1: The regulation of virulence factors in a growth phase dependent manner.....11

Figure 2: A functional Schematic of Two-Component Systems 20

Figure 3: The phosphotransfer reactions of two-component systems 22

Figure 4: The architectural features of histidine kinases 24

Figure 5: The architectural features of response regulators.....25

Figure 6: Predicted topology plot of the IM-HK, SACOL2645 from *S. aureus* 28

Figure 7: The NsaRS/NsaAB locus from *Staphylococcus aureus*.....40

Figure 8: Amino acid sequence alignment of SACOL2525 55

Figure 9: The SACOL2525/2526 operon 57

Figure 10: Transcription of SACOL2525/2526 during growth in standard conditions.....58

Figure 11: Inducibility of SACOL2525/2526 by antibacterial agents.....64

Figure 12: The impact of GraRS and NsaRS on the transcription of SACOL2525/2526 66

Figure 13: SACOL2525/2526 do not present enhanced sensitivity to a number of cell-wall targeting chemicals 68

Figure 14: SACOL2525/2526 aids in the resistance of *S. aureus* to daptomycin 69

Figure 15: Lysostaphin treatment does not lead to increase death in the SACOL2525/2526 mutant 70

Figure 16: SACOL2525/2526 mutants have a weakened cell membrane 71

Figure 17: SACOL2525/2526 mutant protoplasts have elevated sensitivity to daptomycin.....73

Figure 18: SACOL2525/2526 aids in the ability to spontaneously resist the action of a variety of cell-envelope-targeting antibiotics 75

Figure 19: SACOL2525/2526 mutation protects the cell in the presence of free unsaturated fatty acids.....78

Figure 20: Transcription of SACOL2525/2526 in the presence of oleic acid 79

Figure 21: SACOL2525/2526 contributes to resistance against solvent stress 81

Figure 22: SACOL2525/2526 mutants have increased capsule production 82

Figure 23: SACOL2525/2526 mutant cells have altered hydrophobicity 84

Figure 24: SACOL2525/2526 deletion does not effect cell surface charge 86

Figure 25: SACOL2525/2526 mutants have decreased sensitivity to the cell wall targeting agent lysostaphin 88

Figure 26: Schematic representation of peptidoglycan synthesis of *S. aureus*.....91

Figure 27: The extracellular proteases of *S. aureus* and their regulation 105

Figure 28: Activation cascade of *S. aureus* secreted proteases 106

Figure 29: Gelatin zymography of the LAC wild-type and its protease-null mutant 120

Figure 30: Exoprotease deletion does not lead to observable difference in *agr* or *hla* transcript levels 122

Figure 31: Secreted proteases aid in survival during growth in peptide based media and pig serum 123

Figure 32: Secreted proteases play a role in the resistance of *S. aureus* to antimicrobial peptides.....125

Figure 33: Secreted proteases are protective during interaction with the human innate immune system.....127

Figure 34: Secreted proteases contribute to CA-MRSA abscess formation 129

Figure 35: Secreted proteases have contrasting roles in the morbidity and mortality of systemic CA-MRSA infections.....130

Figure 36: Extracellular proteases modulate the stability of a wealth of known secreted virulence factors.....137

Figure 37: Extracellular proteases modulate the stability of a number of surface-associated virulence factors 141

Figure 38: Transcription of *nsaRS*, *nsaAB* and SACOL2525/2526 in response to cell-envelope-targeting drugs.....153

Figure 39: NsaRS regulates NsaAB and SACOL2525/2526 in response to different cell envelope stress.....156

Abstract

S. aureus has 16 predicted two-component systems (TCS) that respond to a range of environmental stimuli, and allow for adaptation to stresses. Of these 16, three have no known function, and are not homologous to any other TCS found in closely related organisms. NsaRS is one such element, and belongs to the intramembrane-sensing histidine kinase (IM-HK) family, which is conserved within the Firmicutes. The regulators are defined by a small sensing domain within their histidine kinase, suggesting that they do not sense external signals, but stress in or at the membrane. Our characterization of NsaRS in this work reveals that, as with other IM-HK TCS, it responds to cell-envelope damaging antibiotics, including phosphomycin, ampicillin, nisin, gramicidin, CCCP and penicillin G. Additionally; we reveal that NsaRS regulates a downstream transporter, NsaAB, during nisin-induced stress. Phenotypically, *nsaS* mutants display a 200-fold decreased ability to develop resistance to another cell-wall targeting antibiotic, bacitracin. Microarray analysis reveals the transcription of 245 genes is altered in a *nsaS* mutant, with the vast majority down-regulated. Included within this list are genes involved in transport, drug-resistance, cell-envelope synthesis, transcriptional regulation, amino acid metabolism and virulence. Using ICP-MS, a decrease in intracellular divalent metal ions was observed in an *nsaS* mutant, when grown under low abundance conditions. Characterization of cells using electron microscopy reveals that *nsaS* mutants also have alterations in cell-envelope structure. Finally, a

variety of virulence related phenotypes are impaired in *nsaS* mutants, including biofilm formation, resistance to killing by human macrophages and survival in whole human blood. Thus NsaRS is important in sensing cell wall damage in *S. aureus*, and functions to reprogram gene expression to modify cell-envelope architecture, facilitating adaptation and survival. Interestingly, in our microarray analysis, we observed a more than 30-fold decrease in transcription of an ABC transporter, SACOL2525/2526, in the *nsaS* mutant. This transporter bears strong homology to nsaAB, and is currently uncharacterized. Exploration of the role of SACOL2525/2526 revealed that, along with NsaRS, it too responds to cell-envelope damaging antibiotics. Specifically, its expression was induced by phosphomycin, daptomycin, penicillin G, ampicillin, oxacillin, D-cycloserine and CCCP. Mutation of this transporter results in increased sensitivity to the antibacterial agent daptomycin, and decreased sensitivity to free fatty acids. These findings are perhaps explained by altered membrane fluidity in the mutant strain, as the transporter null-strain is more readily killed in the presence of organic solvents, such as toluene. In addition, SACOL2525/2526 mutants have a decreased ability to form spontaneous mutants in response to several other peptidoglycan synthesis targeting antibiotics, suggesting a role for SACOL2525/2526 in antibiotic resistance. Inactivation of this transporter alters the cell envelope, and produces similar effects to those observed with the *nsaS* mutant, with increased capsule production, that may provide resistance to lysostaphin. Interestingly, the *nsaS* microarray revealed that this TCS negatively regulates only 34 genes, including 6 out of the 10 major secreted proteases. Despite a number of reports in the literature describing these enzymes as virulence factors, the data is often conflicting. Therefore, the contribution of proteases to CA-MRSA pathogenesis

was investigated, by constructing a strain lacking all 10 extracellular protease genes. Analysis of this strain using murine models of infection reveals secreted proteases significantly impact virulence in both localized and systemic infections. Additionally, inactivation of these enzymes strongly influences survival in whole human blood, and increases sensitivity to antimicrobial peptides. Using a proteomics approach, we demonstrate that the contribution of secreted proteases to pathogenicity is related to differential processing of a large number of surface-associated virulence factors and secreted toxins. Collectively these findings provide a unique insight into the role of secreted proteases in CA-MRSA infections.

Chapter 1: Introduction

Introduction

The Staphylococci. Within the Firmicute phylum and the Staphylococcaceae family is the genus Staphyloccoci. Staphylococci are spherical cells about 1 micrometer in diameter and divide successively in more than one plane, as opposed to their closely related relatives, the streptococci, which form chains. In Greek, staphyle means "bunch of grapes", corresponding to the clustered appearance of the bacteria when viewed under a microscope. They are categorized as Gram-positive bacteria, and have a thick layer of peptidoglycan surrounding a single lipid bilayer membrane (Kloos et al., 1991). The staphylococcal cell wall is distinct due to its high glycine content, which makes them susceptible to cell wall degradation by lysostaphin (Grundling & Schneewind, 2006). This genus is comprised of non-spore forming, non-motile, facultative anaerobes that are catalase-positive and oxidase-negative. They can generate energy through respiration or fermentation (Woese, 1987). Thus far, there have been 36 species and 18 subspecies identified within the Staphylococci genus (Cheung et al., 2006). The Staphylococci are found everywhere in nature, including soil and water, and are able to remain on inanimate objects for long periods of time. Commonly Staphylococci also inhabit the skin and mucus membranes of mammals (Noble & Pitcher, 1978). The majority of this genus is coagulase-negative, including *S. epidermidis* and *S. hominis*. However, one characteristic

that is used to distinguish *S. aureus* from other Staphylococci in the laboratory is that they are coagulase-positive. In addition, most are staphylococci are non-pathogenic, further distinguishing *S. aureus* from its relatives (Klevens et al., 2007).

Staphylococcus aureus. *S. aureus* isolates contain a circular chromosome composed of approximately 2.8 million base pairs, although one or more plasmids may also be included. The chromosome contains about 2700 coding sequences, as well as regulatory RNAs. Many genes have assigned functions through homology with closely related organisms, although only a small portion have been characterized. The genome consists of coding sequences (CDs) involved in cell division and replication (0.5%), chaperones (0.3%), adaptation and protection responses (1.7%), transport proteins (9.2%), macromolecule degradation (1.5%), macromolecule biosynthesis (4.7%), cofactor and carriers biosynthesis (0.6%), central and intermediary metabolism (2.2%), small molecules degradation (3.2%), energy metabolism (1.4%), fatty acid synthesis (0.6%), nucleotide biosynthesis (0.9%), cell envelope metabolism (20.3%), ribosome components (2.4%), mobile genetic elements (7.2%), regulators (4.7%), toxins (0.8%) and pseudogenes (2.6%). In addition, approximately 38% of the entire genome has no known function, and a further 10% has only a tentative function (Suzuki et al., 2012).

The meaning of aureus is golden, which refers to the large colored colonies produced by *S. aureus* on rich media; resulting from the carotenoid pigment staphyloxanthin (Marshall & Wilmoth, 1981). *S. aureus* is found everywhere in nature, forms part of the normal skin flora of warm blooded animals and resides in the anterior nares of one in three people (Schechter-Perkins et al., 2011). *S. aureus* is able to survive on the skin or in the

nose asymptomatically, but can gain access to breaches in the skin that may lead to disease (Foster, 2009). *S. aureus* is commonly transmitted through skin to skin contact with a colonized or infected person. Places that are more crowded, such as military camps, sports teams or dormitories increase the risk of dissemination (Ben-David et al., 2008). This high human prevalence and ease of transmission largely accounts for why *S. aureus* is the most common cause of infection within the Staphylococci (Lowy, 1998).

The diseases of *S. aureus.* *S. aureus* is the most common cause of infection by a single agent in the United States (Kobayashi & DeLeo, 2009; Lowy, 1998). People with diabetes, AIDS, indwelling intravenous catheters, intravenous drug users, surgical and cancer patients have the highest risk of contracting *S. aureus* infections; however as an opportunistic pathogen, all population groups are at risk (Frank, 1997, Zimakoff et al., 1996). Disease normally starts as a localized infection via breaches of the skin, which can result in abscesses or furuncles. These are commonly known as boils and are the most common presentation of *S. aureus* skin infections (Ruhe et al., 2007; Frazee et al., 2005; Miller et al., 2007). Such conditions can subsequently proceed to more serious and deep-seated diseases of the hair follicles, known as carbuncles. In addition, *S. aureus* can also cause cellulitis, hydradenitis, suppurtiva, impetigo, mastitis, pyodermas and pyomyositis (Stack & Sanchez, 2007; Koning et al., 2003). Many of these infections appear red, swollen and painful with liquid or pus exudates. Some may be resolved with antibiotic therapy, but the majority are treated by surgical drainage. Ultimately, *S. aureus* is believed to cause greater than 50% of all skin infections in the United States (Abrahamian & Moran, 2007; Talan et al., 2011).

From these localized sites of infection, *S. aureus* can then enter the bloodstream causing bacteremia. Bacteremia is associated with high mortality and cost, which places a large burden on healthcare systems (Shorr & Lodise, 2006; Steinberg et al., 1996). Percent mortality from *S. aureus* bacteremia ranges from 15% to as high as 80% without treatment (Mortara & Bayer, 1993; Selvey et al., 2000; Shorr,, 2006, Naber, 2009). Once *S. aureus* gains access to the bloodstream it can then disseminate to other organs and cause systemic infection. For example, *S. aureus* can cause osteomyelitis, which is an infection of bone or bone marrow (Lew & Waldvogel, 2004). Acute osteomyelitis is often seen in children, where it infects the long bones of the lower extremities (Waldvogel & Vasey, 1980; Carek et al., 2001). In adults, osteomyelitis is more commonly seen in vertebral bodies (Carek et al., 2001). *S. aureus* is also able to colonize the lungs, causing pneumonia, which is mostly seen in patients that have recently had surgery, have chronic lung disease, are older or are immunocompromised (Kollef et al., 2005). In one study, the mortality rate of patients with pneumonia caused by *S. aureus* was at least 31% (Jeffres et al., 2006). Antibiotic therapy is used to treat *S. aureus* pneumonia, but must be aggressive, and may necessitate the use of harsh drugs, such as vancomycin, for several weeks (Micek et al., 2004).

Surgical site infections are a major health concern since they are associated with high morbidity and health care costs. *S. aureus* is the most common cause of surgical site infections among patients in the ICU (Solomkin, 2001; Grimble et al., 2001). A major reason for this is the presence of *S. aureus* on the skin and mucus membranes of patients and operating room staff (Drinka et al., 2001).

In addition to these infections, which are caused by live, viable *S. aureus*, toxinoses, such as food poisoning, scalded skin syndrome and toxic shock syndrome are caused by toxins that are secreted by this organism. Food poisoning results from ingesting one of several staphylococcal enterotoxins (Holmberg & Blake, 1984), with symptoms usually including nausea, vomiting, headache and diarrhea. The average time of symptom onset after ingesting an enterotoxin is 4.4 hours (LeLoir et al., 2003), and the condition is typically self limiting, and does not require antibiotics or hospitalization. Toxic shock syndrome results from toxic shock syndrome toxin 1 (TSST-1), and can lead to symptoms such as fever, hypotension, rash and hyperemia of the mucus membranes. TSST-1 is a superantigen that elicits the production of multiple cytokines, resulting in a rapid progression of infection that can cause life threatening complications. This syndrome has been associated with high absorbent tampons (Fekety, 1964), and treatment is usually the removal of any material connected to disease, alongside antibiotic therapy.

Virulence factors of *S. aureus*. *S. aureus* is a successful pathogen, causing a wide variety of diseases, in part because of the plethora of virulence factors it produces. Virulence factors are molecules expressed by the pathogen that allow it to survive within the host, evade the immune system, invade and colonize, or obtain nutrients (Casadevall & Pirofski,2009). The virulence process has multiple steps including: adhesion to the surface of host components, escape from the host immune system, and dissemination and tissue invasion (Ferry et al., 2005; Cheng et al., 2011). The ability of *S. aureus* to adhere to extracellular matrix proteins is thought to be essential for colonization and the establishment of infections (Patti et al., 1994; Pohlmann-Dietze et al., 2000). Microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) are anchored

to the peptidoglycan layer of cells, and protrude outwards. These are used to attach to various host extracellular matrices including collagen, fibronectin and vitronectin (Foster & Hook, 1998). Fibronectin binding proteins, FnbA and FnbB, are involved in cell invasion by binding fibronectin, which facilitates uptake into the cell (Massey et al., 2001; Jonsson et al., 1991). Clumping factor, ClfA and ClfB, bind fibrinogen which leads to platelet aggregation (O'Brien et al., 2002). Clumping factor also protects the cell from phagocytosis by macrophages (Palmqvist et al., 2004). Extracellular adhesion protein (Eap) binds to several plasma proteins (fibrinogen, fibronectin, prothrobin and vitronectin), epithelial cells and fibroblasts (Palma et al., 1999; Chavakis et al., 2002; Hussain et al., 2002). Extracellular fibrinogen-binding protein (Efb) is secreted by *S. aureus* and has been shown to prevent platelet aggregation, which is involved in wound healing (Palma et al., 2001). Collectively, these virulence factors aid the cell in adhering to host tissues, and facilitate the creation of infection foci.

Other virulence factors are associated with escape from the host immune system. For example, protein A (Spa) is a cell-wall associated protein that binds to IgG. Normally IgG associates with bacteria so that neutrophils can recognize the foreign entity and facilitate phagocytosis, and destruction of the foreign organism. Protein A binding to IgG attenuates the immune response by blocking the ability of IgG to stimulate neutrophils, and ultimately inhibits phagocytosis. (Peterson et al., 1977). Protein A also binds IgM associated with B cells, which induces apoptosis of these key host defense cells. *S. aureus* also has another virulence factor that serves to bind immunoglobulins and host proteins, Sbi. Sbi, is able to bind the complement protein C3 and blocks the activation of the host complement pathway, allowing for *S. aureus* to evade the host immune system

(Haupt et al., 2008).The majority of *S. aureus* strains produce a capsule, with 11 serotypes thus far identified (Lee et al., 1994; Lin et al., 1994; Sau & Lee, 1996; Ouyang and Lee, 1997); however, serotypes 5 and 8 account for 75-80% of all *S. aureus* strains involved in human infections. Capsule allows *S. aureus* to evade the host immune system by increasing resistance to phagocytosis and preventing it from being killed by neutrophils and monocytes (Watts et al., 2005). Staphylokinase (Sak) is secreted by *S. aureus* and functions to convert human plasminogen bound to the bacterial cells into plasmin. This leads to plasmin activation, and cleavage of IgG and C3b, which inactivates both and prevents phagocytosis (Rooijakkers et al., 2005). Chemotaxis inhibitory protein (CHIPS) binds to human neutrophils and monocytes on the C5a receptors, which represses their chemotaxis and antibacterial activity (Haas et al., 2005; Wright et el., 2007). The staphylococcal complement inhibitor (SCIN) abrogates C3b binding to the bacteria cell, and thus prohibits phagocytosis (Rooijakkers et al., 2005). All of these virulence factors serve as a highly efficient defense to circumnavigate the effects of the host immune system.

Virulence factors are also employed for dissemination and host tissue invasion and include enterotoxins, toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxins, are all staphylococcal superantigens encoded on accessory mobile elements (Ferry et al., 2005). Exfoliative toxin A and B (*eta, etb*) cause a unique array of damage, from localized lesions to extensive exfoliation, and are the primary arbiters behind scalded-skin syndrome (Lee et al.,1987). *S. aureus* also produces extensive cytotoxins that are involved in tissue invasion and the destruction of host cells, causing the lysis of leukocytes, monocytes and/or erythrocytes. Specifically, α-hemolysin associates as a

hexamer to form pores in erythrocytes, resulting in lysis of the cell. Another mechanism *S. aureus* utilizes for such processes are bi-component pore-forming leukotoxins (Menestrina et al., 2003), such as: γ-hemolysin (HlgABC), LukSF-PVL, LukAB and LukED (Alonzo et al., 2012). Further to this, γ-hemolysin has been shown to contribute to *S. aureus* survival in blood and virulence in systemic murine models of infection by targeting polymorphonuclear cells and monocytes (Malachowa et al., 2011). The Panton-Valentine leukocidin (PVL) was found to induce lysis of human polymorphonuclear neutrophils, monocytes and macrophages and seems to contribute to the ability *S. aureus* to cause pneumonia (Voyich et al., 2006; Labandeira-Rey et al., 2007; Diep et al., 2010; Loffler et al., 2010). Recently the LukAB leukotoxins were identified and shown to hinder killing by neutrophils (Dumont et al., 2011). The LukED proteins are the newest identified leukotoxins and have been shown to target and kill murine phagocytes (Alonzo et al., 2012).

In addition to bipartite toxins, another group of proteins, the phenol soluble modulins (PSMs), have also been implicated in tissue invasion. PSMs share a common alpha helical region that is thought to disrupt cell membranes and induce host cell lysis (Wang et al., 2007). Several lipases are also produced by *S. aureus*: the *geh* gene encodes a glycerolester hydrolase that cleaves long-chain and water-soluble triacylglycerols (Lee & Iandolo, 1986; Arvidson, 2000), whilst an esterase, encoded by *lip*, cleaves short-chain triacylglycerides. It has been suggested that lipases are chemotactic to granulocytes and decrease phagocytic killing (Rollof et al., 1988), however the role of these esterases remains unclear. In addition, two phospholipase C proteins have been identified, sphingomyelinase or β-hemolysin, and phosphatidylinositol-specific phospholipase C.

Although these are known to contribute to virulence, their exact role and host target (s) are unknown. Fatty acid modifying enzyme (FAME) catalyzes the esterfication of long-chain free fatty acids to generate cholesterol, allowing enhanced invasiveness in abscesses by diminishing the bactericidal effects of host lipids (Chamberlain and Imanoel, 1996). Additionally, coagulase, a cell surface bound virulence factor, associates with prothrombin and forms staphylothrombin, which converts fibrinogen to fibrin that coagulates serum (Wegrynowicz et al., 1980). *S. aureus* also secretes ten major proteases that aid in dissemination and tissue invasion by cleaving host proteins. Secreted proteases have been shown to cleave the human protease inhibitor α1-proteinase inhibitor (Potempa et al., 1986), the heavy chains of all human immunoglobulin classes (Prokesova et al., 1992) and elastin (Potempa et al., 1988). Staphopain B was shown to cleave human fibronectin, fibrinogen and kininogen (Coulter et al., 1998). These secreted proteases may contribute to the switch from adhesive to invasive phenotypes, as they cleave *S. aureus* surface, as well as other secreted virulence factors (McGavin et al., 1997; Karlsson et al., 2001; McAleese et al., 2001; Lindsay & Foster, 1999). Although secreted proteases have been suggested to be important virulence factors, the scope of proteins that they cleave is currently unclear.

The regulation of virulence factors in *S. aureus*. The success of *S. aureus* in causing disease is attributed not only to these virulence determinants, but in the rapid environmental sensing and adaptation of their expression via precise genetic regulation. The disease process begins with regulatory elements, which *S. aureus* has a multitude of, including sigma factors, DNA binding proteins, regulatory RNAs and two-component systems. Virulence factor production is tightly regulated, and correlates with classical

growth phases experienced by bacteria (Figure 1). Shortly after entry into exponential phase, adhesive surface proteins are expressed, including protein A, fibronectin binding proteins A and B and clumping factors A and B. In addition, expression of the cytotoxin, delta-hemolysin is also activated at this time, and remains expressed throughout post-exponential and stationary growth (Schlievert et al., 2010). Other secreted virulence factor expression correlates with bacterial population density, and is activated by a quorum sensing system, *agr* (accessory gene regulator) later in growth. The *agr* system of *S. aureus* is a global regulatory system that has been extensively studied and regulates most staphylococcal virulence factors (Recsei et al., 1986). The *agr* operon contains four genes, *agrABCD* that are transcribed from promoter 2 (P2, producing RNAII), alongside promoter 3 (P3, producing RNAIII), which is divergent, and produces δ-hemolysin (*hld*) (Novick et al., 1995).

AgrD is a 46 amino acid protein that is cleaved by two proteolytic digestions during the exponential growth phase, before being secreted by AgrB. This results in a cyclic peptide that is seven to nine amino acids in length, depending on the strain, and is referred to as autoinducing peptide (AIP) (Ji et al., 1997). AgrC is a 46 kDa membrane protein that has six transmembrane domains and is the histidine kinase of a two-component system composed of AgrC and AgrA. AgrC contains a sensor domain which recognizes the pheromone AIP, which, once a threshold concentration is reached, leads to AgrC autophosphorylation at a conserved histidine residue in the cytoplasmic portion of this protein. This phosphate is then passed to AgrA, which is a 28 kDa response regulator that belongs to the AlgR/AgrA/LytR family of DNA binding proteins (Nikolskaya & Galperin, 2002). AgrA subsequently binds to both *agr* promoters, although its affinity for

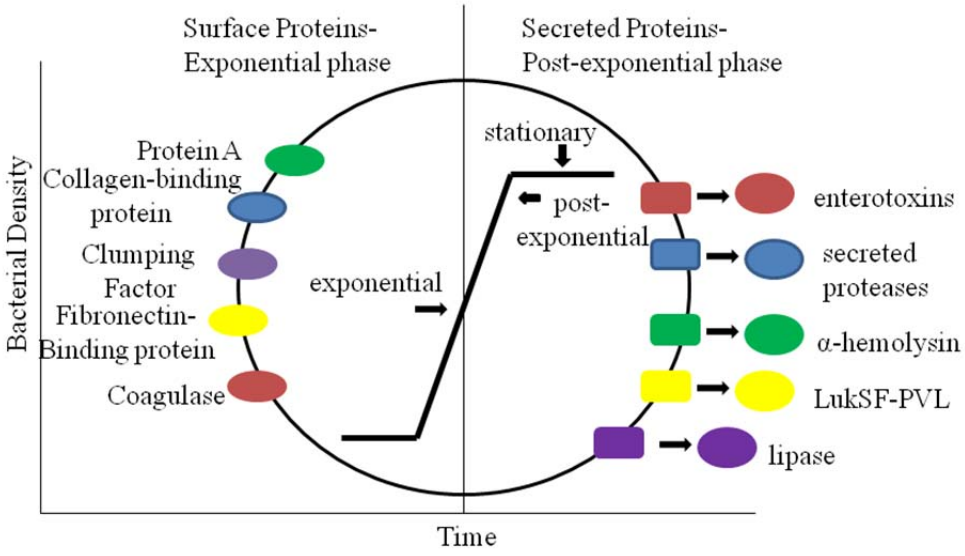

Figure 1. The regulation of virulence factors in a growth phase dependent manner. Virulence factors are regulated in a growth phase dependent manner. During exponential phase several surface-associated virulence factors, including, protein A, collagen-binding protein, clumping factor, fibronectin-binding protein and coagulase are expressed. As bacterial density increases, *S. aureus* switches from expressing adhesive surface proteins to secreted proteins. During the post-exponential/stationary phases secreted virulence factors are produced, including enterotoxins, secreted proteases, α-hemolysin, PVL and lipases.

P3 is considerably greater than for P2 (Koenig et al., 2004). RNAIII, produced from P3, is a 0.5 kb transcript whose levels increase during growth, and is maximally observed during post-exponential phase (Vandenesch et al., 1991). RNAIII ultimately serves as the effector molecule of the *agr* system, seemingly by antisense mechanisms.

RNAIII was previously shown to regulate the α–toxin gene, *hla*, as transcript levels for this hemolysin in a RNAIII mutant are one tenth of those in a wild-type strain, with protein levels concomitantly reduced by 70-fold (Novick et al., 1993). The mRNA of *hla* contains a long 5' untranslated region of 330 nucleotides, which forms a hairpin that contains and blocks the Shine-Dalgarno sequence (Novick et al., 1993). RNAIII binds to this untranslated region, and the Shine-Dalgarno sequence is exposed by RNaseT1. This binding stops the formation of a secondary structure, frees the ribosome binding site, and allows for translation. The observation that both transcription and translation of *hla* were impacted by RNAIII deletion suggests multiple methods of control are exerted by this element. This was shown only recently, upon the determination that RNAIII interacts with the mRNA for the DNA-binding protein Rot. The repressor of toxins (Rot) serves to represses virulence determinant genes regulated by Agr (Said-Salim et al., 2003). To relieve this repression, RNAIII binds to a complementary sequence on the *rot* mRNA, inhibiting its translation (Geisinger et al., 2006; Boisset et al., 2007).

Although Agr contributes greatly to the regulation of virulence factors, it is not the only regulator. The staphylococcal accessory regulatory locus (*sar*) was first identified by screening transposon mutants for decreased exoproteins production (Cheung et al., 1992). The *sar* locus consists of three transcripts that end at the same stem-loop sequence, but

sarA is the only functional product produced (Bayer et al., 1996; Manna et al., 1998). SarA can bind DNA as a dimer, however its target consensus sequence is currently unknown. It has also been suggested that SarA is similar to Fis and IHF proteins of *E. coli*, as SarA has similar characteristics as these proteins. These proteins regulate transcription by mediating structural changes in DNA and supercoiling (Schumacher et al., 2001). SarA has been shown to regulate many virulence factors, both positively and negatively. In addition to binding DNA, SarA regulates mRNA stability, as transcripts for several surface associated and secreted virulence factors have significantly shorter half-lives upon *sarA* deletion (Roberts et al., 2006). In addition to SarA, several Sar homologues have been identified as regulators of virulence factors. Specifically, SarR represses SarA and hence genes regulated by SarA (Manna & Cheung, 2006), whilst SarS is repressed by SarA and Agr (Tegmark et al., 2000; Cheung et al., 2001). SarT, another homologue, has been suggested to repress RNAIII (Schmidt et al., 2001). In total, 12 different members of the SarA family of DNA binding proteins have thus far been identified in the *S. aureus* genome (Schumacher et al., 2001 ; Liet al., 2003).

Agr and Sar-family proteins, although pleiotropic in action, are not the only virulence factor regulators. Another global regulatory element is the Sae system, which influences virulence factors independently of Agr and SarA (Giraudo et al., 1997). In addition to proteins that bind promoters to exert their effects, sigma factors also play a role in regulation. *S. aureus* also has an alternative sigma factor, σ^B, which regulates many virulence factors during stress situations via both direct and indirect mechanisms (Cheung et al., 1999; Nicholas et al., 1999). Collectively, the regulatory network of

virulence factors is multifaceted and all contribute to the success of *S. aureus* as a pathogen.

Antibiotic resistance of *S. aureus.* Genes encoding for antibiotic resistance typically occur on mobile elements, such as genomic islands, transposons, plasmids and phages; and are rapidly and easily transferred. Most antibiotic resistance mechanisms function by either circumnavigating the effects of the drug (e.g. expelling it via efflux pumps) or by modifying the drug directly. *S. aureus* is prolific at acquiring antibiotic resistance cassettes, and thus is a major public health concern due to lack of available treatments. The beta-lactam, penicillin, was the first antibiotic used to treat bacterial infections in 1942. Beta-lactams inhibit penicillin binding proteins (PBP) that perform the last enzymatic reaction in cell wall synthesis (Goffin & Ghuysen, 1998). However, only a year after its clinical introduction, penicillin resistance by *S. aureus* was reported (Rammelkamp et al., 1943). Resistance was achieved by the acquisition of the *blaZ* gene, which encodes a penicillinase that hydrolytically cleaves beta-lactam antibiotics (Kernodle, 1989). Another antibiotic, methicillin, was introduced for clinical use in 1960. Methicillin differs structurally from penicillin by sterically hindering the beta-lactam cleavage site, and hence penicillinases are ineffective against it. A year after its introduction, however, methicillin-resistant *S. aureus* (MRSA) was reported (Jevons 1963*).* Bacteria with methicillin resistance possess PBP2' specified by the *mecA* gene; which is commonly encoded on a mobile genetic element known as the staphylococcal cassette chromosome (SCC*mec*) (Chamber 1997; Ito et al., 2001). Beta-lactams have low affinity to PBP2' and hence do not interfere with its essential function (Pinho et al., 2001). Unlike resistance to penicillin, which is narrow in its activity spectrum, methicillin

resistance is broad, and confers resistance to the entire class of beta-lactam antibiotics, including penicillins, cephalosporins and carabapeneums (McCallum et al., 2009).

S. aureus has also recently gained resistance to glycopeptides antibiotics, such as vancomycin. Vancomycin is considered a last resort antibiotic as it is often the only drug still effective against many strains of *S. aureus* . Vancomycin binds to the D-ala-D-ala residues of the lipid II molecule, which normally cross-links with other lipid II molecules to form peptidoglycan in the cell wall. This binding event by vancomycin inhibits cross linking and peptidoglycan biosynthesis, which results in weakened cell walls and eventually cell death (Watanakunakorn, 1984). Two resistance mechanisms have arisen in *S. aureus* towards glycopeptides. First, isolates with reduced susceptibility to vancomycin were observed and are known as vancomycin intermediate resistant *S. aureus* (VISA). These isolates have an elevated vancomycin minimum inhibitory concentration of 8-16 μl/ml compared to 1-4 μl/ml, which is considered sensitive (Hiramatsu, 2001). VISA isolates are associated with thickened and poorly linked peptidoglycan, which results in an increased number of free D-ala-D-ala dipeptide targets for the antibiotic to bind. Since these extra residues are free and not part of lipid II, this binding event does not inhibit peptidoglycan synthesis (Cui et al., 2006). It has also been proposed that this difference in cell architecture may reduce the diffusion rate of the antibiotic into the cell (Pereira et al., 2007).

True vancomycin resistance was first reported in 1988 in Enterococcus isolates which were found to resist vancomycin by utilizing the *vanA* cluster of genes (Leclercq et al., 1988, Uttley et al., 1988). The first *S. aureus* isolate with vancomycin resistance was

found in Japan in 1997 and mostly likely acquired the necessary genes from *Enterococcus* (Hiramatsu et al., 1997). In 2002, vancomycin resistant *S. aureus* (VRSA) was found in the United States, and contain the gene *vanA* (Clark et al., 2005, Weigel et al., 2003). Since then, other VRSA strains have been isolated in the United States as well as worldwide (Tiwari & Sen, 2006; Hiramatsu et al., 1997). *vanA* encodes for an enzyme that changes the D-ala-D-ala residues in lipid II to D-ala-D-lac (Perichon & Courvalin, 2009). PBPs can still utilize these residues for peptidoglycan construction, but vancomycin has a 1000-fold lower affinity in binding them (Bugg et at., 1991).

Hospital-acquired and community-acquired *S. aureus*. MSRA strains are widespread in hospitals and intensive care units, and are one of the leading causes of bacterial nosocomial infections worldwide (Diekema et al., 2001). In this setting these strains prey upon the immunocompromised and elderly. Such strains are referred to as hospital-acquired MRSA (HA-MRSA), however, in the past decade MRSA strains have been observed in the community infecting young and healthy individuals without any connection to healthcare facilities, and are termed community-acquired MRSA (CA-MRSA) (Johnson et al., 2007; Moran et al., 2005). These, CA-MRSA strains have evolved independently in the community, and possess different combinations of virulence factors and antibiotic resistance traits, which result in hypervirulent and more aggressive isolates (Burlak et al., 2007; Chambers, 2005; Diep et al., 2006; Diep at al., 2008; Kennedy et al., 2008; Vandenesch et al., 2003; Wang et al., 2007).

Multilocus sequence typing and pulsed-field gel electrophoresis (PFGE) has been used to define *S. aureus* isolates according to CDC PFGE types USA100-USA1200 (Enright et

al., 2002; Tenover et al., 2004). HA-MRSA infections, in the United States are commonly caused by USA100, USA200, USA700 and USA800 types, with the former being the most common (Tenover et al., 2004). In contrast, USA300 has become the dominant CA-MRSA clone in the United States in the past 10 years (Moran et al., 2006;Hulten et al., 2010; Talan et al., 2011), replacing USA400 in most regions (Como-Sabetti et al., 2009; Simor et al., 2010).

Recent research has shown multiple differences between HA-MRSA and CA-MRSA. First, the ability of CA-MRSA to invade healthy hosts is the result of a difference in the expression profile of core-genomic elements, as well as newly acquired virulence factors. CA-MRSA strains overproduce several core pathogenic determinants, including α-hemolysin (Hla), delta-hemolysin, proteases and phenol soluble modulins (PSMs) (Burlak et al., 2007; Chambers, 2005; Diep & Otto, 2008; Wang et al., 2007). A large number of these factors are regulated by the two-component system Agr, and thus their overproduction is due, at least in part, to a hyperactive Agr system. The Sae system also positively regulates virulence factors and has been shown to have increased expression in CA-MRSA strains (Giraudo et al., 1997, Goerke et al., 2005; Nygaard et al., 2010),..

Genome analysis of the CA-MRSA isolate MW2 revealed 19 unique virulence factors, including several enterotoxins, leukocidins and surface-associated proteins that are not found in other *S. aureus* genomes (Baba et al., 2002). Other CA-MRSA strains also carry unique pathogenicity islands, such as SaPI5, which encodes two unusual enterotoxins, K and Q (Diep et al., 2006) that are thought to contribute to virulence by stimulating T-cells. Also, the Panton-Valentine leukocidin (PVL) genes are found in 2% of clinical

isolates, however all CA-MRSA strains that cause skin infections possess them (Vandenesch et al., 2003). Of the virulence factor genes acquired by CA-MRSA strains, only the arginine catabolic mobile element (ACME) is completely unique to USA300 (Diep et al., 2006). ACME encodes a complete arginine deaminase system that converts D-arginine to L-ornithine for ATP and ammonia production. It has been proposed that ACME contributes to colonization through ammonification of the acidic skin environment (Diep et al., 2008; Joshi et al., 2011). In addition to toxin production, antibiotic resistance genes also vary between these 2 lineages. Both HA-MRSA and CA-MRSA strains carry *SCCmec,* with eight different types thus far identified. Types I-III contains other drug resistance determinants, whereas types IV-VIII only carry resistance to beta-lactam antibiotics (Carvalho et al., 2010). CA-MRSA typically carry *SCCmecIV*, which means that CA-MRSA strains are commonly susceptible to antibiotics such as clindomycin, macrolids, co-trimoxazole, tetracyclines and fluoroquinolones (Daum et al., 2002). TypeIV *SCCmec* is also smaller in size, and seems to have less of a defect burden on strains, comparead to types I, II and III, which are very large and result in decreased growth rates of harboring isolates (Laurent et al., 2001; Ender et al., 2004; Diep et al., 2008).

The type of infection caused by HA-MSA and CA-MRSA varies greatly. HA-MRSA infections are often associated with invasive diseases such as surgical site infections and bacteremia, which can result in dissemination to the lungs and cause pneumonia (Webb et al., 2009; Selvey et al., 2000). CA-MRSA strains most frequently cause skin infections (Cohen & Kurzrock, 2004), which are characterized by the development of necrotizing lesions of the skin and soft tissues. These lesions can then progress into abscesses or

cellulitis. CA-MRSA can also cause invasive infections, such as bacteremia, endocarditis and necrotizing fasciitis, a condition rarely caused by *S. aureus* (Miller et al., 2005). CA-MRSA is often associated with worse clinical outcomes and has increased sepsis and in-hospital mortality rates (Kempker et al., 2010). On average, patients contracting CA-MRSA are younger and otherwise healthier than those with HA-MRSA (Nair et al., 2011). Recent reports reveal that the number of CA-MRSA infections is increasing in the United States (Limbago et al., 2009; McDougal et al., 2003; Moran et al., 2005), and these strains are being observed in the hospital setting, where they are displacing traditional HA-MRSA isolates (D'Agata et al., 2009; Popovich et al., 2008; Webb et al., 2009).

Two-component systems. The success of *S. aureus* as a pathogen is in part due to precise regulation of genes needed to survive in different environments, including the host. One class of elements used by *S. aureus* to sense the environment and adapt is two-component systems (TCS). The term "two-component" was first used to describe a class of unique regulatory systems found in eubacteria (Hess et al., 1988). However, recently two-component signal transduction systems have been found in Achaea, fungi, slime molds and even plants (Hoch, 1995; Hwang et al., 2002;. As the name suggests, these systems consist of two components, a membrane spanning histidine kinase (HK) and an intracellular response regulator (RR). The histidine kinase acts as a sensor and the response regulator, which is normally a DNA-binding protein, is responsible for changing gene expression to aid in survival of the cell. The chemistry of most TCS consists of three reactions, with two intermediates (Figure 2). The two partners of a TCS communicate through three phospho-transfer reactions after the HK senses its specific

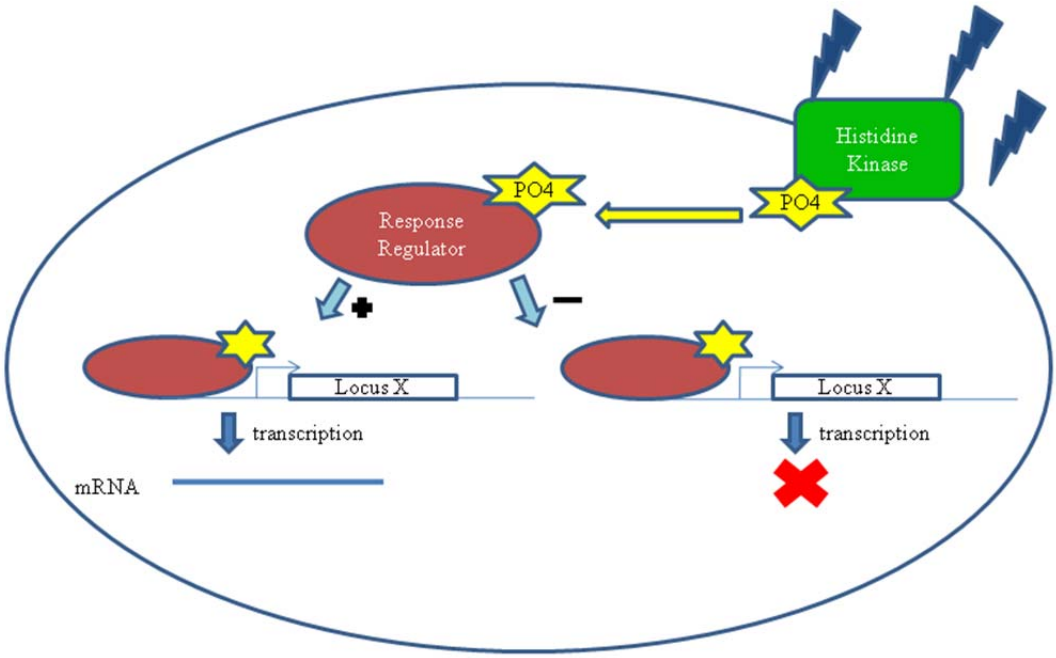

Figure 2. A functional schematic of two-component systems. TCS are utilized by the cell as a way to sense the external environment and alter expression profiles in order to survive. The histidine kinase (HK) is a membrane bound protein that is able to sense specific signals. When the HK senses its target at a threshold level, it autophosphorylates at a conserved histidine residue. The phosphate group is then passed to the cytoplasmically located response regulator (RR) at an aspartate residue. Once phosphorylated, the RR can bind promoters of genes under its control and either increase or decrease transcription in accordance with the stress being sensed.

signal: (1) the histidine kinase (HK) autophosphorylates itself at a conserved histidine residue; (2) the phosphate is transferred to the response regulator (RR) at a conserved aspartate residue (Asp); (3) the RR changes gene expression profiles by binding to promoters under its control (Figure 3). The phosphoryl group is then transferred to a water molecule in a hydrolysis reaction, and is dephosphorylated to reset the system (Parkinson, 1993).

TCS prototypically operate in this way; however some function differently and can contain more than two components, yet still utilize the same sensing and phosphotransfer system. The HK, CheA of *E. coli* can phosphorylate either of two RRs, CheY or CheB (Stock et al., 1988). The sporulation TCS of *B. subtilis* is an example of a multicomponent phosphorelay system. The HK is considered a hybrid HK due to its multistep system and higher complexity. The RR Spo0F receives its phosphate from either KinA or KinB. Then the phosphate is passed to another HK, Spo0B, and then finally to RR Spo0A (Burbulys et al., 1991). Instead of the phosphate being passed to different proteins, as in the sporulation system, it can also be passed to different domains within a single HK. In the anoxic redox TCS from *E. coli*, the HK ArcB contains two domains that autophosphorylate, before the RR, ArcA can be phosphorylated (Ishige et al., 1994). In addition to these, not all kinases are membrane bound; some are actually soluble cytoplasmic HKs. These HKs are regulated by stimuli within the cytoplasm, or interact with the cytoplasmic domains of target proteins. The nitrogen sensing HK, NtrB, is one such example of a soluble HK (MacFarlane & Merrick, 1985).

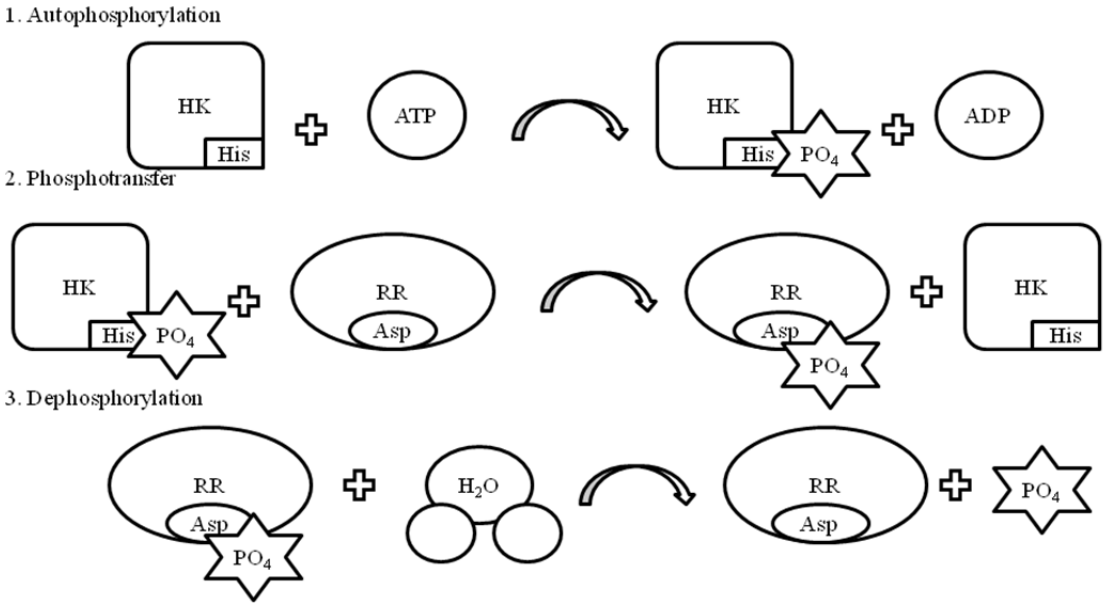

Figure 3. The phosphotransfer reactions of two-component systems. The histidine kinase (HK) and response regulator (RR) of a TCS communicate through three phospho-transfer reactions to ultimately alter gene expression. First, the HK autophosphorylates itself at a conserved histidine residue; then the phosphate is transferred to the response regulator (RR) at a conserved aspartate (Asp); which is followed by the RR changing expression profiles by binding to promoters under its control. The final step consists of the phosphoryl group being transferred to a water molecule in a hydrolysis reaction, leading to dephosphorylation, which resets the system.

Structure and function of histidine protein kinases. The main architectural feature of all HKs is the kinase core, which is composed of a dimerization domain and an ATP/ADP-binding phosphotransfer or catalytic domain (Figure 4) (Stock, 1999). It contains the conserved amino acid motif, called the H box, which houses the histidine residue where phosphorylation occurs (Stock et al., 1989). The ATP-binding pocket is flexible, suggesting that it undergoes a conformational change when associated with ATP (Ban et al., 1999). In addition to the kinase core, HKs contain a sensing domain located at the N-terminus, which is used to sense specific environmental signals. This domain shares little sequence similarity between HKs, supporting the idea that each protein is designed to sense different, and very specific, stimuli. For many HKs the stimulus remains unknown.

Structure and function of response regulators. The majority of RRs consist of two domains: a conserved N-terminal regulatory domain, and a variable effector domain located at the C-terminus (Figure 5). The RR is responsible for transfer of the phosphoryl group from the His residue in the HK to a conserved Asp residue within its own regulatory domain. In addition to the critical Asp residue, many RRs have acidic residue adjacent to this (Stock et al., 1988). The carboxylate side chains of these acidic residues are important in obtaining Mg^{2+}, which is required for phosphoryl transfer (Needham et al., 1993). Some RRs are able to obtain the phosphoryl group from small molecules such as acetyl phosphate, carbamoyl phosphate, imidazole phosphate or phosphoramidate, in addition to the cognate HK (Lukat et al., 1992). This suggests that some RRs can phosphorylate independently of the HK, and that the concentration of these small phosphor-donors can serve as a signal to change gene expression profiles.

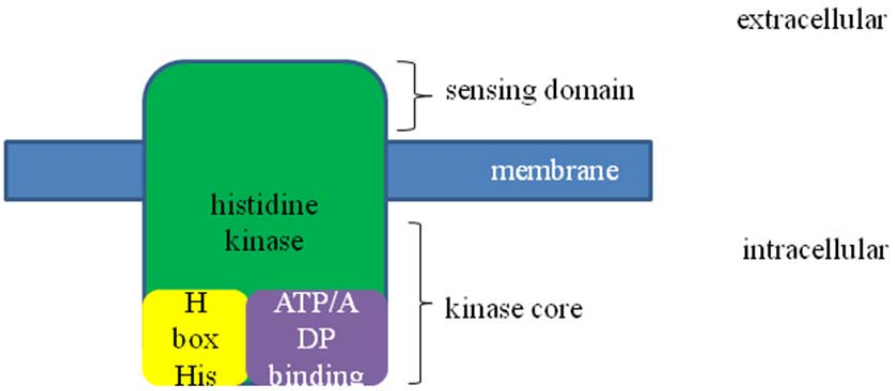

Figure 4. The architectural features of histidine kinases. The histidine kinase is a membrane spanning protein with a sensing domain in the extracellular environment and a kinase core that is cytoplasmically located. The sensing domain of each HK is different as each recognizes a unique signal. The kinase core is composed of an ATP/ADP-binding phosphotransfer domain and the H box. The ATP/ADP-binding phosphotransfer domain uses the hydrolysis of ATP for energy, which creates a conformational change in the protein. This leads to autophosphorylation at a conserved histidine residue in the H box**.**

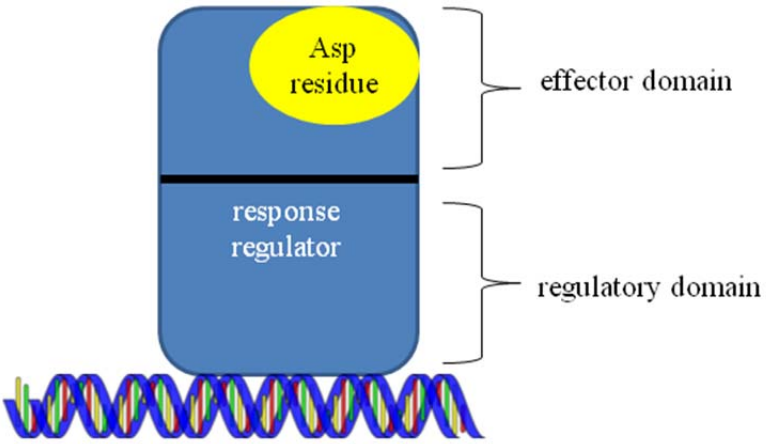

Figure 5. The architectural features of response regulators. The response regulator (RR) of a two-component system is a cytoplasmic located protein and is composed of an effector domain and a regulatory domain. The effector domain contains a conserved aspartate (Asp) residue where the phosphate group is received from the histidine kinase. Once phosphorylated the RR can bind to genes under its control via its DNA-binding or regulatory domain. This leads to either increased or decreased transcription of the target gene(s).

Phosphorylation ultimately promotes a conformational change in both the regulatory domain, as well as the effector domain. The effector domain, the DNA sequences that it binds to, the arrangement of the binding sites and the exact mechanism of transcriptional regulation varies greatly amongst different RRs. It is thought that RRs exists in equilibrium between the active and inactive state, with the phosphorylation event shifting the equilibrium to the active state. In the active state the RR can promote dimerization (McCleary, 1996), higher-order oligomerization (Webber et al., 1997), or interactions with other proteins or DNA (Aiba et al., 1989). The majority of effector domains have DNA-binding activity, which can either activate or repress genes under its control. However, phosphorylation does not equate to activation, and for some RRs, activation is the result of relieving inhibition. In this scenario, phosphorylation creates a conformational change and modifies the binding of the RR to DNA. In certain situations the unphosphorylated RR can bind DNA and inhibit transcription, under normal conditions. Upon phosphorylation, the RR is no longer bound and thus repression is relieved.

The occurrence of TCS in prokaryotes. With the availability of whole genome sequencing, the frequency of TCSs among bacteria is readily determined. Homology suggests that the total number of TCSs differs vastly between bacteria, and range from 0 in *Mycoplasma genitalium* to 80 in *Synechocystis* sp., which is a total of ~2.5% of its entire genome (Mizuno et al., 1998). Analysis of other bacterial genomes reveals that *Bacillus subtilis* possesses 70 TCSs; *Haemophilus influenza*, 9 ; *Helicobacter pylori*, 11; (Mizuno et al., 1998) while *E. coli* has 30 HKs and 32 RRs (Mizuno, 1997). The exact number of TCSs associated within a bacterium, however, changes as new ones are

acquired. Indeed, many newly discovered TCSs are the result of horizontal gene transfer, and are involved in sensing and responding to antibiotics, or affecting virulence processes.

Intramembrane-sensing histidine kinases. Several different classes of TCS have been identified in recent years, including the HWE (based on the H,W and E residues in the kinase core domain), YwpD-like and PleD-like TCS (Karniol & Vierstra, 2004, Wietzorrek & Bibb, 1997; Galperin, 2006) Recently, a class of histidine kinases was identified and termed the intramembrane-sensing histidine kinases (IM-HKs) (Mascher, 2006). IM-HKs are small in size, are no more than 400 amino acids in length, and the N-terminus contains two transmembrane helices (TMH) with less than 25 amino acids between them (Figure 6). This small linker region between TMHs is thought to be the lone extracellular fragment, meaning that the majority of the kinase is cytoplasmic. Therefore it is likely that these IM-HKs do not sense an external signal, but rather sense a stimulus either inside or at the surface of the membrane (Mascher, 2006). From approximately 350 sequenced genomes, 5000 HKs were identified. Of these 5000, 147 contain the architecture and characteristics of IM-HKs, with the majority of them encoded by Gram-positive bacteria with low G+C content (Macher, 2006). Indeed, when analyzing 79 different Gram-positive genomes, 110 proteins were identified as belonging to the IM-HK family.

Subgroups of intramembrane-sensing histidine kinases. Two distinct subgroups of IM-HKs have been characterized, and are almost exclusive to the Firmicutes. The major characteristic of the first subgroup is that they belong to the HPK7 subclass, and the RR

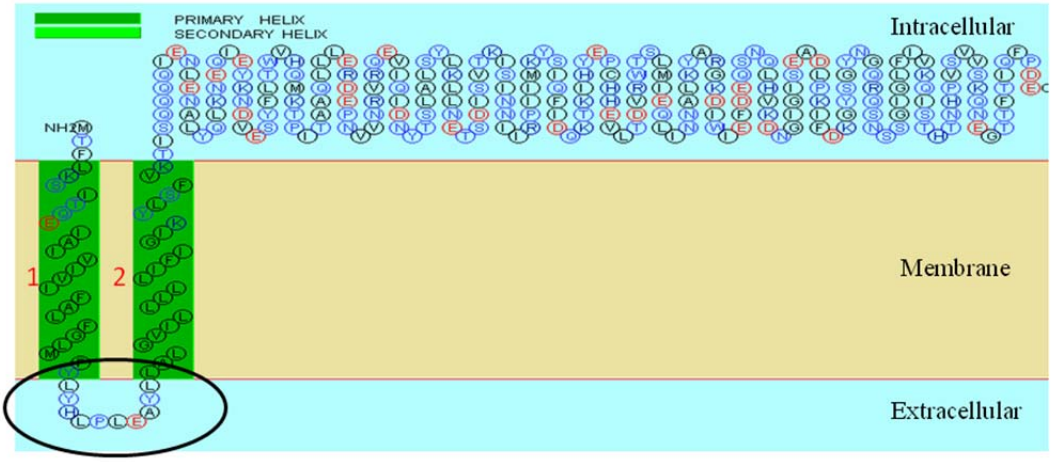

Figure 6. Predicted topology plot of the IM-HK, SACOL2645 from *S. aureus*. IM-HKs are characterized by having two transmembrane domains and an extracellular sensing domain (circled) less than 25 amino acids, suggesting they sense changes at the surface or in the membrane. *S. aureus* has 4 IM-HK's, SACOL 2645, GraS, VraS, and SaeS. This depicts the projected transmembrane domains and sensing domain of the uncharacterized IM-HK, SACOL 2645. Produced using the SOSUI website.

and HK proteins utilize an additional, conserved transmembrane protein to exert their effects. Thus far 25 IM-HKs belonging to this group have been identified (Mascher, 2006). A well studied example is the LiaRS TCS from *B. subtilis*, which is encoded on the *liaIHGFSR* operon. LiaRS senses and responds to lipid II-interacting antibiotics, including bacitracin, vancomycin, ramoplanin and nisin. The LiaRS system is also activated by cationic antimicrobial peptides, alkaline shock, organic solvents, detergents and ethanol (Petersohn et al., 2001; Hyyrylainen et al., 2005; Pietiainen et al., 2005). In the absence of these stimuli, LiaRS is inactive due to the membrane protein LiaF. In a *liaF* deletion mutant, the LiaRS is constitutively activated, and additional stimuli are not required. Therefore, LiaRS and LiaF form a three component system, and thus full function relies on the presence and activity of all three elements, although the exact functional mechanism of this three component system is unknown (Jordan et al., 2006). The second group is the largest class, and is characterized by being genetically associated with, and an adjacent ATP-binding cassette transporter (ABC transporter). ABC transporters are transmembrane proteins that hydrolyze ATP for energy to translocate various substrates across the membrane (Higgins, 2001). IM-HKs encompassing this group belong to the HPK3i subfamily and regulate, and utilize, the adjacent ABC transporter to pump various substrates across the membrane in response to the stimulus sensed by the HK.(Mascher, 2006). The cognate TCS is often constitutively expressed, and senses cell-wall-targeting compounds, or the damage they cause. Upon induction, the RR is activated, and in turn induces transcription of the adjacent ABC transporter. A total of 70 proteins belong to this subgroup, with 65 of them found in Gram-positive bacteria with low a low G+C content (Mascher, 2006). Of note, almost all of these (53 of 55) are

very closely related from an evolution perspective, and gather tightly in a phylogenetic tree.

Two-component systems of *S. aureus*. Through homology and domain comparisons, *S. aureus* is thought to encode 16 TCSs within its relatively small genome. These TCSs mediate the response to changing environments and facilitating survival in the presence of a multitude of stresses (Table 1). In addition, these TCSs are a major contributor to the success of this bacterium as an invasive and effective pathogen. The TCSs of *S. aureus* sense a variety of signals covering a range of different processes. Only one TCS has been shown to be essential for growth, and is involved in cell wall homeostasis. This TCS, YycFG, has been shown to activate the expression of cell wall and fatty acid metabolism genes, and has also been implicated in daptomycin resistance and vancomycin resistance (Dubrac et al., 2007). Other TCSs are also involved in the cell wall stress response, including GraRS and VraRS. GraS, VraS and a further unstudied protein that is the focus of this work, SACOL2645, belong to the IM-HK class, which are known to sense damage of the cell wall. Further to this, LytRS controls the rate of autolysis by regulating genes involved in autolytic processing and cell wall metabolism by altering murein hydrolase activity (Brunskill & Bayles, 1996). Similar to this, ArlRS regulates 114 genes, many of which are also involved in autolysis, as well as cell division processes, growth and pathogenesis. ArlRS also has been shown to regulate other TCS, including AgrAC, LytRS and KdpDE (Liang et al., 2005).

TCS in *S. aureus* can also respond to elemental changes in the environment, including potassium, iron, phosphate, oxygen and nitrogen. The activation of KdpDE is dependent

Table 1. *S. aureus* possesses 16 two-components systems.

COL #	MW2 #	Gene name	Function
0019/20		*yycFG/walkRS*	Essential
0201/2	0199/8		Anaerobiosis (Preliminary investigations)
0246/5	0236/7	*lytRS*	Autolysis regulation
0716/7	0621/2	*graRS*	Cell-wall/vancomycin resistance
0766/5	0668/7	*saeRS*	Virulence determinant regulation
1355/4	1208/9		LuxR family
1451/0	1305/4	*arlRS*	Virulence determinant regulation and autolysis
1535/4	1446/5	*srrRS*	Oxygen sensing regulation
1740/39	1637/6	*phoPR*	Alkaline phosphatase regulation
1905/6	1790/1	*yhcSR*	Essential/cell wall/phosphomycin resistance
1942/3	1825/4	*vraRS*	Cell-wall synthesis
2026/5	1962/3	*agrCA*	Accessory gene regulation
2070/1	2002/3	*kdpDE*	Potassium transport regulation
2358/9	2282/3	*hssRS*	Iron transport and metabolism
2389/90	2313/4	*nreBC*	Nitrate reductase regulation
2646/5	2545/4	*nsaRS*	

on external levels of potassium, and inactivation of this system has been shown to decrease the expression of several virulence factors. This suggests that *S. aureus* determines its infectious state, in part, by sensing external potassium levels (Xue et al., 2011). The HssRS TCS is essential for the adaptive response to iron-containing heme within the host. HssRS responds to iron levels; in environments with low iron it upregulates the heme regulated transporter efflux pump, HrtAB. Although iron is a nutritional requirement for *S. aureus*, which it obtains in a host from heme, metabolizing this molecule can lead to reactive oxygen species and membrane damage. HssRS regulates HrtAB to alleviate hemin toxicity (Stauff et al., 2007). *S. aureus* uses the TCS NreBC to senses nitrogen in the external environment. In the presence of low oxygen or nitrogen, the RR of this system binds to genes that are involved in nitrate, nitrite reduction and fermentative metabolism (Fedtke et al., 2002). This system is required for the transport of nitrogen, as mutations in *nreBC* inhibits the use of nitrogen and force the cell to use fermentative pathways (Schlag et al., 2008). Although the PhoPR TCS has not been studied in *S. aureus*, its function can be inferred through comparison to a similar system in *B. subtilis*, where it senses limiting phosphate levels, and regulates genes accordingly (Martin et al., 1999). It is also implicated in cell wall homeostatsis, as peptidoglycan synthesis is affected by low phosphate levels. SrrAB is involved in global energy changes in response to oxygen availability. SrrAB directly regulates virulence factors in response to this, and a mutation in *srr* results in decreased virulence (Yarwood et al., 2001; Bae et al., 2004). Another TCS AirSR (formally known as YhcRS) is also important in sensing low oxygen levels. This TCS uses a redox active Fe-S cluster within the histidine kinase to sense oxidation signals and responds accordingly. This system has

been shown to be important for resistance to H2O2, vancomycin, norfloxin and ciprofloxacin (Sun et al., 2012).

Using comparative genomics, four of the sixteen TCSs of *S. aureus* appear to contain IM-HKs. One of these is associates with a membrane protein (VraRS), two belong to the subfamily associated with ABC transporters (GraRS and SACOL 2645/2646), and the final one seems to be an outlier IM-HK that does not fall into this 2 sub-family structure (SaeRS).

The SaeRS two-component system of *S. aureus*. The *sae* locus in *S. aureus* is composed of four open reading frames: *saeP, saeQ, saeR* and *saeS*. The latter two genes encode the HK (*saeS*) and the RR (*saeR*) of the two-component system. SaeS is 351 amino acids in length and has two transmembrane domains. The external or sensor domain is nine amino acids long, suggesting that the SaeRS does not sense external stimuli, as with other IM-HKs. A microarray analysis performed in the clinical isolate MW2 showed that SaeRS regulates the expression of 212 genes, ranging in function from energy and metabolism processes, ion transport, DNA repair and virulence (Voyich et al., 2009). This TCS is required for the expression of several virulence factors including cell wall-associated proteins, and secreted proteins (Giraudo et al., 1997, Giraudo et al., 1994, Goerke et al., 2005). In addition, the *sae* locus is vital to the survival of *S. aureus* in animal models (Voyich et al., 2009), although the exact conditions it responds to are currently unknown.

The VraRS system of *S. aureus*. VraRS was first identified in a VISA isolate as being upregulated in a strain with increased vancomycin resistance (McAleese et al., 2006). It

was originally termed vancomycin resistance associated regulator and sensor (VraRS) as a result of a microarray analysis of VSSA, VISA and VRSA strains which showed upregulation of VraRS in VISA and VRSA. In addition to vancomycin, the presence of cell wall-targeting antibiotics, including glycopeptides, beta-lactams, bacitracin, D-cycloserine, daptomycin and mersacidin also results in the upregulation of this TCS (Gardete et al., 2006; Kuroda et al., 2003). While the exact signal for the activation of VraRS is unknown, the fact that it is an IM-HK with a small sensing domain, and that it responds to antimicrobial agents that target multiple steps in cell wall biosynthesis, suggests that VraRS responds to general cell wall damage and not that caused by specific antibiotics (McAlleese et al., 2006; Gardete et al., 2006). Functionally, VraRS controls the expression of the cell wall stress stimulon, which includes many genes involved in cell wall biosynthesis. This is suggested, at least in part, to be the reason for altered resistance to cell-wall-targeting agents in *vraRS* mutants (Fan et al., 2007; McCallum et al 2006). A microarray performed on *S. aureus* in the presence of vancomycin resulted in 139 genes with altered expression suggesting a requirement for these genes in vancomycin resistance. However a *vraRS* mutant grown in the presence of vancomycin had only 93 genes altered in expression, suggesting that 46 genes utilized by *S. aureus* to combat vancomycin stress are dependent upon VraRS (Gardete, 2006).

The GraRS two-component system of *S. aureus*. The glycopeptides resistance associated (Gra) TCS operon consists of three genes, GraXSR. *graS* encodes an IM-HK that is 346 amino acids in length and has only four amino acids on the exterior of the membrane. *graR* encodes the cognate response regulator, while *graX* specifies a protein of unknown function that has been shown to be important for the function of this TCS

(Falord et al., 2011). The GraSR TCS has been shown to be important in resistance to vancomycin, a glycopeptide, which is where the name came from. In a microarray that was performed on a VISA strain, GraSR was found to be upregulated (Cui et al., 2005). GraSR has been shown to regulate approximately 248 genes, with 115 being upregulated and 133 down regulated. A large portion of these genes are involved in cell wall biosynthesis, however other regulators (Rot and MgrA) are contained in this regulon, thus some of the 248 genes may be indirectly regulated by GraRS.

A mutation of *graR* was found to produce increased susceptibility to vancomycin (Meehl et al., 2007). In correlation with these observances, overexpression of this TCS leads to an increase in MIC for vancomycin. Directly downstream of *graXSR* is an ABC transporter termed VraFG, which is regulated by this TCS, and also seems to play a role in vancomycin resistance (Falord et al., 2011), however the exact mechanism by which this is mediated is currently unknown. One of the proposed reasons for the change in susceptibility to glycopeptides is the D-alanylation of teichoic acids which is controlled by the *dltABCD* operon. Specifically, a mutation in this operon results in increased vancomycin susceptibility (Peschel et al., 2000). This operon is regulated by GraRS, and seemingly, GraXSR, VraFG, as well as other genes involved in cell wall biosynthesis work together to maintain cell wall homeostasis when in the presence of cell-wall-targeting compounds.

One of the main functions of GraSR is cationic antimicrobial peptide (CAMP) resistance; and in fact this system has an alternate name, ApsRS (antimicrobial peptide sensor) as a result of this. CAMPs are produced by all living organisms, and are a major

component of the innate immune system. They are positively charged, and kill bacteria by binding to their negatively charged cell wall, where they form pores, or enter the cell and inhibit DNA, RNA or protein synthesis (Hale et al., 2007). The presence of CAMPs induces GraSR, which changes gene expression to facilitate resistance by the D-alanylation of teichoic acids and lysylination of phosphatidylglycerol. D-alanylation of teichoic acids increases the overall positive charge of the cell and decreases the affinity of CAMP binding. D-alanylation of teichoic acids is performed by the DltABCD enzymes, while MprF is responsible for the lysylination of phosphatidylglycerol. GraRS helps in the infection process by limiting the activity of host CAMPs and has shown to be important in several animal models of infection including drosophila, murine and silkworm larvae (Kraus et al., 2008; Tabuchi et al., 2010; Kurokawa et al., 2007).

Project Aim: TCS have been shown to be important regulatory elements for understanding both the virulence process as well as antibiotic resistance. *S. aureus* has sixteen TCS sensing a variety of signals and covering a range of functions. Thirteen of the sixteen TCS have definable roles, either through homology with closely related organisms, or by their study in *S. aureus*. IM-HKs are a group of histidine kinases that have been suggested to sense damage at the cell surface as they have small sensing domains. GraRS and VraRS are highly important IM-HKs, involved in antibiotic resistance and the evaluation of these systems is generating insight into resistance mechanisms of *S. aureus*. One IM-HK, SACOL 2645/2646 has not been studied, and does not have homology with any TCS in closely related organisms. This suggest that the role of this TCS may be unique to *S. aureus*, and thus understanding its role may elucidate important information regarding drug resistance mechanisms, as well as provide

a better understanding of regulatory elements *S. aureus* utilizes during pathogenesis. Accordingly, the aim of this project is to characterize SACOL 2645/2646 and determine, as with other IM-HKs, if it is involved in cell wall homeostatsis. Further to this, identifying genes under the control of SACOL 2645/2646 and determining their contribution to the overall function of this TCS will provide exclusive unique insight into the lifestyle of *S. aureus*.

Chapter 2: The role and regulation of NsaRS: a cell-envelope stress-sensing two-component system of *S. aureus*

Introduction

Bacteria are exposed to a variety of environments in which they must adapt in order to survive. As such, they have developed multiple mechanisms to adapt to, and survive these stresses. While it may be considered that it is only the genes directly involved with adaptation that are important, the regulators of these elements are a vital component to survival and proliferation. Bacteria possess a variety of regulatory elements, including sigma factors, DNA-binding proteins, regulatory RNAs and two-component systems. Understanding the function and control of these elements commonly provides an important insight into the behavior of bacteria, and, in the case of pathogens, may gives a greater understanding of virulence mechanisms.

Bacteria have specialized regulatory elements to detect when they are in a host, and when virulence genes need to be expressed. One example from Group A *Streptococcus* (GAS) is the TCS SilAB, which aids in the virulence process via a quorum sensing mechanism, allowing GAS to sense the level of other GAS bacteria that are in the immediate environment (Belotserkovsky et al., 2009). These systems have been shown to be important in the virulence of GAS, as the allow for a delay in expression of virulence factors until a large concentration of bacteria are present (Eran et al., 2007). *S .aureus* also has a similar system, *agr*, which has been shown to be important to the

virulence process. Agr is a quorum sensing TCS that regulates many virulence factors; and mutants in this system are attenuated in murine models of infection (Mayville et al., 1999; Schwan et al., 2003; Blevins et al., 2003).

While several TCS have been shown to be important in virulence, another aspect of the infection process is treatment with antibiotics. TCS are also imperative in sensing either the antibiotic itself, or the damage caused by these agents. In *Enterococcus faecalis* the TCS, VanRS, is vital for resistance to glycopeptides, including vancomycin. In the presence of glycopeptides this system is induced, leading to the upregulation of genes including *vanHAX*, which alters the binding of glycopeptides to the cell wall, and ultimately resulting in resistance (Bugg et al., 1991). *S. aureus* also has TCSs that responds to glycopeptides, VraRS, and to a lesser extent GraRS (McAlleese et al., 2006; Gardete et al., 2006; Meehl et al., 2007; Peschel et al., 2000).

S. aureus has 16 predicted TCS that respond to a range of environmental stimuli, and allow for adaptation to various stresses. Of these 16, three have no known function, including SACOL2645/2646, which is the focus of this work. Even using homology analysis with closely related organisms, no function can be inferred for this TCS, suggesting that it may be unique to the Staphylococci. SACOL2645/2646 was recently termed nisin susceptibility associated response regulator and sensor (NsaRS) (Blake et al., 2011), and thus this nomenclature will be used throughout. The *nsaRS* operon consists of 3 genes that are expressed from one promoter (Figure 7). The first gene is small, consisting of only 201 base pairs, and theoretically producing a protein of 67 amino acids. This is followed by the response regulator and then the histidine kinase.

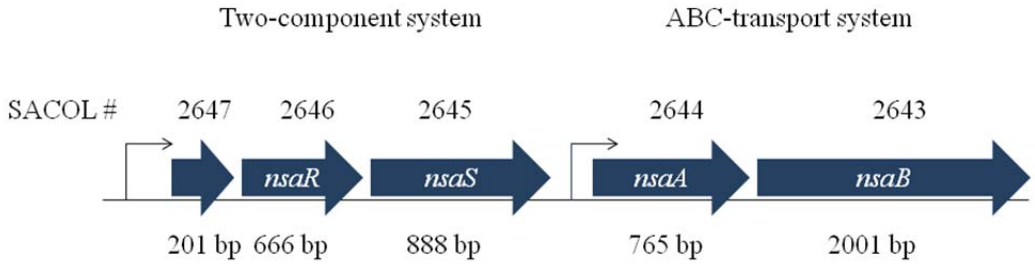

Figure 7. The NsaRS/NsaAB locus from *Staphylococcus aureus*. The two-component system, NsaRS, is expressed from one promoter, and exists in an operon with another small gene, SACOL 2647. NsaS is an IM-HK, belonging to the sub-family that uses an adjacent ABC transport system to exert its affects. This is located 3' of the regulatory genes, and is termed NsaAB. *nsaAB* are putatively expressed from one promoter as shown.

Analysis of the histidine kinase revealed that NsaS is an IM-HK and has typical characteristics of this family, bearing 2 transmembrane domains, and a sensing domain of only 9 amino acids in length. This suggests that this TCS may sense damage at the cell surface, rather than external signals. NsaS belongs to the group of IM-HKs that regulate and utilize an adjacent ABC transporter to exert its affects. For NsaRS, this transporter is NsaAB, which is located directly upstream of the TCS, and form an ABC transport system of currently unknown function. It also remains unknown whether this transporter is regulated by NsaRS. In addition, both GraRS and VraRS, two other IM-HKs in *S. aureus* contribute to antibiotic resistance in some manner, and sense cell wall disruption. Therefore, elucidating the role of NsaRS may better our understanding of the mechanisms *S. aureus* uses to combat cell wall stress, and/or antibiotic resistance.

Therefore, the focus of this chapter is the characterization of NsaRS, which we hypothesize is employed by *S. aureus* to combat cell-wall stress. As such the utilization of NsaRS by *S. aureus* will be evaluated, and its response to antibiotics and cell-envelope-targeting agents determined. Since NsaRS is a regulator, identifying components of its regulon will likely provide information leading to the discovery of its function. The impact of NsaRS on the virulence of *S. aureus* will also be addressed, and may generate insight into the mechanisms used by *S. aureus* during pathogenesis.

Results

Note to Reader

These results have been previously published (Kolar et al., 2011) with permission of the publisher and the published manuscript can be found in Appendix 1.

Chapter 3: The role and regulation of the ABC transporter, SACOL2525/2526 of *S. aureus*

Introduction

NsaRS is involved in sensing cell-envelope stress, and exerts its effects by regulating over 200 different genes. The majority of these are positively regulated, and cover a range of functions, including cell wall biosynthesis genes (*tagB*, *fmhA*, and *scdA*), osmoprotectants (*opuCA* and *cudT*), as well as other regulators (*sarS and rex*). The ontology grouping that contains the most genes within the NsaRS regulon is transporters, with 55 such genes being affected. One particular transporter, SACOL2525/2526, not only had the largest decrease in expression of the transporters, but in fact had the most alteration in transcription of all 245 genes. SACOL2525/2526 encodes an ABC-transporter, and homology analysis suggests that it is involved drug resistance. Since the appearance and clinical use of penicillin in 1942, antibiotics have been used to treat diseases caused by many bacterial pathogens, including *S. aureus*. However, due to antibiotic resistance, some previous treatable infections are now major public health threats. One mechanism bacteria utilize in antibiotic resistance is the extrusion of the drug by either an efflux pump or carrier (Paulsen et al., 1996). Bacteria typically have multiple drug resistance (MDR) transport proteins that they use to protect against antimicrobial agents. MDR proteins have effectively been divided into two

subgroups: the small multidrug resistance family (SMR) and the multidrug and toxic compound extrusion (MATE) family.

The MATE family is the larger group of the two, and members expunge compounds by using the proton motive force. The QacA protein of *S. aureus* is a classic example of a MATE transporter, conferring resistance to more than 30 lipophilic antimicrobial compounds (Tennent et al., 1989; Mitchell et al., 1998). The SMR group also contributes to antibiotic resistance, but does so by utilizing ABC transporters. ABC transporters contain a membrane spanning protein that creates a translocation pathway for specific substrates. In addition, 2 cytoplasmic adenosine triphosphate-binding cassettes (ABCs) are associated with the membrane protein, and hydrolyze ATP to generate energy for export. This action causes a conformational change that allows the transporter to move substrates from one side of the membrane to the other (Higgins, 1992). ABC transporters are involved in moving a variety of molecules, nutrients, ions and antimicrobial agents across the membrane. ABC transporters contribute to antibiotic resistance by directly pumping out drugs, and this action by the transporter can be specific for only one antibiotic, or be broad in spectrum, and transport several different drugs.

In the context of this work, SACOL2526/2526 form an SMR ABC transporter. SACOL2525 encodes the ATP-binding protein, while SACOL 2526 is the membrane-spanning protein. In the literature SACOL2525 is referred to as *stpC* and SACOL2526 *smpC (*Ross et al., 1996). This naming is due to homology with the *stpA* and *smpA* genes from *S. epidermidis*. In *S. epidermidis*, StpA and SmpA interact with the protein

MsrA, which has been found in coagulase negative staphylococci to confer macrolide resistance (Schmitz et al., 2000). Although *stpA* and *smpA* have high similarity to *stpC* and *smpC*, with 85% and 65% respectively, their functions appear to be divergent. *S. aureus* does not have a chromosomally encoded *msrA* gene, and mutation of *stpC* and *smpC* does not affect macrolide resistance (Ross et al., 1996). Although it has been shown that SACOL2525/2526 does not have the same function as *stpA* and *smpA*, its actual role is currently unknown.

In addition to our work with NsaRS, a microarray performed on a *graRS* mutant of *S. aureus* also showed decreased expression of SACOL2525/2526 (Herbert et al., 2007). This of significant interest as both NsaRS and GraRS are IM-HKs associated with an ABC transporter, both regulate SACOL2525/2526, and both are involved in cell wall homeostasis. While NsaRS regulates the downstream transporter, NsaAB, this effect is only seen in the presence of nisin. Conversely, NsaRS regulates SACOL2525/2526 under standard conditions, during exponential growth. As such, understanding the function of this transporter will provide better insight into mechanisms NsaRS utilizes to respond to cell-envelope stress, and potentially drug resistance.

Materials and Methods

Bacterial strains, plasmids and growth conditions. *E. coli* was grown in Luria-Bertani (LB) medium at 37 °C. *S. aureus* was grown in 100 ml Tryptic Soy Broth (TSB) (1:2.5 flask/volume ratio) at 37 °C with shaking at 250 rpm, unless otherwise indicated. For all experiments one ml of relevant overnight *S. aureus* cultures were used to inoculate fresh media, and allowed to grow for 3h. These cultures were sub-cultured into new media at an OD_{600} of 0.05 and allowed to grow for as long as the experiment needed. When necessary, antibiotics were added at the following concentrations: ampicillin 100 mg/ml (*E. coli*), erythromycin 5 mg/ml (*S. aureus*), lincomycin 25 mg/ml (*S. aureus*), and chloramphenicol 5 mg/ml (*S. aureus*).

Construction of the SACOL2525/2526 mutant strain. Fragment A was amplified using OL1299/OL1300 (Table 2), is ~ 1.5 kb upstream of SACOL 2525 and a Mlu1 restriction site was added. Fragment B was amplified using OL1301/OL1672, is ~ 1.5 kb downstream of SACOL 2526 with an added Mlu1 site to the beginning. These fragments were ligated together cloned into the pJB38 plasmid creating pSLL2. This plasmid was electroporated into the *S. aureus* strain RN4220 and clones were selected for using TSA containing chloramphenicol (plasmid encoded). The resulting isolates were confirmed by genomic extraction and PCR analysis. The plasmid was extracted and a confirmed isolate electroporated into SH1000, again selecting for chloramphenicol. The resulting isolates were confirmed by genomic extraction and PCR analysis. A confirmed isolate was streaked onto a TSA plate containing chloramphenicol and incubated overnight at 43°C. Isolates were passaged for 5 days

Table 2. Strains, plasmids and primers used in this study.

Strain, plasmid or primer	Genotype or description	Reference or source
E. coli		
DH5α	Φ80 Δ(*lacZ*)*M15* Δ(*argF-lac*)*U169 endA1 recA1* hsdR17 ($r_K^-m_K^+$) deoR thi-1 supE44 gyrA96 relA1	Lab Stocks
S. aureus		
RN4220	Restriction deficient transformation recipient	Lab Stocks
SH1000	Wild-Type Laboratory Strain *rsbU* functional	Horsburgh et al., 2002
SLL1	SH1000 *SACOL 2525/2526-lacZ* fusion	This Study
SLL2	SH1000 *SACOL 2525/2526* mutant	This Study
SLL3	SH1000 *SACOL 2525/2526* mutant complemented with pSLL3	This Study
SLL4	SH1000 *graR* mutant	This Study
Plasmids		
pAZ106	Promoterless *lacZ erm* insertion vector.	Kemp et al., 1991
pMK4	*cm* shuttle vector	Sullivan et al., 1984
pJB38		
pSLL1	pAZ106 containing a 1.5 kb *SACOL2525/2526* promoter fragment	This study
pSLL2	pJB38 containing an 1.5 kb *SACOL2525/2526* AB fragment	This study
pSLL3	pMK4 containing a 2.5kb *SACOL2525/2526* complementation fragment	This study
Primers		
OL1299	ATG ATG GAA TTC GTC ACG AGA GGA ATA ATT CCG C	
OL1300	ATG ATG ACG CGT CAT TCG CTC CAA CGA GAC CAA C	
OL1301	ATG ATG ACG CGT CCC TTC ACC CAA ATA ATG GTG CG	
OL1584	ATG GGA TCC CCG CCA ACT GTC GCA TTT ATC C	
OL1672	ATG ATG GGT ACC TGA TCG ACG ACT AAG CGT TGT ACG G	
OL1673	ATG ATG GAA TTC TAC CTG CAC CAT TCG CTC C	
OL1703	AGG CAC ATT TGT TGG CGT	
OL1704	AGC AAT GGC TAC TCC CGT	

onto TSA containing chloramphenicol and incubated at 43°C to ensure incorporation of the plasmid into the chromosome. Isolates were confirmed by genomic extraction and PCR analysis and an 80α phage lysate created for transduction into a clean SH1000 and selected by chloramphenicol. A confirmed isolate was grown in TSB containing chloramphenicol at 30°C overnight. The resulting culture was diluted 10,000 times and 100 µl plated on TSA containing 1µg/ml of anhydrous tetracycline and incubated at 30°C overnight. Resulting isolates were streaked onto TSA and TSA containing chloramphenicol. Isolates that grew on TSA only were selected and confirmed by Southern blotting creating strain SLL2.

Construction of the SACOL2525/2526 reporter fusion strain. The putative promoter region of the SACOL 2525/2526 loci was amplified as ~1.5 kb PCR fragments using primer pair OL1584/OL1673 . The PCR fragment was cloned into pAZ106 creating plasmid pSLL1 (SACOL 2525/2526-*lacZ*). *S. aureus* RN4220 was transformed with the resulting plasmid, with clones selected for using TSA containing erythromycin (plasmid encoded). The resulting strains were confirmed by genomic DNA extraction and PCR analysis. An 80α phage lysate was created and used for the transduction of *S. aureus* SH1000, with clones again confirmed by PCR analysis. This created strain SLL1 (SACOL 2525/2526-*lacZ*).

Construction of SACOL2525/2526 complemented strain. The entire SACOL2525/2526 locus was PCR amplified as a 2.0 kb fragment using primer pair OL-1584/OL-1926. This fragment was cloned into the shuttle vector pMK4, creating

pSLL3. *S. aureus* RN4220 was transformed with this construct, with clones selected for on TSA containing chloramphenicol (plasmid encoded). Isolates were confirmed by genomic DNA extraction and PCR analysis. A representative clone was selected to generate an 80α phage lysate, which was used to transduce the SH1000, SACOL2525/2526 mutants of *S. aureus*. Clones were again confirmed by PCR analysis, creating strain SLL3.

Plate-based assay for determination of altered transcription from external stress. This assay was performed as described previously (Shaw et al., 2008). TSA plates were overlayed with TSA top agar (0.7% w/v) containing X-GAL, and the *lacZ* reporter-fusion strain. Sterile filter discs were placed onto these plates (3 per plate), before being inoculated with 10 µl of the following stress inducing chemicals: 6M HCl, 85% phosphoric acid, 100% TCA, 88% formic acid, 0.2M acetic acid, 6M sulphuric acid, 6M nitric acid, 6M sodium hydroxide, 2M NaCl, 1M glucose, 95% ethanol, 100% methanol, 100% isopropanol, 10% SDS, 10% Triton X-100, 10%Tween-20, 1M N-lauroyl sarcosine, 30% hydrogen peroxide, 1M methyl viologen, 1% menadione, 2 mg ml^{-1} pyrogallol, 1M sodium nitroprusside, 1M 4-MMS, 5 mg ml^{-1} penicillin G, 5 mg ml^{-1} vancomycin, 2 mg ml^{-1} phosphomycin, 5 mg ml^{-1} spectinomycin, 100 mg ml^{-1} ampicillin, 5 mg ml^{-1} tetracycline, 50 mg ml^{-1} kanamycin, 50 mg ml^{-1} neomycin, 10 mg ml^{-1} chloramphenicol, 20 mg ml^{-1} puromycin, 2 mg ml^{-1} bacitracin, 2 mg ml^{-1} mupirocin, 500 mM diamide, 12.8 mg ml^{-1} berberine chloride, 4.21M peracetic acid, 0.1M EDTA, 1M DTT. Plates were incubated for 24 h at $37^{O}C$ and screened for a blue halo around the perimeter of the filter discs indicating the induction of expression.

Real-time PCR. One ml of an overnight culture was laced into fresh TSB and allowed to grow for 3 hours at 37°C, shaking. Cultures were standardized to OD_{600} of 0.05 in TSB or TSB with sub-inhibitory concentrations of various chemicals. After the specified hour, 3 ml samples were collected, mixed with ethanol and acetone (50:50) and placed at -80°C. Samples were centrifuged at 4150 rpm for 10 minutes and cells lysed using bead beating. RNA was extracted using the Qiagen RNeasy kit. Resulting RNA was used to make cDNA using the iSCRIPT from Bio-Rad. cDNA was measured on a nanodrop and standardized for qRT-PCR. SYBR green from Takara was mixed with cDNA and the appropriate primers and qRT-PCR was performed on an Eppendorf Mastercycler.

Microtitre MIC assay for cell-wall targeting drugs. Cultures of SH1000 and SACOL2525/2526 mutant were diluted 1:1000 in fresh TSB and 200 µl was applied to the wells of a 96-well plate. Chemicals were added to the wells in decreasing concentrations and mixed by pipetting. Plates were incubated at 37°C and the minimum inhibitory concentration determined by visual examination for the well containing the lowest concentration producing no growth.

Survival in the free unsaturated fatty acid, oleic acid. Bacterial overnight cultures were sub-cultured in fresh TSB and allowed to grow for 3 hours. Cells were washed three times with sodium phosphate buffer before being standardized to an OD_{600} reading of 0.05 in 5 ml sodium phosphate buffer. Oleic acid was added to a final concentration of 0.01% and 30 µl sample removed every 15 minutes, serial diluted and plated to determine cfu/ml.

Survival in toluene. Bacterial overnight cultures were sub-cultured in fresh TSB and allowed to grow for 3 hours. Cells were standardized to an OD_{600} reading of 0.05 in 100 ml TSB. Toluene was added to a final concentration of 1.5% and 30 µl samples removed every 15 minutes, serial diluted and plated to determine cfu/ml.

Negative staining electron microscopy. Aliquots of cells grown in TSB for 3h were initially fixed in an equal volume of 2% osmium tetroxide in distilled water for 1h at 4˚C. Following fixation, cells were rinsed in distilled water and pelleted 3 times at 5000 RPM for 10 minutes. Dilutions were performed to obtain approximately 2000-3000 cells per drop, before one drop of each sample was applied to a carbon-formvar coated copper grid. Grids were allowed to air dry and photographed by electron microscopy.

Hydrophobicity assay using hexadecane. The hexadecane assay was performed at described by Greene et al., 1992. Synchronized bacterial cultures were standardized to an OD_{540} of 0.5 in 1ml of PBS. To each 150 µl of hexadecane was added, mixed thoroughly and left at room temperature for 20 minutes for phase separation. The aqueous phase was removed and the OD_{540} collected. Percent adherence was calculated using

% adherence = (initial OD_{540} – final OD_{540} / initial OD_{540}) * 100

Cytochrome C binding assay. The cytochrome C binding assay was performed at described by Peschel et al., 1999. Bacteria were grown for 3 hours and harvested by centrifugation. Cells were washed three times in MOPS buffer (20mM, pH 7) and resuspended to a final OD_{600} of 7. Cytochrome C was added (0.5 mg/ml) and incubated

at room temperature for 10 minutes. Cells were pelleted by centrifugation and supernatants analyzed at 530 nm.

Peptidoglycan lysis kinetics assay. The lysis kinetic assay was performed as described by Shaw et al., 2005. Cells were harvested from exponential-phase cultures, washed three times with sodium phosphate buffer before being resuspended to an OD_{600} of 1.0. Excess lysostaphin (50μg/ml) was added and OD_{600} values were recorded every 5 minutes using a plate reader.

Survival in daptomycin. Overnight cultures were synchronized and then standardized to an OD_{600} of 0.05 in 5 ml TSB. Daptomycin was added to a final concentration of 0.75 μg/ml and samples taken every hour, serially diluted and cfu/ml calculated.

Lysostaphin treatment for removal of the cell envelope. Overnight cultures were subcultured into fresh TSB and allowed to grow for 3 hours. Cells were pelleted by centrifugation, washed three times in TSM buffer (50 mM Tris-HCl, 0.5 M sucrose, 10 mM MgCl2) before being resuspended in TSM buffer to an OD_{600} of 0.05. Lysostaphin was added at a concentration of 5 μg/ml and incubated at 37°C for 45 minutes. Aliquots were taken and gram stained to ensure removal of the cell envelope but not cell lysis by observing pink cocci under the microscope. The resulting protoplasts were washed three times with sodium phosphate buffer and resuspended in either TSB or TSB with $CaCl_2$, daptomycin (0.4 μg/ml), kanamycin (1.0 μg/ml), or CCCP (0.005 μg/ml).

Spontaneous mutation frequency. Overnight cultures (100 μl) of SH1000 or the SACOL2525/2526 mutant were plated on TSA plates containing 3X the MIC of bacitracin, nisin, vancomycin, teicoplanin, daptomycin, CCCP, phosphomycin and

penicillin G at incubated at 37°C overnight. The overnight culture was also serially diluted and cfu/ml determined for mutation frequency calculation.

Results

In silico analysis of SACOL2525/2526. Since the function of SACOL2525/2526 has not yet been investigated, other than they do not fulfill the same role as StpA and SmpA, an in silico analysis was performed to identify any conserved domains and homology to other proteins. The family that SACOL2525 belongs to was determined by analyzing amino acid sequences using the NCBI site. This revealed that SACOL2525 is similar to other ATP-binding proteins that are part of the galliderm-class lantibiotic ABC transporter family. This family of ABC transporters is known to transport lantibiotics, including nisin (Tomii & Kanehisa, 1998). SACOL2525 is the ATP-binding protein that comprises part of the ABC transporter system and as such has the attributes/domains of similar proteins, including an ATP-binding domain, an ABC transporter signature motif, a Walker A/P loop and no transmembrane domains. Using the ClustalW2 software, the amino acid sequence of SACOL2525, EpiF from *Staphylococcus epidermidis*, MutL from *Streptococcus mutans*, and two other predicted ATP-binding proteins that have similarity to members of the galliderm-class lantibiotic ABC transporter family, from *S. hominis* and *S. haemolyticus* were compared (Figure 8). EpiF from *S. epidermidis* is part of an ABC transport system that mediates protection from epidermin, by pumping it outside of the cell (Peschel & Gotz, 1996). Interestingly, SACOL2525 was found to have 84% similarity to EpiF at the amino acid level. Similarly, MutL from *S. mutans* is the ATP-binding protein of an ABC transport system that confers resistance to mutacin (Chen et al., 1999). SACOL2525 only had 40% similarity with MutF at the amino acid level.

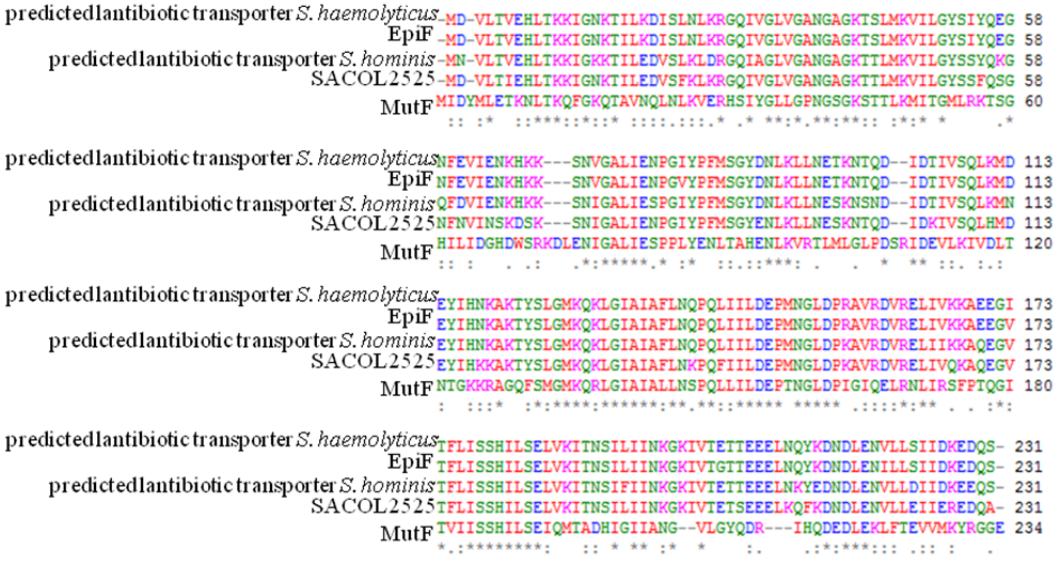

Figure 8. Amino acid sequence alignment of SACOL2525. The amino acid sequence of SACOL 2525; the lantibiotic transporter ATP-binding protein, EpiF, from *Staphylococcus epidermidis*; the lantibiotic transporter ATP-binding protein MutL from *Streptococcus mutans*; and two other predicted ATP-binding proteins (from *S. hominis* and *S. haemolyticus*) that have similarity to the galliderm-class of lantibiotic ABC transporters were compared using ClustalW2 software. The asterisks represent a conserved residue in all sequences, whilst a colon indicates a strong similarity between residues. A period signifies conservation between residues with similar biochemical properties. Colors are designating according to the physicochemical properties of amino acids: red (small and hydrophobic); blue (acidic); magenta (basic) and green (hydroxyl + sulfhydryl + amine).

Analysis of the integral membrane protein, SACOL2526, shows that it does not have domains specific for lantibiotic transport. SACOL2526 has six transmembrane domains, which several other lantibiotic drug transporters have, including SpaG and NisG of *B. subtilis* (Dawson & Locher, 2006; Kuipers et al., 1993). These proteins bind as dimers, creating a 12 transmembrane channel for the passage of drugs. Collectively this data suggests that SACOL2525/2526 may form the two components of an ABC transport system that is specific to lantibiotics and/or other drugs. When one analyzes the coding region for SACOL2525/2526 it is apparent that they are likely transcriptionally linked, as only 15bp separates the 2 open reading frames. Upstream of SACOL2525 is a putative consensus sequence for recognition by the housekeeping sigma factor, σ^A (Figure 9). Specifically, we determined a -35 sequence of TTcACA, followed by a 17 bp spacer, and a -10 sequence of TATAAT. Since this predicted ABC transporter contains the consensus sequence recognized by σ^A, this may suggest that SACOL2525/2526 is utilized by the cell under standard conditions.

Transcriptional profiling of the SACOL2525/2526 ABC transporter. To analyze the utilization of SACOL2525/2526 by *S. aureus*, its transcriptional profile was determined. Using qRT-PCR, expression was observed during both the exponential and post-exponential phases of growth (Figure 10). Primers specific for SACOL2525 or SACOL2526 were utilized, however both produced similar results as they are predicted to be transcribed under one promoter. As such, the data for primers specific to SACOL2526 are presented herein, and throughout, even though SACOL 2525 primers were also used, and corroborated findings in each case. We determined that expression was highest at hour 3, which declined 2.4-fold by hour 5, and 3.2-fold by hour 15.

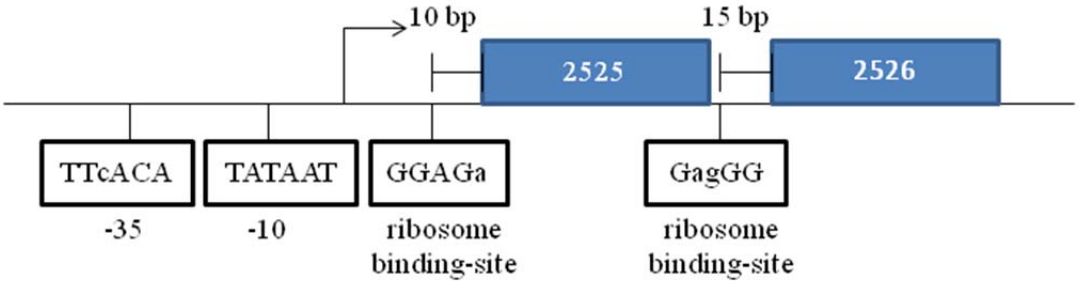

Figure 9. The SACOL2525/2526 operon. The operon structure of SACOL2525/2526. Shown are the consensus sequences for a lone predicted promoter found upstream of SACOL2525 that appears to control both open reading frames. Also shown are relative spacing between elements, and predicted ribosome binding-sites.

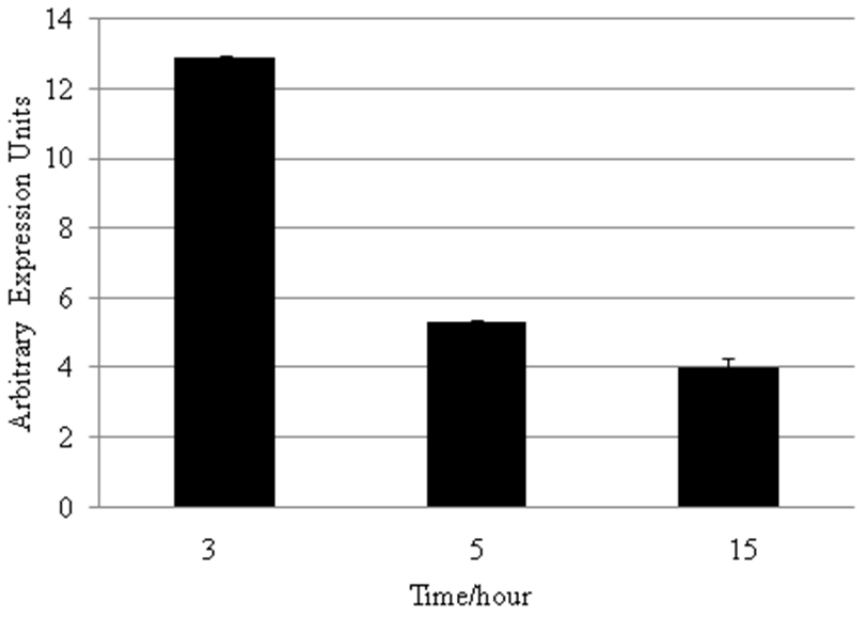

Figure 10. Transcription of SACOL2525/2526 during growth in standard conditions. qRT-PCR was performed on SACOL2525/2526 at hours 3 (exponential phase), 5 (post-exponential phase) and 15 (stationary phase) in SH1000 grown under standard conditions. The data presented are from three independent cultures and error bars are shown +/- SEM.

Given that SACOL2525/2526 has homology to other SMR multi-drug transporters, we next set out to determine if any modulation in transcription of this transporter is seen in the presence of stressor compounds. This was performed using a *lacZ* fusion in strain SH1000, which was constructed by cloning the entire predicted promoter region upstream of SACOL2525 upstream of the *lacZ* gene in the suicide vector pAZ106 (see Methods). As such, each time SACOL2525/2526 is transcribed from its promoter, the *lacZ* gene is also, which is then translated into β-galactosidase. When β-galactosidase and X-Gal come into contact, the X-Gal is cleaved and a blue color is visualized. This strain was then utilized in a plate based assay as described by ourselves and others (Cao et al., 2002; Shaw et al., 2008), with a variety of antibacterial chemicals (Table 3). An overlay containing the *lacZ* fusion strain, and the β-galactosidase substrate X-Gal at 40 μg/ml, which would not ordinarily result in blue coloration, was inoculated onto TSA plates. A sterile filter disk was placed on the plates and 10 μl of each chemical was added and allowed to dry. The plates were incubated overnight at 37°C and the appearance of a blue ring around the zone on inhibition signified an increase in transcription, as a result of X-Gal cleavage by β-galactosidase. We determined that several cell-envelope-targeting agents, including penicillin G, phosphomycin, bacitracin, chloropromazine, CCCP, oxacillin and ampicillin produced a blue ring in this analysis, suggesting an increase in SACOL2525/2526 transcription (Table 4). In addition, triclosan, which inhibits fatty acid synthesis by targeting FabI, also increased transcription. Novobiocin, a drug that inhibits DNA replication by targeting the gyrase enzymes produced the same effect. To quantitatively confirm these results, qRT-PCR was conducted on the SH1000

Table 3. Stressor compounds used in the plate-based assay to determine the inducibility of SACOL2525/2526 expression.

Stress	Chemical	Concentration
Cell-envelope-targeting Antibiotics	**Daptomycin***	10 µg
	Gramicidin	50 µg
	CCCP*	1 µg
	Chloropromazine*	50 µg
	D-cycloserine	50 µg
	Penicillin G*	20 µg
	Oxacillin*	50 µg
	Cefotaxime	50 µg
	Phosphomycin*	20 µg
	Vancomycin	20 µg
	Ampicillin*	1 mg
	Bacitracin	20 µg
Other Antibiotics	Nalidixic Acid	50 µg
	Ciprofloxacin	50 µg
	Novobiocin*	50 µg
	Triclosan*	10 µg
	Chloramphenicol	50 µg
	Phosphomycin	20 µg
	Spectinomycin	50 µg
	Tetracycline	50 µg
	Erythromycin	50 µg
	Lincomycin	250 µg
	Kanamycin	500 µg
	Neomycin	500 µg
	Puromycin	250 µg
	Mupirocin	20 µg
Acid	Hydrochloric Acid	6 M
	Phosphoric Acid	10 M
	Formic Acid	12 M
	Acetic Acid	1 M
	Sulphuric Acid	12 M
	Nitric Acid	6 M
	Trichloroacetic Acid	12 M
Alkali	Sodium Hydroxide	3 M
Osmotic	Sodium Chloride	1 M
	Glucose	1 M

Table 3 continued

Alcohol	Ethanol	100%
	Methanol	100%
	Isopropanol	100%
Detergent	Sodium Dodecyl Sulfate	10%
	Triton X-100	1%
	Tween-20	1%
	N-lauroyl Sarcosine	1%
Oxidative	Hydrogen Peroxide	30%
	Methyl Viologen	2 M
	Menadione	1%
	Pyrogallol	4 mg
Nitrosative	Sodium Nitroprusside	1M
DNA Damage	Methyl Methansulfonate	50 mM
	Ethyl Methanesulfonate	50 mM
Miscellaneous	Berberine Chloride	128 µg
	Peracetic Acid	4.2 M

*And in bold = chemicals that increased transcription of SACOL2525/2526

Table 4. Cell-envelope-targeting antibiotics utilized to determine the transcription of SACOL2525/2526 under cell-envelope stress

Drug	Target	Results in damage of
phosphomycin*	intracellular peptidoglycan subunit assembly	cell-wall biosynthesis pathway
D-cycloserine*	intracellular peptidoglycan subunit assembly	cell-wall biosynthesis pathway
nisin	transport and anchoring via lipid II	cell-wall biosynthesis pathway
teicoplanin	transport and anchoring via lipid II	cell-wall biosynthesis pathway
ampicillin*	extracellular cross-linking of peptidoglycan subunits	cell-wall biosynthesis pathway
penicillin G*	extracellular cross-linking of peptidoglycan subunits	cell-wall biosynthesis pathway
oxacillin*	extracellular cross-linking of peptidoglycan subunits	cell-wall biosynthesis pathway
bacitracin	re-entry of bactoprenol carrier	cell-wall biosynthesis pathway
gramicidin	pore forming	cell-membrane
daptomycin*	pore forming	cell-membrane
CCCP *	loss of the proton motive force	cell-membrane
chloropromazine*	ion flux across the cell membrane	cell-membrane

* denotes chemicals shown to increase transcription of SACOL2525/2526

wild-type strain during a window of peak SACOL2525/2526 expression (3h), grown in either TSB or TSB with sub-inhibitory concentrations of the compounds shown to increase transcription. In addition, drugs that increase the transcription of *nsaRS*, but did not produce an increase in SACOL2525/2526 on the plate based assay, including daptomycin, D-cycloserine, nisin and gramicidin, were also included (Figure 11). The largest effect was observed with CCCP, which produced a 10.2-fold increase in transcription. Further to this, penicillin G produced a 7.8-fold increase, followed by chloropromazine which showed a 6.3-fold elevation. Daptomycin and phosphomycin produced a 3.9 and 2-fold increase respectively. The fatty acid targeting drug triclosan produced a 2-fold increase, however the other, non cell-envelope-targeting drug, novobiocin, did not alter expression. The remaining drugs, oxacillin, vancomycin, bacitracin, nisin, novobiocim, ampicillin and D-cycloserine produced fold changes below 2-fold, suggesting that they result in limited differences in SACOL2525/2526 transcription, at least using the concentrations and conditions tested herein.

NsaRS, but not GraRS, regulates transcription of SACOL2525/2526 in response to a variety of cell-envelope-targeting compounds. NsaRS and GraRS have both been shown to regulate SACOL2525/2526. As stated previously, SACOL2525/2526 transcription increases in the presence of a multitude of cell-envelope-targeting drugs that affect various stages in the cell wall biosynthesis pathway. To determine if the observed increase in transcription is influenced by either NsaRS or GraRS, qRT-PCR was conducted for SACOL2525/2526 in either an *nsaS* or *graR* mutant. These strains were grown in sub-inhibitory concentrations of the same drugs that increase

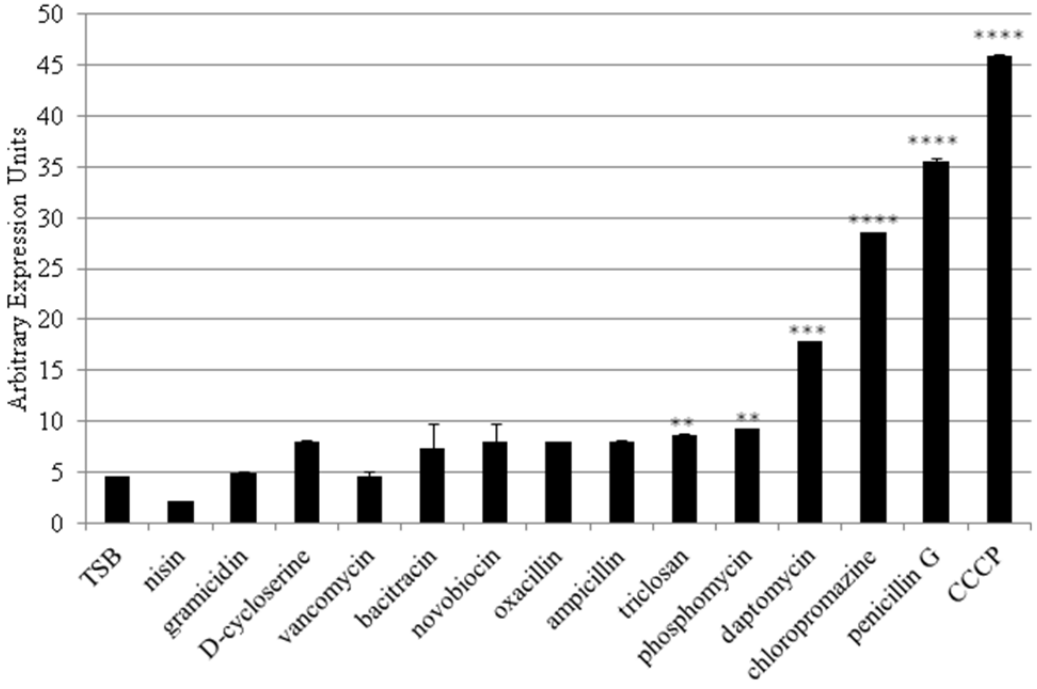

Figure 11. Inducibility of SACOL2525/2526 by antibacterial agents. qRT-PCR was performed on SACOL2525/2526 in SH1000 at hour 3 under standard conditions, or in the presence of sub-inhibitory concentrations of the chemicals indicated. The data presented are from three independent cultures and error bars are shown +/- SEM. A Student's T-Test was used with a 5% confidence level to determine statistical significance, ** = p□0.01, *** = p< 0.001, **** = p□0.0001.

SACOL2525/2526 transcription, and were compared to expression in the SH1000 wild-type (Figure 12). Interestingly, transcription of SACOL2525/2526 was not significantly altered in a *graR* mutant in the presence of any drug tested. However, a decrease in transcription was observed for several compounds in the *nsaS* mutant. Specifically, mutation of *nsaS* caused a 2.7-fold reduction in expression in the presence of daptomycin. In addition, a 5.2-fold reduction was observed for penicillin G. The largest alteration was in the presence of the membrane targeting drug CCCP, with a 7.1-fold decrease observed compared to the parental strain. All of the other drugs tested generated a fold change less than 2, and thus NsaRS does not strongly influence the increases in transcription produced by ampicillin, oxacillin, D-cycloserine, phosphomycin and chloropromazine. Collectively, this suggests that NsaRS regulates SACOL 2525/2526 in response to a variety of cell-envelope- targeting antimicrobial agents, although other factors also appear to be at work.

SACOL2525/2526 aids in resistance to daptomycin. Several cell-envelope-targeting drugs increase the transcription of SACOL2525/2526, and hence a SACOL 2525/2526 mutant was constructed to determine the sensitivity of this strain to such agents. In addition, the mutation was complemented by introducing SACOL2525/2526, along with its native promoter, on the pMK4 shuttle vector into mutant strains (see methods). The sensitivity of the mutant to a variety of chemicals was determined via MIC analysis for phosphomycin, ampicillin, penicillin G, D-cycloserine, oxacillin, daptomycin, chloropromazine, bacitracin, nisin, teicoplanin and gramicidin. No difference in sensitivity was found between the two strains for any of these compounds. MIC assays are performed overnight, and thus it is possible

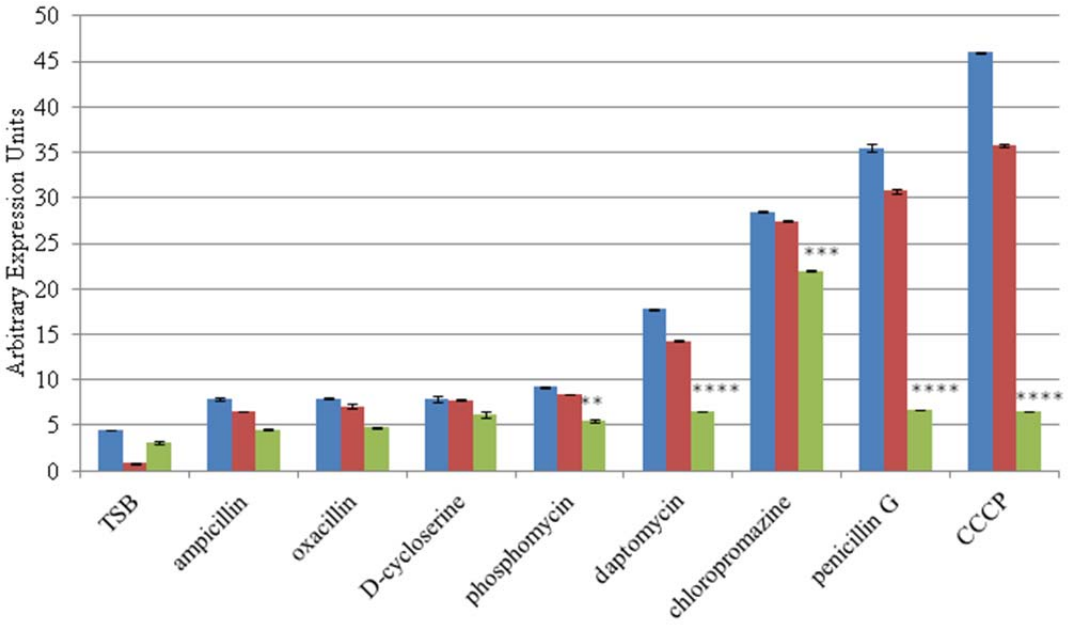

Figure 12. The impact of GraRS and NsaRS on the transcription of SACOL2525/2526. qRT-PCR was performed on SACOL2525/2526 at hour 3 under standard conditions, or in the presence of sub-inhibitory concentrations of the chemicals listed. The strains used were the wild-type (blue), *graR* mutant (red) and *nsaS* mutant (green). The data presented are from three independent cultures and error bars are shown +/- SEM. A Student's T-Test was used with a 5% confidence level to determine statistical significance, ** = p□0.01, *** = p< 0.001, **** = p□0.0001.

that the mutant might be more sensitive to a given compound initially, but it ultimately does not impact final growth yields. To eliminate this possibility, death curves were performed during 5 hours of incubation in the presence of lethal concentrations of several of these compounds. No difference in the rate of death was observed with penicillin G, bacitracin, gramicidin or CCCP (Figure 13). We did note, however, that in the presence of daptomycin, the SACOL2525/2526 mutant had decreased survivability compared to the wild-type and complemented strains (Figure 14). Daptomycin affects the cell membrane, and must diffuse through any external barriers, including teichoic acids, peptidoglycan and capsule, to impact its target. As *S. aureus* produces has many such impediments when grown under standard conditions (O'Riordan & Lee, 2004), we sought to determine if the decrease in survivability of the SACOL2525/2526 mutant in the presence of daptomycin could be enhanced by the removal of these elements. As such, strains were first treated with 5μg/ml of lysostaphin for 45 minutes to remove the cell wall, along with all proteins and molecules external to the membrane. After treatment, samples of the resulting protoplasts were Gram stained to ensure the removal of peptidoglycan, and that cell lysis had not occurred. In addition, the cfu/ml of cells before and after lysostaphin treatment was determined to ensure that treatment did not affect one strain more than the other (Figure 15). After treatment, the resulting protoplasts were washed to remove the lysostaphin, before being subject to death curve kill studies. First, control experiments were performed to determine if either TSB or TSB with 1 mM $CaCl_2$, had an effect on cell viability (Figure 16). For these analyses, $CaCl_2$ was used as a control because it is required for daptomycin to exert its activity (Jung et al., 2004;

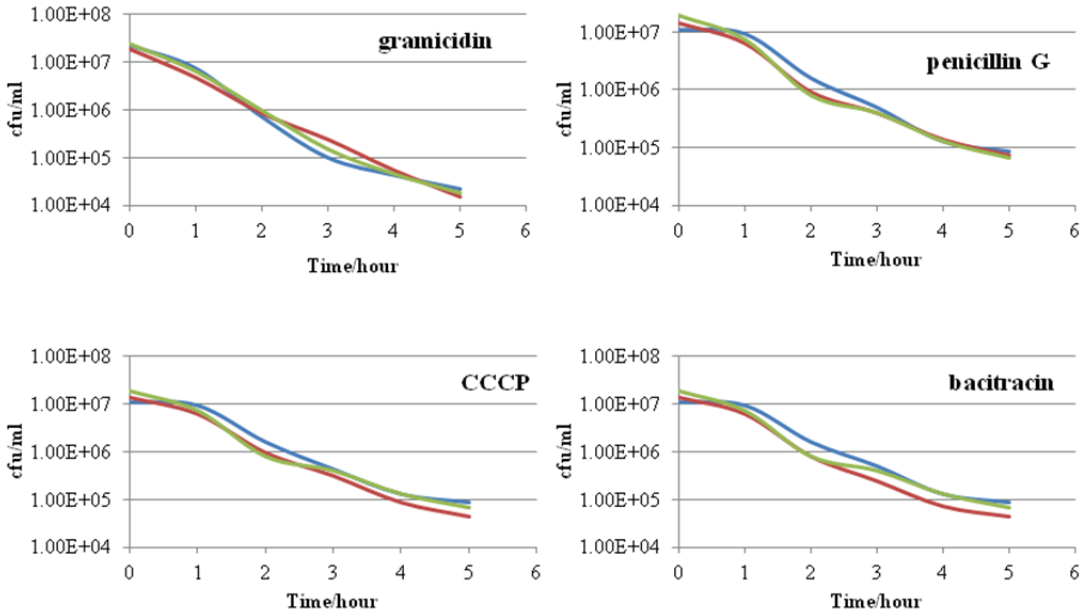

Figure 13. SACOL2525/2526 do not present enhanced sensitivity to a number of cell-wall targeting chemicals. Growth of the SH1000 wild-type (blue), SACOL2525/2526 mutant (red) and complemented (green) strains was analyzed over 5 hours in the presence of lethal concentrations of: gramicidin (15 μg/ml), penicillin G (0.5 μg/ml), CCCP (0.05 μg/ml) or bacitracin (25 μg/ml). The results presented are representative of three independent cultures that show less than 10% viability.

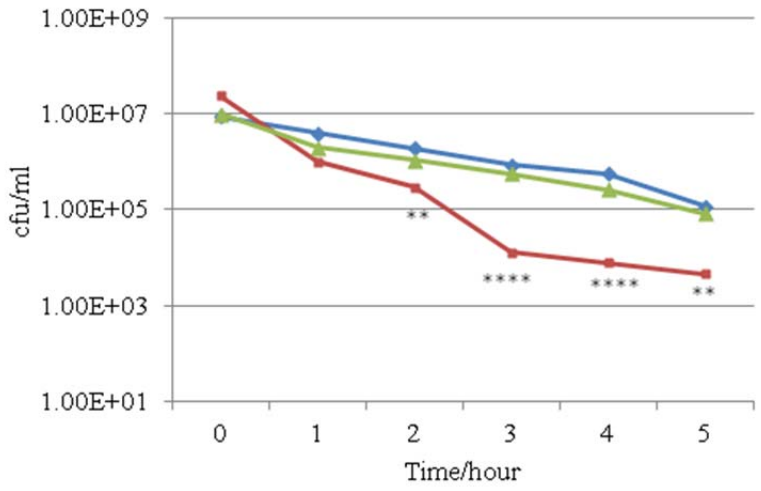

Figure 14. SACOL2525/2526 aids in the resistance of *S. aureus* to daptomycin. Growth of the SH1000 wild-type (blue), SACOL2525/2526 mutant (red) and complemented (green) strains was analyzed over 5 hours in the presence of a lethal concentration of daptomycin (0.75 μg/ml + 1mM $CaCl_2$). The results presented are representative of three independent cultures that show less than 10% viability. A Student's T-Test was used with a 5% confidence level to determine statistical significance, ** = $p<0.01$, **** = p□0.0001.

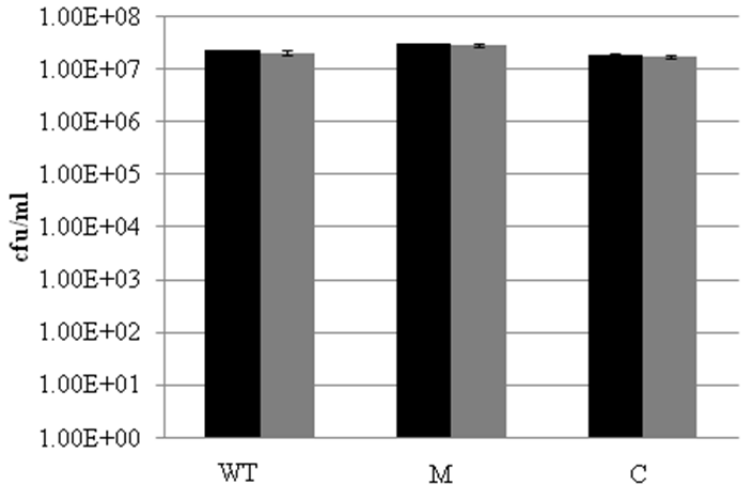

Figure 15. Lysostaphin treatment does not lead to increase death in the SACOL2525/2526 mutant. Lysostaphin was added to the wild-type (WT), SACOL2525/2526 mutant (M) and its complemented strain (C)at 5 μg/ml for 45 minutes at 37°C. cfu/ml was calculated by serial dilution and plating before (black) and after (grey) lysostaphin treatment. The error bars are shown as +/- SEM.

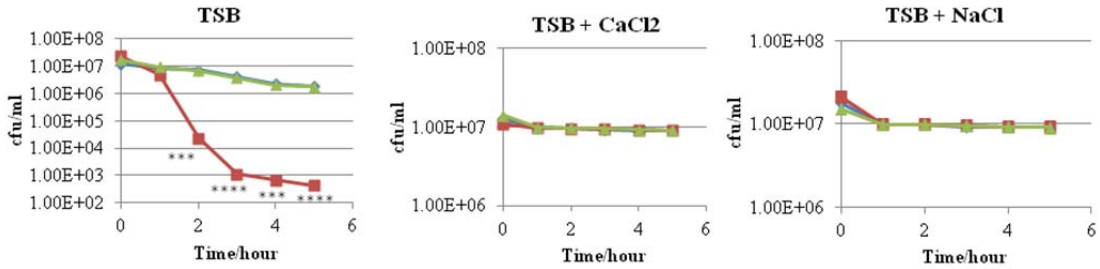

Figure 16. SACOL2525/2526 mutants have a weakened cell membrane. The SH1000 wild-type (blue), SACOL2525/2526 mutant (red) and complemented (green) strains were treated with 5 μg/ml of lysostaphin and the resulting protoplasts were incubated in either TSB, TSB with $CaCl_2$ (1 mM) or TSB with NaCl (1 mM). Growth was analyzed over the course of 5 hours; the results presented are representative of three independent cultures that show less than 10% viability. A Student's T-Test was used with a 5% confidence level to determine statistical significance, *** = $p < 0.001$, **** = $p < 0.0001$.

Ho et al., 2008). In TSB alone, the SACOL2525/2526 mutant had decreased survivability when compared to the wild-type and complemented strain. Specifically, a 31.3-fold decrease in the SACOL 2525/2526 mutant viability was observed at hour 2 that declined further at hour 3 (391-fold), and continued through hours 4 (343-fold) and hour 5 (442-fold). Significantly, this phenotype was completely reversed upon the addition of $CaCl_2$, with all three strains surviving equally well upon its addition to TSB. To determine if this effect was specific to $CaCl_2$, survival in 1 mM NaCl was also evaluated. Again, the decreased survival in TSB by the mutant strain was completely reversed, as all three strains grew equally. Next, the survival of the three strains was analyzed in TSB with 1 mM $CaCl_2$ and 0.4 μg/ml of daptomycin (Figure 17). It should be noted that this concentration is not normally lethal to cells, but is however, toxic to protoplasts. Despite our findings when used by itself, the addition of $CaCl_2$ in the presence of daptomycin was unable to rescue viability of the SACOL 2525/2526 mutant compared to the wild-type. Specifically, a significant difference was seen at hour 2, which produced a 17.2-fold decrease in survival. This effect was further enhanced at hours 3 (159-fold), hour 4 (209-fold) and hour 5 (264-fold). To determine if this effect is specific to daptomycin, the same analysis was performed for CCCP (5.0 ng/ml), gramicidin (10 μg/ml) and kanamycin (1.0 μg/ml). CCCP works by altering the proton motive force of membranes, whilst gramicidin produces similar damaging effects to daptomycin by inserting into the membrane. Kanamycin was used as a control as it does not target the membrane or cell wall, but instead inhibits protein synthesis. Neither CCCP, gramicidin or kanamycin produced the same effect as daptomycin when tested, as all three strains had similarly declining viability upon

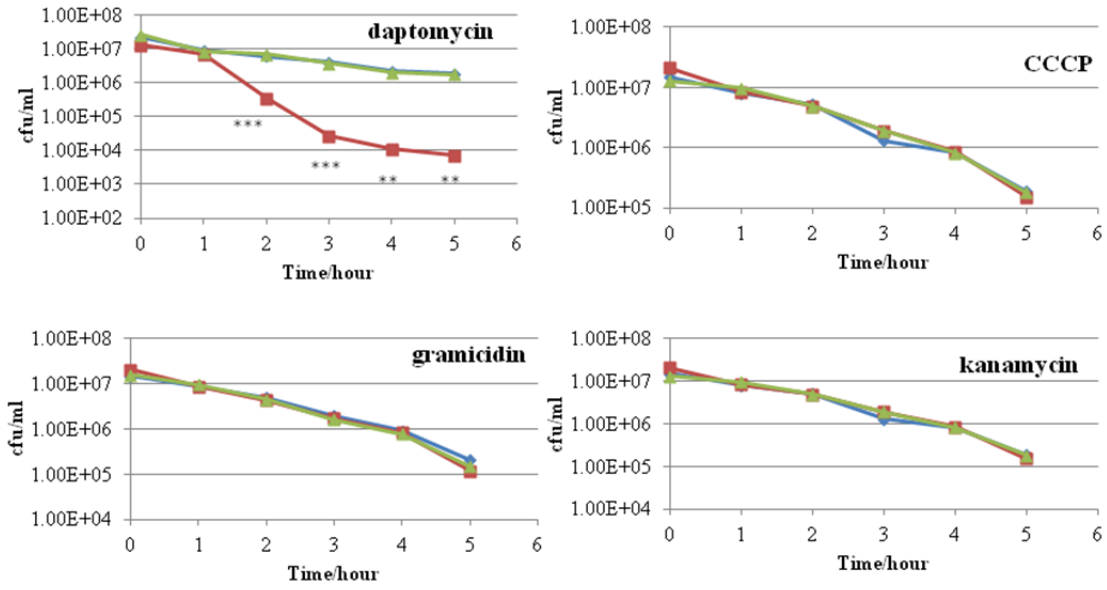

Figure 17. SACOL2525/2526 mutant protoplasts have elevated sensitivity to daptomycin. The SH1000 wild-type (blue), SACOL2525/2526 mutant (red) and complemented (green) strains were treated with 5 µg/ml of lysostaphin. The resulting protoplasts were incubated in either TSB with 1 mM $CaCl_2$ and 0.4 µg/ml daptomycin, 5 ng/ml CCCP, 10 µg/ml gramicidin or 1.0 µg/ml kanamycin. Growth was analyzed over the course of 5 hours and the results presented are representative of three independent cultures that show less than 10% viability. A Student's T-Test was used with a 5% confidence level to determine statistical significance, ** = $p<0.01$, *** = $p< 0.001$.

analysis. This suggests that SACOL2525/2526 aids *S. aureus* in resistance to daptomycin specifically.

SACOL2525/2526 aids in the ability of *S. aureus* to spontaneously resist the action of a variety of cell-envelope targeting antibiotics. Work by ourselves and others reveals that NsaRS has a role in the resistance to nisin and bacitracin. NsaRS regulates SACOL2525/2526, and their mutants share several phenotypic commonalities. In addition, SACOL2525/2526 is predicted to be a multi-drug transporter based on homology analyses, and disruption of this transporter leads to increased sensitivity towards the cell-membrane-targeting antibiotic, daptomycin. Thus the ability of SACOL2525/2526 to mediate resistance to cell-envelope-targeting drugs was investigated. The spontaneous mutation frequency for bacitracin, nisin, vancomycin, teicoplanin, daptomycin, CCCP, phosphomycin and penicillin G were analyzed using 10 different replicates from 3 independent cultures of either the wild-type or SACOL2525/2526 mutant (Figure 18). Interestingly, no alteration in frequency was observed for nisin, vancomycin, CCCP or phosphomycin. We did, however, observe differences for the remaining 4 drugs, which all produced a significant difference in the number of isolates that were able to spontaneously resist the action of these compounds. The SH1000 wild-type produced a mutation frequency of 5.03×10^{-8} for teicoplanin, while the SACOL2525/2526 mutant displayed a 3.1-fold decrease, with a frequency of only 1.58×10^{-8}. The spontaneous mutation frequency for daptomycin produced similar results, with the wild-type generating a resistance frequency of 1.85×10^{-9}, while the SACOL2525/2526 mutant showed a 3.6-fold decrease of only 5.04×10^{-10}. The observed frequency for the mutant in response to penicillin G had a larger discrepancy,

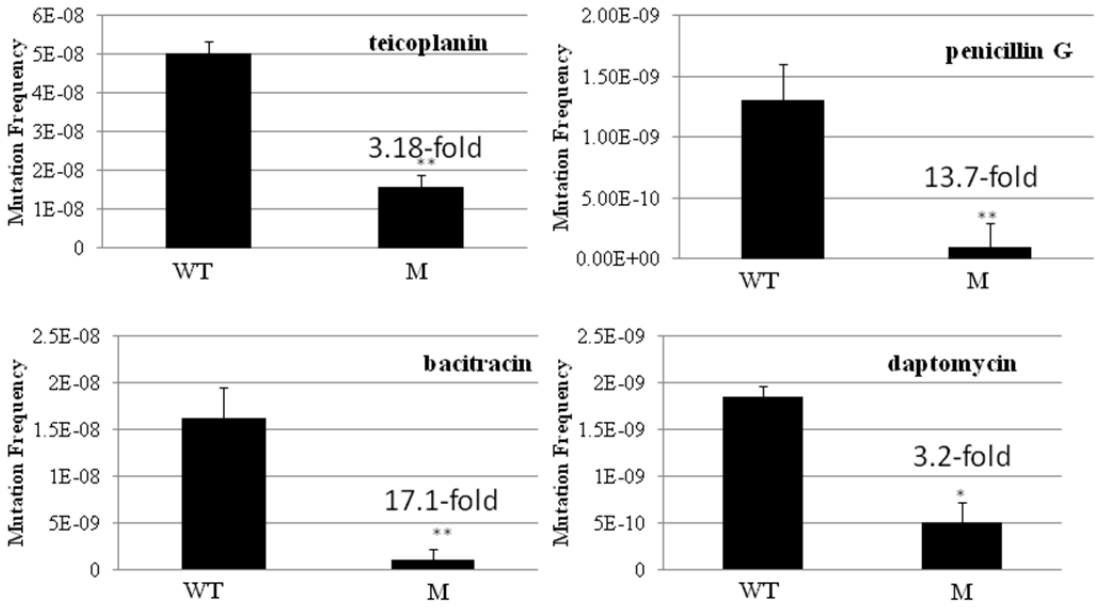

Figure 18. SACOL2525/2526 aids in the ability to spontaneously resist the action of a variety of cell-envelope-targeting antibiotics. The SH1000 wild-type (WT) and SACOL2525/2526 mutant (M) were grown on TSA containing 3X the MIC of the antibiotics indicated. Plates were incubated overnight, and spontaneous mutants were allowed to develop. Mutation frequencies were calculated with respect to the initial inoculum. Data is presented from three independent cultures and at least 10 replicates. Error bar are shown as +/- SEM. A Student's T-Test was used with a 5% confidence level to determine statistical significance, * = $p<0.05$, ** = $p<0.01$.

with a 13.7-fold decrease in mutability. Specifically, we observed a frequency for the wild-type of 1.31 x 10^{-9} towards penicillin G, and 9.52 x 10^{-11} for the mutant. The largest difference was seen with bacitracin, with a 17.1 decrease in the mutant, with the parent generating a 1.63 x 10^{-8} mutation frequency, and the SACOL 2525/2526 mutant resulting in a frequency of 9.25 x 10^{-10}.

SACOL2525/2526 is part of the *S. aureus* resistance mechanism against toxic non-antibiotic chemicals that target the cell membrane. In contrast to many of the other agents analyzed above, triclosan increases transcription of SACOL2525/2526, but does not target the bacterial cell envelope. Instead, triclosan impacts fatty acid synthesis via inhibition of FabI (Slater-Radosti et al., 2001). To determine if the mutant was sensitive to triclosan, the minimum-inhibitory concentration (MIC) determined for this strain compared to the wild-type. Upon analysis it was found that both strains have an MIC for this compound of 0.1μg/ml. Next, growth of the wild-type and mutant strain in the presence of a sub-inhibitory concentration of this agent (0.05 μg/ml) was evaluated over 5 hours, with no difference again observed in either growth rates or yields. Fatty acids are synthesized and inserted into the membrane to control fluidity, among other roles (Zhange & Rock, 2008). Since the mutation of SACOL2525/2526 had no effect on sensitivity to the inhibition of fatty acid synthesis, we examined the effect of free fatty acids on the SACOL2525/2526 mutant. Oleic acid is an unsaturated free fatty acid that inserts into the membrane of bacteria causing a variety of damage, including interference with proteins needed for energy and metabolism, the inhibition of biosynthetic pathways, peptidoglycan precipitation, altered membrane fluidity, cell leakage and eventually lysis (Galbraith & Miller, 1973; Knapp & Melly., 1986; Speert

et al., 1979; Chamberlain et al., 1991; Xiong & Kapral., 1992). As such, the wild-type, mutant and complement strains were standardized to an OD_{600} of 1.0 in PBS containing a lethal concentration of oleic acid (0.01%) and the cfu/ml was evaluated every 15 minutes for 90 minutes. What was found was that the viability of all strains vastly decreased compared to the inoculum, however the SACOL2525/2526 mutant survived considerably better than the other strains (Figure 19). Specifically, after 15 minutes 0.002% of the wild-type, 0.08% of the SACOL2525/2526 mutant, and 0.005% of the complemented strain inoculums was recovered; resulting in a 30-fold increase in survivability of the mutant. Additionally, no cells from the wild-type or complemented strain could be recovered after 15 minutes, while viable SACOL2525/2526 mutant cells were found up to 90 minutes post-inoculation. The increased survivability of the SACOL2525/2526 mutant clearly shows that it is more resistant to unsaturated free fatty acids than either the parental or complemented strains. Gram-positive bacteria often resist the action of free fatty acids by decreasing the fluidity of their membrane (Desbois & Smith, 2010). Therefore, we hypothesized that, in the presence of unsaturated free fatty acids, *S. aureus* decreases fluidity of its membrane to cope with this stress, and may do so by decreasing the expression of SACOL2525/2526. In order to determine if this in fact the case, qRT-PCR for SACOL2525/2526 transcript levels in the presence of oleic was performed. Upon analysis, we observed a 5-fold decrease in expression of the transporter for SH1000 grown in 1.5% Oleic acid, compared to unsupplemented TSB (Figure 20). Surprisingly when *S. aureus* is grown in rich media (TSB) it is able

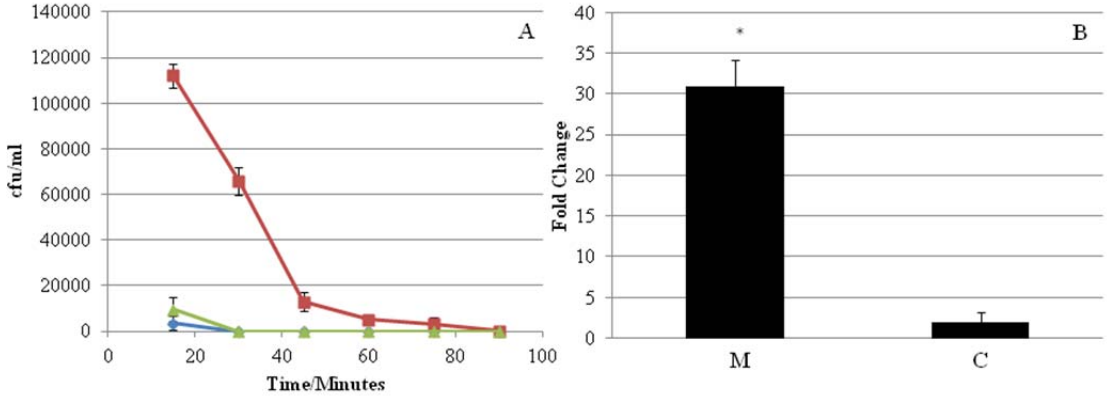

Figure 19. **SACOL2525/2526 mutation protects the cell in the presence of free unsaturated fatty acids. A).** The SH1000 wild-type (blue), SACOL2525/2526 mutant (red), and SACOL2525/2526 complement (green) strains were standardized in PBS containing 0.01% oleic acid. The survivability of strains was then evaluated over time by serial diluting and plating. Error bars are shown as +/- SEM. **B).** Fold change in viability of the mutant (M) and complemented (C) strains compared to the wild-type. Data is presented from three independent cultures, with a student's T-Test using a 5% confidence level used to determine statistical significance, * = $p<0.05$. The error bars are shown as +/- SEM.

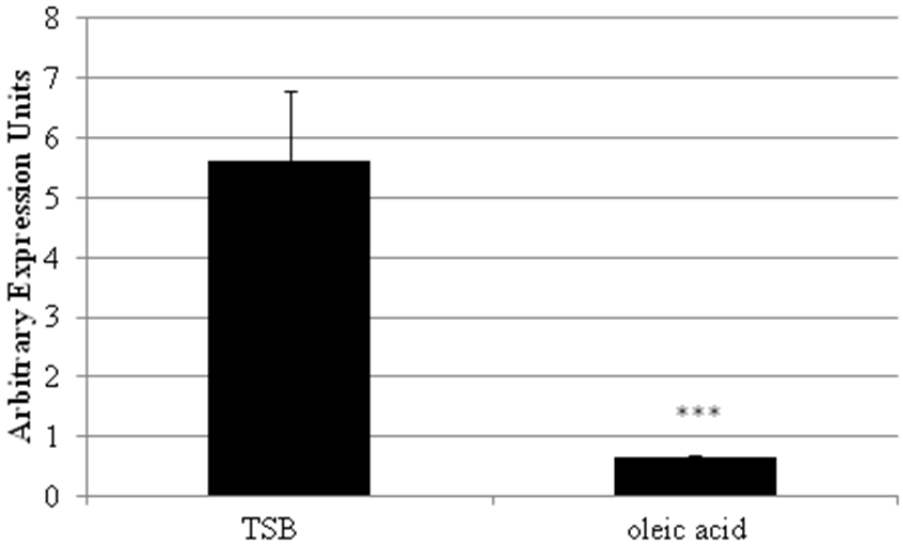

Figure 20. Transcription of SACOL2525/2526 in the presence of oleic acid. qRT-PCR was performed on SACOL2525/2526 in SH1000 cells grown for 3h in TSB under standard conditions, or in the presence of a sub-inhibitory concentration of oleic acid (1.5%). The data presented are from three independent cultures, with error bars are shown as +/- SEM. A Student's T-Test was used with a 5% confidence level to determine statistical significance, *** = $p < 0.001$.

to withstand much higher concentrations of oleic acid than in PBS. As such, despite the fact that 1.5% oleic acid is a very high concentration, it was not toxic and did not affect growth of SH1000 in TSB. To further corroborate this finding, growth in toluene was investigated as Gram-positive bacteria that have increased membrane fluidity also have higher solvent tolerance (Nielsen et al., 2005). Death in 1.5 % toluene was evaluated over a 120 minute time span for SH1000 and its SACOL2525/2526 mutant and complemented strains (Figure 21). While all strains decreased over time, the SACOL2525/2526 mutant declined far more rapidly than the parent. Specifically, after 30 minutes a 4.2-fold decrease was observed in mutant cell numbers, followed by a 5.8-fold reduction after 60 minutes when compared to the wild-type. Collectively this data suggest that SACOL2525/2526 contributes to the membrane composition by putatively aiding in membrane fluidity.

SACOL2525/2526 mutants of *S. aureus* have altered cell envelope architecture. Our data thus far suggests that SACOL2525/2526 mutants potentially have altered membrane architecture. In addition, a microarray performed on *S. aureus* grown in the presence of linoleic acid, another unsaturated free fatty acid, which disrupts the cell wall in a manner akin to oleic acid, revealed decreased SACOL2525/2526 transcription, and an increase in the expression of capsule genes (Kenny et al., 2009). This is intriguing as *nsaS* mutants have both decreased SACOL2525/2526 transcription, and increased capsule production. As such, negative staining TEM analysis was performed on the SH1000 wild-type, SACOL2525/2526 mutant and the complemented strains grown in TSB for 3 hours (Figure 22). Interestingly, the SACOL2525/2526 mutant showed increased encapsulation when compared to the other strains. It has been

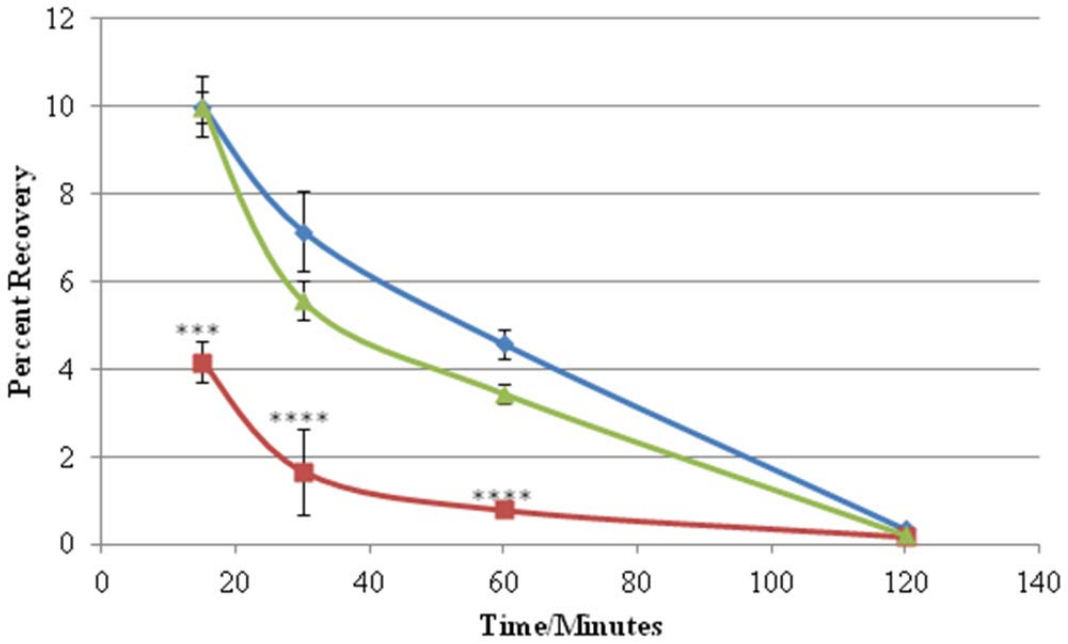

Figure 21. SACOL2525/2526 contributes to resistance against solvent stress. The SH1000 wild-type (blue), SACOL 2525/2526 mutant (red) and complemented (green) strains were inoculated into TSB containing 1.5 % toluene. Their survivability was evaluated by serial diluting, plating and calculation of the percent recovery compared to the initial inoculum. The data presented are from three independent cultures. A Student's T-Test was used with a 5% confidence level to determine statistical significance, *** = $p < 0.001$, **** = p□0.0001. Error bars are shown as +/- SEM.

Figure 22. SACOL2525/2526 mutants have increased capsule production. SH1000, along with the SACOL2525/2526 mutant and complemented strains were grown in TSB (3 h), and prepared for negative staining analysis. Presented are TEM images that are representative of more than 10 separate frames, from three independent cultures.

previously been shown that along with decreasing membrane fluidity for resistance to fatty acids, *S. aureus* also increases capsule production in an attempt to construct a barrier to lessen the contact of free fatty acids with the membrane (Kenny et al., 2009). When the cell changes the composition or abundance of fatty acids in the membrane, either increasing or decreasing fluidity, the phosphatidylglycerol outer polar head groups attached to the fatty acid, commonly stay the same, and only the fatty acid tails are altered (Mishra et al., 2011). For example, by placing more saturated fatty acids in the membrane, the cell can decrease fluidity because fatty acids do not contain any double bonds, and therefore pack together tightly; however the polar head group remains the same. Using this mechanism, the outer polar head groups of the membrane lipids still come into contact with any environmental chemicals (free fatty acids) no matter the composition. Therefore, increasing capsule production acts as a barrier to try to limit the contact of the free fatty acids with the membrane (Kenny et al., 2009). As a result, increased capsule production has been shown to increase the overall hydrophilicity of the bacterial cell (Greene et al., 1992). This change in hydrophilicity is a result of the proteins that are added to the cell envelope, including capsule proteins. To determine if excess capsule seen with the TEM analysis alters the overall hydrophilicity of the cell, a hexadecane assay was employed using the wild-type, SACOL2525/2526 mutant and complemented strains (Figure 23). Bacterial suspensions were mixed with hexadecane and the two phases were allowed to separate for 20 minutes. Hydrophilic encapsuled cells do not bind to hexadecane, and thus remains in the aqueous layer upon exposure, which can be then be measure photometrically. Upon analysis we observed that the SH1000 wild-type and

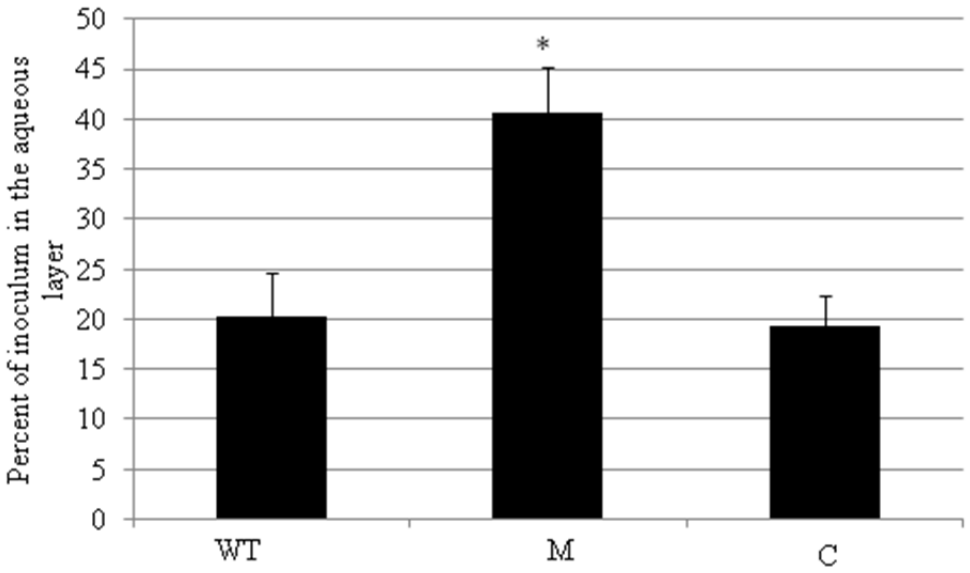

Figure 23. SACOL2525/2526 mutant cells have altered hydrophobicity. A hexadecane assay was performed on the SH1000 wild-type (WT), SACOL2525/2526 mutant (M) and complemented (C) strains. Bacterial suspensions were mixed with hexadecane and the two phases were allowed to separate. The aqueous layer was removed, the OD_{600} read and the percentage of cells in the aqueous layer, indicating increased hydrophilicity, calculated. The data presented are from three independent cultures and error bars are shown as +/- SEM. A student's T-Test with a 5% confidence level was used, * = $p<0.05$.

SACOL2525/2526 complemented strains only retained 20% and 18% of the total number of cells, respectively, in the aqueous layer. The SACOL2525/2526 mutant, however, displayed a higher amount, with 31% of cells not binding to hexadecane, and remaining in the aqueous layer. This suggests that the SACOL2525/2526 mutant cells are more hydrophilic than the parent and complemented strains, which is perhaps explained by its increased encapsulation. Another aspect of the cell envelope is surface charge, which is controlled in *S. aureus* by a number of factors, including GraRS (Sass & Bierbaum, 2009; Herbert et al., 2007). GraRS not only regulates SACOL2525/2526, but also alters the expression of the *dlt* operon, which ultimately changes cell charge to be more positive, in order to decrease the binding of harmful charged elements such as antibiotics and antimicrobial peptides. Changing the overall surface charge differs from altering cellular hydrophobicity. Hydrophobicity results from proteins and molecules deposited on the outside of the cell membrane, whilst cell charge in *S. aureus* is altered by introducing a positively charged lysyl group into the phosphatidylglycerol head group of membrane lipids (Peschel et al. 2001). Since the SACOL2525/2526 mutant has altered hydrophilicity, the cell surface charge was also evaluated. To determine if the SACOL2525/2526 mutant has altered cell surface charge, a cytochrome C binding assay was performed with the SH1000 wild-type, SACOL 2525/2526 mutant and the *graR* mutant, which was included as a control (Figure 24). Cytochrome C is positively charged and therefore binds to the negatively charge *S. aureus* cell. We determined that SH1000 and its SACOL2525/2526 mutant bound very similar, low amounts of cytochrome C (17% and 22%, respectively). In contrast, the *graR* mutant had a

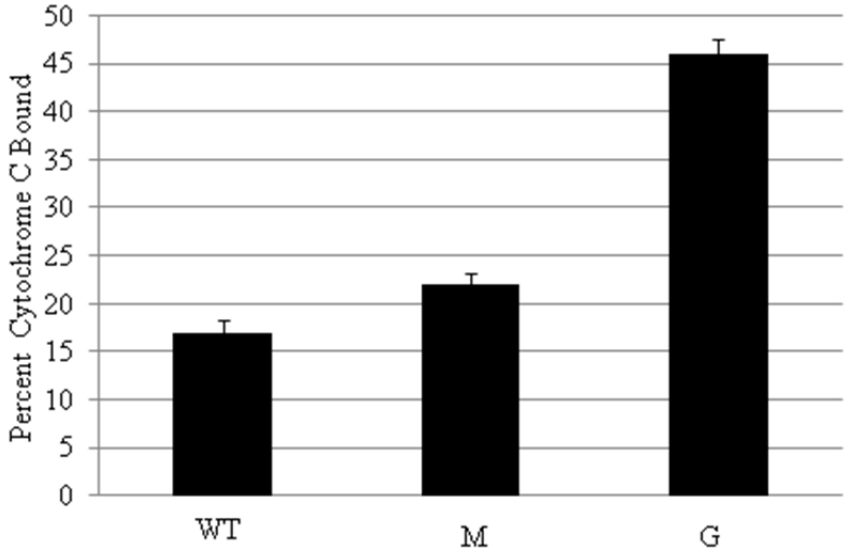

Figure 24. SACOL2525/2526 deletion does not effect cell surface charge. A cytochrome C assay was performed on the SH1000 wild-type (WT), SACOL2525/2526 mutant (M) and *graR* mutant (G) strains. Cytochrome C was added to cells and allowed to bind. The cells were pelleted and the OD_{540} of the supernatant was used to determine the percentage of cytochrome C bound. The data presented are from three independent cultures and error bars are shown as +/- SEM.

2.7-fold increase, with 46% of the cytochrome C bound, suggesting that SACOL2525/2526 mutants do not have altered surface charge and that the phosphatidylglycerol head groups of the lipids are likely similar.

SACOL2525/2526 contributes to protection against lysostaphin. An altered cell-envelope has been reported to affect the interaction between antimicrobial compounds and bacterial cells; and ultimately their activity and potency (Cui et al., 2006; Pillai et al., 2007). Given that our data thus far shows that the SACOL2525/2526 mutant has altered cell envelope architecture, particularly with regard to increased capsule production, a lysostaphin kinetics assay was performed to determine if this alteration affects resistance to lytic bacterial agents (Figure 25). The *nsaS* mutant was also included in this analysis due to its increased capsule production. SH1000 wild-type, its SACOL2525/2526 mutant and complemented strains, alongside and the *nsaS* mutant, were standardized to an OD_{600} of 1.0 in PBS, before 50 μg/ml of lysostaphin was added. OD_{600} readings were taken every 5 minutes, and the percent lysis, compared to the initial inoculum, was determined. While the viability of all strains declined rapidly, the SH1000 wild-type and complement strains lysed far faster than the SACOL2525/2526 or *nsaS* mutant strains. Specifically, after 15 minutes, 77% and 78% of the wild-type and complement strains had lysed, yet only 53% and 48% of the SACOL2525/2526 and *nsaS* mutants lysed, respectively. After 25 minutes of incubation, almost all of the wild-type and complement strains were lysed, whilst the 2 mutant strains, took an additional 10 minutes each to reach similar levels of lysis. This suggests that alterations in the cell envelope of the SACOL2525/2526 and *nsaS* mutant strain perhaps decrease the sensitivity to lysostaphin.

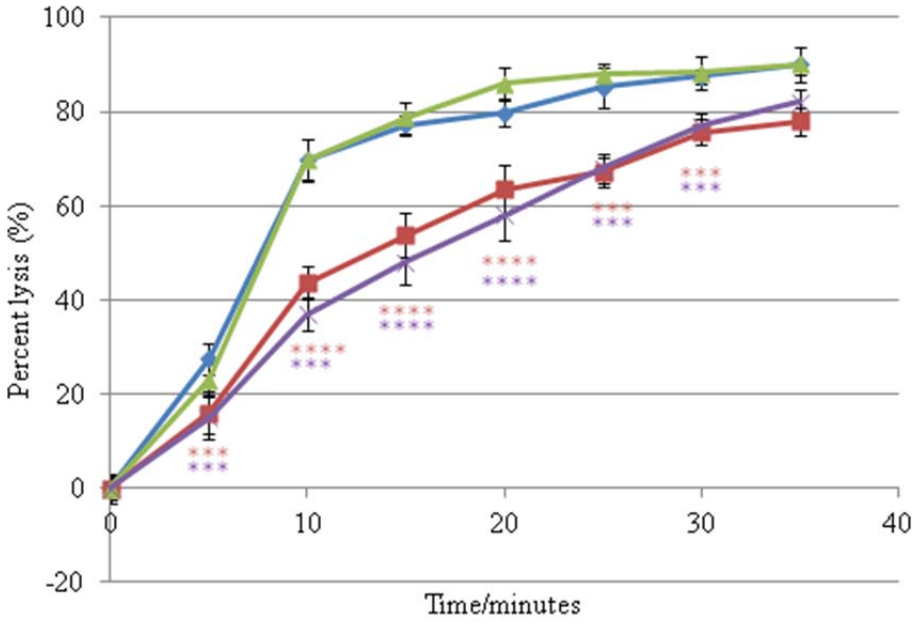

Figure 25. SACOL2525/2526 mutants have decreased sensitivity to the cell wall targeting agent lysostaphin. Lysostaphin lysis reaction kinetics for the SH1000 wild-type (blue), SACOL2525/2526 mutant (red), complemented strain (green) and the *nsaS* mutant (purple) were performed using excess lysostaphin (50 μg/ml). The OD_{600} for each strain was recorded every 5 minutes, and the percentage of cell lysis was calculated in relationship to cultures pre-exposure (T=0). The data presented are from three independent cultures and error bars are shown as +/- SEM. A Student's T-Test was used with a 5% confidence level to determine statistical significance, *** = p< 0.001, **** = p□0.0001.

Discussion

In silico analysis demonstrates that SACOL2525 is an ATP-binding protein that is similar to members that transport galliderm-class lantibiotics. Only a few of these proteins have been studied, but many have been found to aid in immunity of the cell to lantibiotics by exporting these peptides (Peschel & Gotz, 1996; Chen et al., 1999). Analysis of SACOL2526, located downstream of SACOL2525, revealed that it is an integral membrane protein, although no major homology was observed with other transporters in the database. Together these two proteins form an ABC-transport system that may serve a role in the protection of *S. aureus* from lantibiotics and /or peptide antibiotics. Lantibiotics are peptides that are ribosomally synthesized and have been shown to contain unusual amino acids, such as lanthionine and dehydrated amino acids. These agents of antimicrobial defense have largely been divided into 2 classes based on their mode of action, with type A defined by inserting into the membrane and forming pores, whilst type B inhibits peptidoglycan synthesis (Chatterjee et al., 2005; Sieber & Marahiel, 2005). Interestingly, lantibiotics are not the only antibacterial agents to contain unusual amino acids; for example daptomycin is a lipopeptide antibiotic that contains the unusual amino acid L-kynurenine (Baltz et al., 2005; Wessels et al., 1996). In our study, we found the SACOL2525/2526 mutant was not susceptible to nisin (type A), the only lantibiotic tested. This effect implies that SACOL2525/2526 has no role in type A lantibiotic detoxification, but may suggest that SACOL2525/2526 transports lantibiotics belonging to different classes, or that perhaps it more generally transports peptide antibiotics. It is also plausible that this transporter broadly translocates any peptide-containing molecule, not just those considered antibiotics, across the

membrane, such as perhaps lipid II. This observation is not entirely implausible as has been shown that the membrane protein FtsW translocates Lipid II via flippase activity in *S. aureus* (Weidenmaier & Peschel, 2008). In addition, the substrate for the GatD/MurT ABC transporter from *S. aureus*, has recently been suggested to be lipid II (Munchet al., 2012) Therefore, *S. aureus* may have multiple transport systems that can shuttle lipid II transport across the membrane, which could include SACOL2525/2526. This is made further possible by the observation that SACOL2525/2526 is transcribed under standard conditions; however antibiotic transporters tend to be induced only during specific stresses (Muthaiyan et al., 2008; Ouyang et al., 2010; Meehl et al., 2007). Additionally, compounds that induce Lipid II stress do appear to upregulate expression of SACOL2525/2526.

Both IM-HKs associated with ABC transporters in *S. aureus* (NsaRS and GraRS) have been shown to regulate SACOL2525/2526 (Herbert et al., 2007; Kolar et al., 2011). The expression of both *graRS* and *nsaRS* respond to a number of antimicrobial agents that target the cell wall, or perhaps to the damage caused by these agents. NsaRS has a role in sensing and adapting to changes caused by chemicals that affect every step in the cell wall biosynthesis pathway. The transcription of SACOL2525/2526 in response to cell-envelope-targeting compounds revealed that several drugs, including phosphomycin, daptomycin, chloropromazine, penicillin G, ampicillin, oxacillin, D-cycloserine and CCCP caused an increase in transcription. The majority of these agents affect cell wall biosynthesis, including the making of lipid II (phosphomycin, D-cycloserine) and peptidoglycan synthesis (penicillin G, chloropromazine, ampicillin, oxacillin,) (Figure 26). The daptomycin mode of action is currently contentious, although it does appear to

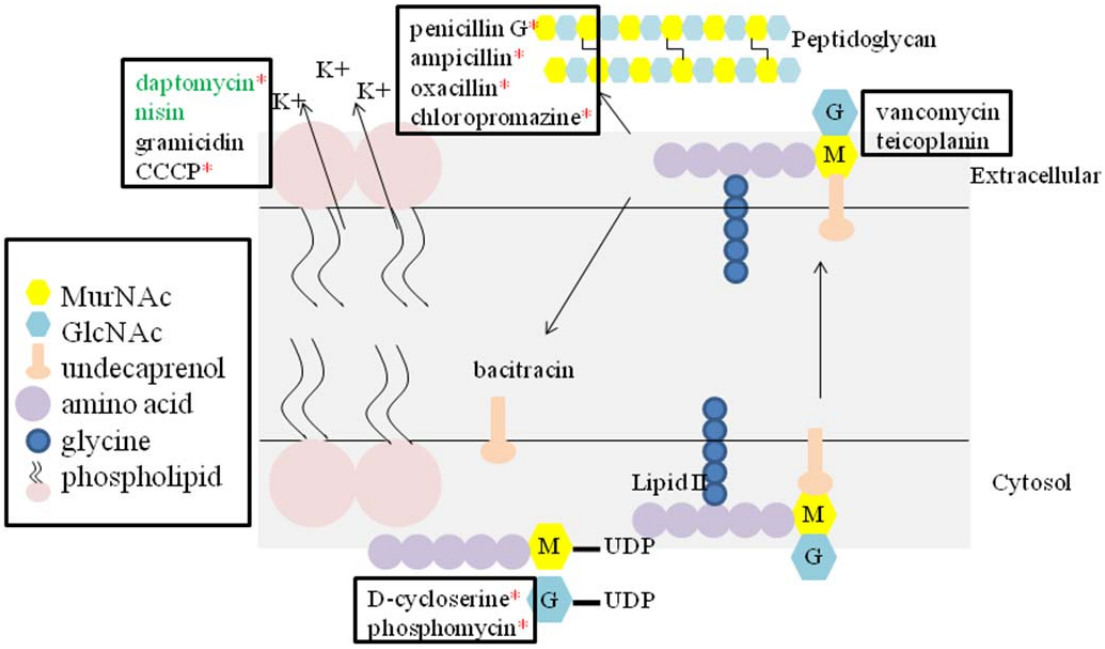

Figure 26. Schematic representation of peptidoglycan synthesis of *S. aureus*. Antibiotics target specific steps in the peptidoglycan synthesis pathway are shown, including: intracellular peptidoglycan subunit assembly (D-cycloserine and phosphomycin), transport and anchoring of the Lipid II molecule (vancomcyin, teicoplanin and nisin), cross-linking of peptidoglycan subunits (penicillin G, ampicillin, chloropromazine and oxacillin) and finally re-entry of the bactoprenol carrier (bacitracin). In addition, other drugs (Gramicidin and CCCP) target the membrane instead of peptidoglycan synthesis. Drugs highlighted in green have been shown to target both the membrane and the peptidoglycan biosynthesis pathway. *Indicates compounds shown to increase transcription of SACOL2525/2526.

target both the membrane and peptidoglycan synthesis (Canepari et al., 1990; Silverman et al., 2003; Cotroneo et al., 2008). Of note, the only compound that increased transcription of SACOL2525/2526, and that solely impacts membrane stability, is CCCP. CCCP alters the proton motive force, which effects ATP levels and ultimately impacts every process that utilizes ATP, which can also include peptidoglycan synthesis (Dimroth & Cook, 2004). Therefore, the observed increase in expression of SACOL2525/2526 in response to CCCP may be explained by the fact that the peptidoglycan synthesis pathway is impacted, albeit as a downstream effect. Collectively, this data suggests that SACOL2525/2526 overwhelmingly responds to cell wall biosynthesis targeting compounds.

When comparing the transcription of SACOL2525/2526 to that of *nsaRS,* it is clear that the expression profiles of these 2 elements is very similar. Seven of the ten drugs tested (phosphomycin, ampicillin, penicillin G, D-cycloserine, CCCP, daptomycin and oxacillin) resulting in increased expression of both loci, whilst only 3 drugs (nisin, gramicidin and chloropromazine) show opposing effects. These 2 systems do not both respond to every antimicrobial agent tested, suggesting some specificity, and the likelihood of divergent regulation. Overall, this suggests that NsaRS relies heavily on SACOL2525/2526 to exert its effects under standard conditions, as well as during cell-envelope stress. NsaRS has been shown to respond to several cell-envelope-targeting drugs, but not to all of them. This is consistent with the literature on other IM-HKs, and may be explained by the overlapping function of a number of other TCSs that respond to cell wall stress, including VraRS, GraRS and WalKR (Li et al., 2007; Kraus et al., 2008; Pietainen et al., 2009; Delaune et al., 2011). Also, SACOL2525/2526 only

responds to certain cell-envelope-targeting drugs, which may be the result of differential signaling through NsaRS, and GraRS, which has also been shown to regulate this transporter (Herbert et al., 2007). The other two TCSs that respond to cell wall stress, VraRS and WalKR, have not yet been shown to regulate SACOL2525/2526, however the available transcriptomic data is currently limited, thus not discounting such a possibility. Although NsaRS and GraRS both positively regulate SACOL2525/2526 under standard conditions, it is NsaRS, not GraRS, which mediates the increase in transcription in response to cell-wall-targeting chemicals. Interestingly, while expression of SACOL2525/2526 in a *nsaS* mutant in the presence of cell envelope antibiotics decreased compared to the wild-type, these were not to the levels of unstimulated cells. This suggests that, while NsaRS does positively regulate SACOL2525/2526 in response to cell-envelope-targeting drugs, it is not the only regulator. This is not surprising, as it has been demonstrated that resistance to antibiotics is often modulated by a number of regulatory elements (Howden et al., 2010; McAleese et al., 2006). For example, the WalKR TCS has been shown to contribute to methicillin and glycopeptide resistance in a variety of *S. aureus* strains (Dubrac et al., 2007; Martin et al., 2002). In addition, MgrA is a regulator of multidrug transporters, and has also been shown to impact resistance to methicillin (Chen et al., 2006; Truong-Bolduc et al., 2005). As WalKR is essential in *S. aureus*, defining its regulon has proven complex, however work by Luong et al. reveals that SACOL2525 is contained within the MgrA regulon (Luong et al., 2006) Therefore, it is likely that NsaRS utilizes SACOL2525/2526 under cell wall stress conditions; however other regulators, responding to different types of damage may also employ SACOL2525/2526 for

cellular detoxification. This is supported by microarray findings that demonstrate SarA, Rot, ArlRS and the alternative sigma factor, σ^B, all influence SACOL2525 and/or SACOL2526 expression (Bischoff et al., 2004; Cassat et al., 2006; Said-Salim et al., 2003; Liang et al., 2005).

Transcription of the IM-HK GraRS TCS has also been shown to respond to cell-wall-targeting agents, although again, to a select number, likely suggesting specificity (Cui et al., 2005). Interestingly, GraRS and NsaRS produced opposing effects in response to the cell-wall biosynthesis targeting compounds bacitracin, vancomycin, nisin and phosphomycin (Meehl et al., 2007; Kolar et al., 2011). This may suggest that GraRS may utilize SACOL2525/2526 either only under standard conditions, or in a manner that is contrasting to NsaRS. Indeed, the response and resistance to antimicrobial agents is commonly pleiotropic, with signaling occurring through multiple regulatory pathways. For example, the GraRS TCS contributes to glycopeptide resistance by regulating genes required to alter the cell surface charge in order to limit the binding of glycopeptides to the cell (Meehl et al., 2007). In addition, the VraRS system regulates genes involved in cell wall biosynthesis to repair the damage to the peptidoglycan layer (Blake et al., 2009). Together, these 2 TCS systems regulate genes important in different aspects of the vancomycin resistance mechanism. Therefore, NsaRS and GraRS likely follow a similar model with regards to SACOL2525/2526, where NsaRS regulates this transporter in response to cell-wall stress, while GraRS performs alternative functions. Since GraRS is able to regulate SACOL2525/2526 under standard conditions, it is possible that under cell-wall stress, a hierarchy exists between these 2 systems, where NsaRS has a higher capacity for SACOL2525/2526 regulation, and

ultimately influences the expression of SACOL2525/2526 more so than GraRS. In addition, the regulation of SACOL2525/2526 by GraRS may be indirect, and occur through the action of the regulator MgrA. Indeed, GraRS has been shown to positively regulate MgrA, which is a master regulator of multi-drug resistance efflux pumps (Herbert et al., 2007; Luong et al., 2006).

Human sebum is the oil found on the skin, and contains free fatty acids to help limit bacterial infections and carriage (Kligman, 2006). Free unsaturated fatty acids from human sebum insert in bacterial membranes leading to cell death. Some proposed models of how this occurs include peptidoglycan precipitation, peroxidative stress, alterations of metabolism, inhibition of certain major biosynthesis pathways, modulation of membrane permeability or alterations in membrane fluidity (Galbraith & Miller, 1973; Knapp & Melly., 1986; Speert et al., 1979; Chamberlain et al., 1991; Xiong & Kapral., 1992). Surprisingly, when the SACOL2525/2526 mutant was exposed to oleic acid, a free fatty acid found in human sebum, it was better able to survive than the wild-type. Of note, the *nsaS* mutant, which has a ~30-fold decrease in SACOL2525/2526 transcription, also survives better than the wild-type during this type of stress, although not to the levels of the transporter mutant (preliminary data). Although the exact mechanism that mediates this is unclear, it has been shown that Gram-positive bacteria resist free fatty acids by decreasing membrane fluidity (Desbois & Smith, 2010). Specifically, it has been shown that enhanced production of the carotenoid pigment staphyloxanthin is positively correlated with increased resistance to fatty acids (Chamberlain et al., 1991). It has been suggested that the observed resistance to fatty acids is not due to the pigment itself, but is the result of a decrease in membrane

fluidity produced by its overproduction. (Xiong & Karpal.,1992). While enhanced staphyloxanthin production decreases membrane fluidity, *S. aureus* can also change the fluidity of its membrane in other ways, including altering the fatty acid composition of membrane phospholipids (Mrozik et al., 2004). This may suggest that in the presence of free fatty acids, *S. aureus* may decrease membrane fluidity for survival by limiting the expression of SACOL2525/2526. To corroborate this, qRT-PCR was performed on cells grown in the presence of oleic acid, revealing transcription of SACOL2525/2526 to be decreased as expected. Following this, if an SACOL2525/2526 mutant does have decreased membrane fluidity, then it should also have decreased survival in the presence of stresses that *S. aureus* resists by increasing membrane fluidity. Certain solvents, such as toluene, have been shown to force Gram-positive cocci to change their membrane to become more fluid (Nielsen et al., 2005). Thus, survival in toluene was evaluated, and, as hypothesized, the SACOL2525/2526 mutant showed a decrease in survivability. Collectively, these data suggest that the membrane of the SACOL2525/2526 mutant does in fact have decreased fluidity. This is perhaps explained by our finding that SACOL2525/2526 responds to chemicals that disrupt peptidoglycan synthesis; perhaps suggesting that the substrate it transports is important in this process. It has been shown that disruptions in the peptidoglycan layer cause a weaken cell wall, and decreases in cellular integrity (Rohrer & Berger-Bachi, 2003). Furthermore, alterations in the cell membrane, such as decreased carotenoid production, have been shown to result in decreased membrane fluidity in an attempt to provide increased cellular integrity (Mishra et al., 2011). Therefore, it seems likely that in the SACOL2525/2526 mutant, peptidoglycan synthesis is impaired, leading to the potential

for decreased cellular integrity. In response to this, the SACOL2525/2526 mutant seemingly decreases membrane fluidity as a coping mechanism to these alterations, by an as yet unknown mechanism.

Daptomycin was approved for skin and soft tissue infections caused by *S. aureus* and other Gram-positive pathogens in 2003 (Arbeit et al., 2004). At this time one survey showed that only 0.1% of *S. aureus* isolates were resistant to daptomycin, suggesting that it was a promising antibiotic with high clinical relevance. Since then, reports of daptomycin resistant *S. aureus* isolates have surfaced, and hence determining the resistance mechanism has become a priority (Streit et al., 2004; Marty et al., 2006). Daptomycin binds to the bacterial membrane in a calcium-dependent manner, and then oligomerizes producing a pore, resulting in a flood of ions from inside the cell to the extracellular environment, eventually leading to cell death (Steenbergen et al., 2005). While the SACOL2525/2526 mutant did not show a difference in sensitivity to daptomycin overnight, survivability in lethal concentrations of daptomycin was decreased when compared to the wild-type. Interestingly, a study on daptomycin resistant strains showed that the cells had altered cell surface charge as a result of increased MprF expression, controlled by GraRS (Pillai et al., 2001;Mishra et al., 2009). MprF alters surface charge by changing lysinylation of membrane phosphotidylglycerol, which decreases binding of daptomycin to the cell membrane, limiting its effects (Jones et al., 2008; Peschel et al., 2001; Weidenmaier et al., 2005). The SACOL2525/2526 mutant did not have altered cell surface charge, as determined by a cytochrome C assay, which suggests that the decrease in survivability is not the result of altered binding capacity of daptomycin to the cell membrane. Daptomycin

resistant strains have also been shown by others to have increased membrane fluidity (Mishra et al., 2009). Interestingly, as suggested above, the SACOL2525/2526 mutant may possess a membrane with decreased fluidity, which perhaps could explain the decrease in survival. In addition, cationic antimicrobial peptides, which area chemically similar to daptomycin, insert into the bacterial membrane forming pores; and resistance to these agents is also influenced by increased membrane fluidity (Bayer et al., 2000; Bayer et al., 2006).

Another explanation for these findings is that the cell envelope may limit the interaction of daptomycin with the membrane of *S. aureus* cells. As such, the cell wall and capsule was removed from wild-type and mutant strains using lysostaphin, to determine if the observed decrease in survivability could be enhanced. As expected, the daptomycin-mediated decrease in survival of the SACOL2525/2526 mutant was exacerbated upon lysostaphin treatment. Of note, the SACOL2525/2526 mutant produced no difference in survivability to other membrane targeting drugs, such as CCCP or gramicidin, suggesting that this effect is specific to daptomycin. An explanation for this might be that daptomycin is the only compound to form pores in the membrane, as CCCP effects the proton potential and gramicidin binds to bacterial membranes before eventual translocation into the cytoplasm (Hancock & Chapple, 1999; Silverman et al., 2003; Cotroneo et al., 2008). Conversely, daptomycin can insert into the membrane, where it forms pores that allow for the leakage of ions, leading to cell death. The SACOL2525/2526 mutant has been suggested to have decreased membrane fluidity, which may influence the stability of the formed pores, allowing for increased ion leakage and more rapid death. A situation akin to this was observed *in E. faecalis*, as

increased membrane fluidity decreased the stability of pores caused by the peptide antibiotic, alamethicin (Cafiso, 1994).

Although daptomycin was the only chemical where the mutant had decreased survivability, SACOL2525/2526 contributes to the ability of *S. aureus* to generate spontaneous mutants in the presence of several cell-wall targeting agents, including penicillin G, bacitracin, teicoplanin and daptomycin. These four drugs, having produced lower mutation frequencies in the mutant compared to the wild-type, all inhibit cell wall biosynthesis, although each at a different step of the pathway. It has been previously shown that a *nsaRS* mutant is sensitive to bacitracin, and that the *nsaS* mutant produced a decrease in mutation frequency compared to the wild-type in the presence of bacitracin (Kolar et al., 2011; Matsuo et al., 2010). Because of the similarities between NsaRS and SACOL2525/2526, this finding is therefore perhaps unsurprising. The SACOL2525/2526 mutant produced a decrease in spontaneous mutation frequency when exposed to daptomycin, which corroborates our data showing decreased survival of the mutant in the presence of this agent. Teicoplanin and penicillin G both target peptidoglycan cross-linking, and the decreased mutation frequencies observed for the SACOL2525/2526 mutant may further suggest a role for this ABC transporter in the response to peptidoglycan damage. As stated previously, SACOL2525/2526 does not respond to every cell-wall-targeting agent. The other drugs tested either did not stimulate SACOL2525/2526 expression (nisin and vancomycin), or they did not impact the cell wall biosynthesis pathway (CCCP). This may therefore explain the similar mutation frequencies observed for these agents between the mutant and wild-type strains. An explanation for these findings may again come from the putative

involvement of this transporter in peptidoglycan biosynthesis. If SACOL2525/2526 transports a substrate important for this process, then its peptidoglycan layer is likely weakened in some aspects in the mutant strain. Therefore, it is possible that such alteration in peptidoglycan result in more rapid death in the mutant when exposed to these compounds, presenting limited opportunity to spontaneously mutate before cell death. The reason that we observed differences in mutation frequency but not sensitivity to some of these agents may be due the high concentration of antibiotic used in this experiment, which is 3 times the MIC. In other experiments using these compounds, the SACOL2525/2526 mutant may not produce a difference in survival at lower concentrations, due to its ability to compensate for the difference in peptidoglycan and/or membrane composition, and thus the action of the compound.

S. aureus strengthens its peptidoglycan cell wall by cross-linking lipid II molecules via a pentaglycine bridge (Schleifer & Kandler, 1972). Lysostaphin, a lytic agent produced by the closely related species *S. simulans*, cleaves these pentaglycine bridges, leading to lysis of the cell. (Grudling & Schneewind, 2006). Strains resistant to lysostaphin have been shown to lower the glycine content by changing glycine to serine in the bridge, and as such cleavage does not occur (Kumar, 2008). In addition, FmhB is responsible for the attachment of the first glycine residue to the lysine of the lipid II molecule (Rohrer et al., 1999), while the proteins FemAB aid in the successive addition of the second through fifth glycine residues. Accordingly, resistance to lysostpahin is commonly mediated through mutation of FemA or FemB, resulting in a lone glycine residue available for cross-linking; and such monoglycines cannot be cleaved by lysostaphin (Ehlert et al., 1997; Stranden et al., 1997) Given the alterations in cell

superstructure demonstrated for the SACOL2525/2526 mutant in this study, we performed lysostaphin lysis kinetics to assess how these changes impacted cell lysis rates. Unexpectedly, we found that the mutant strain was actually more resistant to the action of this agent than the parental strain. Additionally, given the similarity of phenotypes for the transport mutant and that of *nsaS*, we also performed these studies with the TCS deficient strain, and revealed similar findings. Although both mutants took longer to achieve complete, or near-complete, lysis, both did eventually reach the same levels as that of the parent and complemented strains. These final lysis data may suggest that the glycine content in the pentaglycine bridges are not changed in the mutant strains. An alternative explanation might come from our analysis of the *nsaS* and SACOL2525/2526 mutant using electron microscopy. Negative staining of the SACOL2525/2526 mutant revealed an altered cell envelope, similar to that observed with the *nsaS* mutant. The observed difference may be increased capsule production, as the microarray for the *nsaS* mutant showed a decrease in expression of several *cap* genes. Thus, increased capsule production may contribute to lysostaphin resistance by limiting the contact of lysostaphin with peptidoglycan, and ultimately reducing the rate of lysis. Collectively, these data suggest that the SACOL2525/2526 mutant has increased capsule production, and an altered cell envelope that allows for a decrease in susceptibility to lysostaphin. The increased capsule produces a protective effect for lysostaphin, as no difference in glycine content is suggested. It is also likely that the increased production of capsule may limited the interaction of daptomycin with the mutant membrane, perhaps explaining why we see no difference in MIC during overnight growth. However we do show at very high concentrations, or in the absence

of capsule and peptidoglycan that the transporter mutant is more susceptible to daptomycin, which is likely explained, as suggested above, by the stabilization of pores resulting from decreased membrane fluidity.

Chapter 4: Extracellular Proteases are Key Mediators of *S. aureus* Virulence via the Global Modulation of Virulence Determinant Stability

Introduction

Of the 245 genes modulated by NsaRS, only 34 are negatively regulated. These included those involved in capsule production, such as *capGOIHED*; the virulence genes β-hemolysin, hyaluronate lyase, fibronectin binding protein A; and six of the 10 major secreted proteases. Proteases are enzymes that catalyze the cleavage of amide linkages in proteins and peptides. Proteases can be divided into families based on the functional group at their active site; with the major catalytic categories being serine, threonine, aspartate, metallo and cysteine proteases. It has been shown that the secreted proteases of *S. aureus* are inactive until they are secreted, and are processed outside of the cell, with the exception of the Spl proteins (Rice et al., 2001 and Shaw et al., 2004; Reed et al, 2001). Upon activation, these enzymes modify proteins by breaking peptide bonds, which can lead to activation or degradation. As this modification can only occur once the proteases have been activated outside of the cell, it suggests that their targets must be: 1) other secreted proteins, 2) cell surface associated proteins, or 3) host proteins. Included within this list are self-derived virulence determinants secreted by *S. aureus*, which may serve as targets of the proteases to help mediate their level in response to the environment. Indeed, such a theory was proposed with regards to these enzymes more than ten years ago (Lindsay & Foster, 1999). Since the presentation of this theory, only a

few studies have actually been conducted investigating this phenomenon (Karlsson et al., 2001; McAleese et al., 2001; McGavin et al., 1997; Zielinska et al., 2011).

The *S. aureus* genome encodes 129 putative proteases, with 10 being secreted enzymes. These include a metalloprotease (aureolysin, *aur*), a V8 or SspA serine protease, two cysteine proteases (staphopain A (ScpA) and staphopain B (SspB), and six serine-like proteases that are SspA homologues (SplABCDEF) (Reed et al, 2001; Shaw et al., 2004). These ten proteases are located in four different operons, all of which are polycistronic, except aureolysin (Figure 27). It has been shown that these extracellular proteases are activated by Agr, and strongly negatively affected by SarA, with the exception of the Spl proteins (Gustafsson & Oscarsson, 2008; Horsburgh et al., 2002). In addition, the proteases are also regulated by σ^B, SarR, SarZ, Rot and MgrA, although some of these regulators most likely act through Agr or SarA (Karlsson & Arvidson, 2002; McNamara et al., 2000; Tamber & Cheung, 2009). In addition to transcriptional regulation, the proteases are regulated at the post-translational level, as each is secreted as a proenzyme, with the exception of the Spl proteins. Each needs to be cleaved in order to gain enzymatic activity in a cascade of activation that is thought to keep these proteases inactive until secreted (Figure 28). First, aureolysin is able to activate V8 protease, which then processes and activates staphopain B (Drapeau, 1978; Rice et al., 2001). The activation of aureolysin and Staphopain A resides outside of this cascade, and occurs through autocatalysis (Nickerson et al., 2007). In addition, both of the Staphopain enzymes have specific, cytoplasmic inhibitory proteins (the staphostatins) encoded downstream of the protease (Rzychon et al., 2003). The regulation of secreted proteases

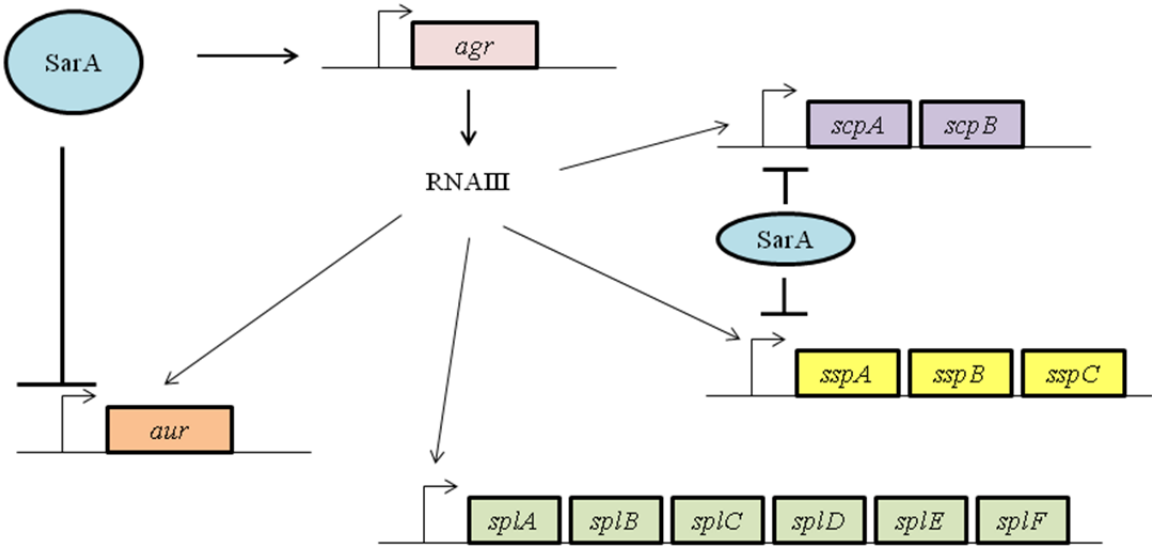

Figure 27. The extracellular proteases of *S. aureus* and their regulation. *S. aureus* has 10 major secreted proteases: aureolysin or metalloprotease (Aur), a V8 or serine-protease (SspA), two cysteine proteases (ScpA and SspB) and 6 serine-protease like enzymes (SplABCDEF). The two cysteine protease, ScpA and SspB are transcribed with their specific inhibitor, staphostatin A (ScpB) and staphostatin B (SspC), respectively. The secreted proteases are located on 4 different operons, which are positively regulated (arrows) by RNAIII (Agr), and negatively regulated (lines) by SarA.

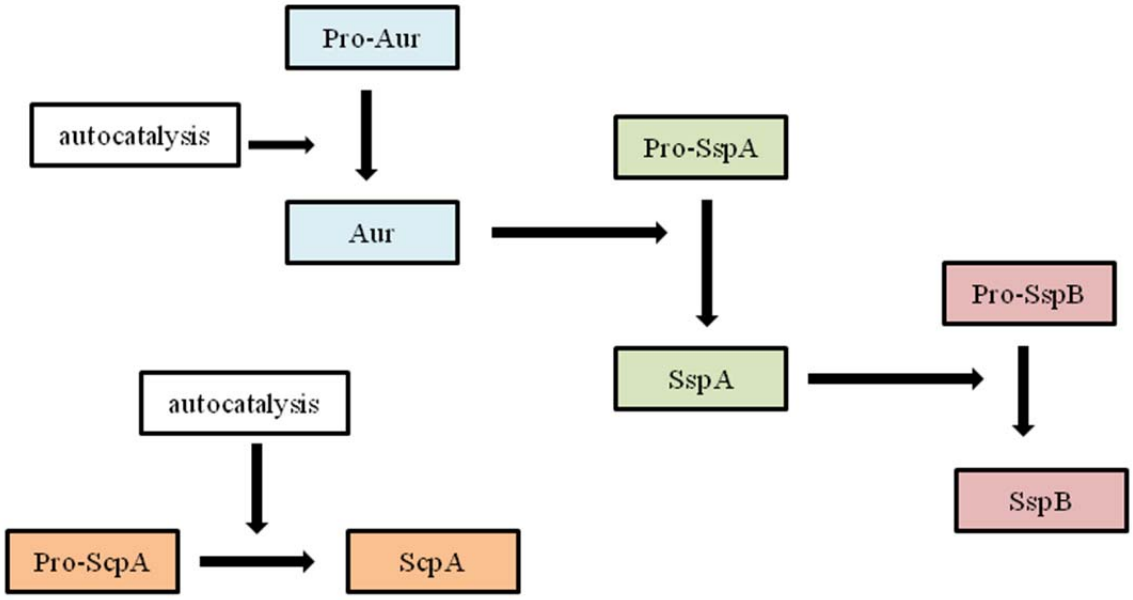

Figure 28. Activation cascade of *S. aureus* secreted proteases. Secreted proteases are regulated at the post-translational level, with each secreted as a proenzyme, apart from the Spl proteins. Each needs to be cleaved in order to gain enzymatic activity in a cascade of activation. First, aureolysin (Aur) activates the V8 protease (SspA), which then processes and activates Staphopain B (SspB). The activation of aureolysin and Staphopain A (ScpA) occur through autocatalysis.

is complex and occurs at the transcriptional as well as at the post-translational level. This intricate level of control suggests that secreted proteases are important elements of *S. aureus* and understanding their role may give a unique insight into the pathogenesis of *S. aureus*.

S. aureus is a dangerous pathogen causing infection in every part of the body. Recently, MRSA strains have been found infecting the young and healthy, which appear to have evolved independently within the community (Johnson et al., 2007; Moran et al., 2005). Community-acquired Methicillin Resistant *S. aureus* CA-MRSA strains have increased transmission and are hypervirulent, presenting a serious public health threat. The ability of CA-MRSA to invade healthy hosts is the result of not only newly acquired virulence factors, but alterations in the expression profile of key, core-genomic elements (Diep et al., 2006; Diep et al., 2008; Kennedy et al., 2008; Vandenesch et al., 2003; Wang et al., 2007). Included within this latter class is α-hemolysin (Hla), delta-hemolysin, phenol soluble modulins (PSMs), and secreted proteases (Burlak et al., 2007; Chambers, 2005; Diep & Otto, 2008; Wang et al., 2007). Most if not all of these factors are regulated by the two-component system Agr, and thus their overproduction is due, at least in part, to a hyperactive Agr locus (Li et al., 2009). While others have focused on identifying the role of cytolytic toxins, such as Hla and Psms in CA-MRSA hypervirulence, the impact of extracellular proteases has thus far been overlooked (Wang et al., 2007; Kobayashi & DeLeo, 2009; Li et al., 2009; Montgomery et al., 2008; Burlak et al., 2007; Chambers, 2005; Diep & Otto, 2008). Whilst a number of studies on secreted proteases are contained within the literature, their findings are contradictory. As such, the role of these enzymes and their involvement in pathogenesis is currently unclear.

Therefore, the focus of this chapter is to better understand the pathogenesis of CA-MRSA infections and determine if secreted proteases are in fact key virulence factors. Specifically, we will determine if secreted proteases are vital to the infection process using both systemic and localized infection models. Additionally, we will explore the theory that *S. aureus* secreted proteases modulate the stability of self-derived secreted and surface-associated virulence factors.

Manuscript Introduction

S. aureus is a highly successful and diverse pathogen causing an array of diseases. *S. aureus* infections typically proceed from localized sites (e.g. wound), and can proliferate via bacteremia to life-threatening systemic diseases, such as osteomyelitis, endocarditis and septic arthritis. This diversity and pathogenic success can be attributed, largely, to its vast array of temporally and environmentally regulated virulence factors (Lowy, 1998; Novick, 2006). Formerly, *S. aureus* infections were confined to the healthcare setting, afflicting the immunocompromised and elderly. Recently there has been a shift in *S. aureus* epidemiology, with increased incidences of severe invasive disease in healthy subjects lacking predisposing factors (Johnson et al., 2007; Moran et al., 2006). This trendshift is the result of emerging, hypervirulent strains of methicillin-resistant *S. aureus* (MRSA) that have evolved within the community (CA-MRSA). Of considerable concern, these CA-MRSA strains appear to be moving into clinical settings and displacing existing hospital-associated MRSA strains (D'Agata et al., 2009; Popovich et al., 2008; Webb et al., 2009).

Several CA-MRSA lineages have appeared in the last decade (Diep & Otto, 2008; Limbago et al., 2009; McDougal et al., 2009), with USA300 now representing the major clone in the United States (Tenover et al., 2008). The reason for the surprising success of this strain as the primary CA-MRSA, and perhaps MRSA, isolate in the USA is somewhat unclear; however work by a number of groups suggests it may be attributable to the differential expression of core genomic elements (Li et al., 2009), including the PSMs, hemolysins, enterotoxins and extracellular proteases (Diep & Otto, 2008; Li et al.,

2009; Adem et al., 2005; Kobayashi & Otto, 2009; Montgomery et al., 2008; Wang et al., 2007). With regards to this latter class of enzymes, *S. aureus* possesses 10 major secreted proteolytic enzymes. These include a metalloprotease (aureolysin, *aur*), a V8 or SspA serine protease, two cysteine proteases (staphopain A (ScpA) and staphopain B (SspB)), and six serine-like proteases that are SspA homologues (SplABCDEF) (Reed et al., 2001; Shaw et al., 2004).

A number of studies have been conducted to determine the contribution of extracellular proteases to the disease process; however many have been contradictory. In the RN6390 background, a SspA mutant displayed attenuated virulence in three different animal models of infection (Coulter et al., 1998). Similarly, *sspABC* and *sspBC* mutations in the 8325-4 background also showed reduced virulence in a murine wound model (Shaw et al., 2004). In addition to these findings, it has been shown that both *S. aureus* cysteine proteases induce vascular leakage and shock in a guinea pig model of infection (Imamura et al., 2005). Furthermore, it was shown that the ability of Newman to evade killing by primary human macrophages is dependent on a functional aureolysin gene (Kubica et al., 2008; Burlak et al., 2007). Finally, a number of studies have shown that Aur, SspA and SspB are produced upon engulfment by human neutrophils, and that antibodies are generated against these enzymes during infection (Burlak et al., 2007; Calander et al., 2008; Holtfreter et al., 2009). In contrast, several other studies have produced opposing results regarding the pathogenic role of extracellular proteases. Specifically, single mutations in *aur* and *scpAB* using strain 8325-4 had no effect on wound formation (Shaw et al., 2004), whilst a non-polar *sspA* mutant in the RN6390 background displayed enhanced virulence in a similar model (Rice et al., 2001). Additionally, mutants in

sspABC, *sspB, aur*, and *scpAB*, in the SH1000 background produced no attenuation in virulence in a murine septic arthritis model (Calander et al., 2004). It was also observed that a *splABCDEF* deletion mutant showed no significant difference in virulence in a murine peritonitis infection model (Reed et al., 2001).

In addition to these more general functions, *S. aureus* exoproteases have been shown to cleave specific host proteins. Staphopain B can degrade human fibronectin, fibrinogen and kininogen, and may contribute to the ability of *S. aureus* to disseminate (Imamura et al., 2005; Massimi et al., 2002). Secreted proteases can also cleave human α1-proteinase inhibitor (Potempa et al., 1986), the heavy chains of all human immunoglobulin classes (Prokesova et al., 1992), and elastin (Potempa et al., 1988), which aids in tissue invasion. Beyond their interaction with the host, it has been demonstrated that secreted proteases modulate the stability of self-derived virulence determinants. Specifically, SspA was shown to cleave surface proteins, including fibrinogen-binding protein (McGavin et al., 1997) and surface protein A (Karlsson et al., 2001). In addition, Aur cleaves the surface associated protein clumping factor B (McAleese et al., 2001). Cleavage of these proteins by extracellular proteases is thought to affect the transition from an adhesive to invasive phenotype. It has also been suggested that extracellular proteases can cleave secreted toxins in order to regulate the abundance of virulence factors depending on the specific niche encountered within the host (Lindsay & Foster, 1999). Indeed, our group has recently shown that aureolysin modulates the stability of both α-toxin and phenol soluble modulins in CA-MRSA strains (Gonzalez et al., 2012; Zielinska et al., 2011).

Consequently, whilst there is a wealth of information on the role of secreted proteases in *S. aureus* disease causation, the specific role of these enzymes as virulence factors remains unclear. Therefore, in the present study, we sought to define the collective impact of this class of enzymes on pathogenesis and virulence determinant stability. This was achieved using the CA-MRSA strain USA300, which is known to hyperproduce secreted proteases, and a strain genetically lacking all 10 of these enzymes.

Materials and Methods

Ethics statement. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of South Florida (Permit Number: A-4100-01).

Bacterial strains, plasmids and growth conditions. The CA-MRSA USA300 LAC isolate AH1263 served as the wild-type strain for analysis in this study. A derivative of this has been generated and described previously (Wormann et al., 2011) that lacks all 10 major secreted proteases (strain AH1919). Strains were grown in TSB as documented (Shaw et al., 2008), using the following protocol: 1 ml of overnight *S. aureus* cultures were used to inoculate fresh medium and allowed to grow for 3 h. These exponentially growing cultures were used to seed new medium at an OD_{600} of 0.05. These exponentially growing test cultures were then allowed to grow for the necessary time periods. Growth in milk broth was performed as previously described (Carroll et al., 2012). Briefly, exponentially growing cultures of the LAC wild-type and protease null strain were washed three times with PBS and resuspended in 100 ml 10% skimmed milk. The initial inoculum of each strain was also determined at this time by serial dilution and plating on TSA. Cultures were incubated at 37°C with shaking and the cfu/ml of each strain determined at the times indicated, again by serial dilution and plating. All were performed in triplicate, and significance in growth alterations was determined using a Student T-Test with a 5% confidence limit. For growth in pig serum, exponentially growing cultures of the LAC wild-type and protease null strain were washed three times

with PBS and resuspended in 1 ml of pig serum (Sigma). Cultures were incubated at 37°C with shaking and the cfu/ml of each strain was determined by serial dilution and plating, every hour for five hours. The initial inoculum of each strain was also determined from the original culture in the same manner. Data are presented as percentage survival of each strain compared with initial inocula. These experiments represent three independent biological replicates. Statistical significance was evaluated using a Student T-Test with a 5% confidence limit.

Real-time PCR. Quantitative real-time PCR analysis was conducted as described previously (Livak & Schmittgen, 2001; Riordan et al., 2010) using primers specific for RNAII (F- ATGCGCTGATGATATACCACG, R- GTTGATAGACCTAAACCACGACC), RNAIII (F- ATTTGTTCACTGTGTCGATAATCC , R- GGAGTGATTTCAATGGCACAAG) and *hla* (F- CGAAAGGTACCATTGCTGGTCAGT, R - AAATGCTGAAGGCCAGGCTAAACC). The control primers were for the 16s rRNA gene, as described elsewhere (Koprivnjak et al., 2006).

Whole human blood survival assay. Survival in whole human blood was performed as previously described (Kolar et al., 2011). These experiments were performed with three separate, de-identified blood samples (purchased from Bioreclamation) and represent nine independent replicates. Data are presented as percentage survival of each strain compared with initial inocula. Statistical significance was evaluated using a Student T-Test with a 5% confidence limit.

Antimicrobial peptide sensitivity assay. Liquid cultures of LAC and its protease null mutant were grown in Luria-Bertani (LB) media without NaCl. These were diluted

1:1000 in fresh LB, again lacking NaCl, and 200 µl was applied to the wells of a 96-well plate. The AMPs LL-37, Indolicidin or Histatin-5 were added to these wells in decreasing concentrations and mixed by pipetting. Plates were incubated at 37°C overnight, followed by the measurement of culture density by OD_{600} readings. LD_{50} values were determined as the concentration required to generate a 50% reduction in OD_{600} compared to controls wells, inoculated with *S. aureus* cells, but lacking AMPs.

Murine model of wound formation. These experiments were conducted as described previously (Shaw et al., 2004; Bunce et al., 1992). Briefly, 6 week old, female SKH1-E nude-mice were purchased from Charles River Laboratories, and housed at the vivarium in the College of Medicine, University of South Florida. *S. aureus* strains LAC and LAC-protease null were grown for 15h in TSB as described above. After this time, aliquots of these bacterial suspensions were stored at -80°C, and their cfu/ml determined retroactively by serial dilution and viable cell counts. For infection purposes cultures were thawed, washed twice in PBS, and diluted in PBS containing 20 µg of sterile Cytodex microcarrier beads to 5×10^8 cfu/ml. Ten mice per strain were inoculated subcutaneously between the scapula with 200 µl bacterial suspension, giving a final inocula of 1×10^8 cfu/ml. Mice were monitored for 6 days during the infectious process, before being sacrificed, and any abscesses harvested and stored at -80°C. Each abscess was subsequently homogenized in 3 ml sterile PBS, and the cfu/abscess determined via serial dilution and viable count enumeration. The statistical significance of bacterial recovery was evaluated using a Student T-Test with a 5% confidence limit.

Murine model of sepsis and dissemination. These experiments were conducted as described previously (Li et al., 2009; Voyich et al., 2006). Briefly, 6 week old, female CD-1 Swiss mice were purchased from Charles River Laboratories, and housed at the vivarium in the College of Medicine, University of South Florida. *S. aureus* strains LAC and LAC-protease null were prepared as for the wound formation model. For infection purposes, cultures were thawed, washed twice in PBS, and diluted in PBS to 1 x 10^9 cfu/ml. Thirty mice per strain were inoculated by tail vein injection with 100 µl bacterial suspension, giving a final inocula of $1x10^8$ cfu/ml. The infection was allowed to proceed for 6 days, or until mice reached a pre-moribund state (used as a measure of mortality). Mice were then euthanized and the brain, liver, kidneys, heart, lungs and spleens collected and stored at -80°C. Any mouse sacrificed before day 6 was recorded for mortality, but their organs were not analyzed for bacterial burden. Each organ was subsequently homogenized in 3 ml sterile PBS, and the cfu/organ determined via serial dilution and viable count enumeration. The statistical significance of bacterial recovery was evaluated using a Mann-Whitney Test with a 5% confidence limit. Mortality was measured using a Log Rank and Chi Squared Test with 1-degree of freedom.

Proteomic analysis of surface and secreted proteins. Stationary phase (15h) cultures of wild-type and mutant strains were prepared in TSB, and their secretomes were harvested and purified as described previously (Rivera et al., 2011). Surface proteins were extracted by methods previously described (Gatlin et al., 2006). Briefly, wild-type and LAC-protease null mutant cells were grown to stationary phase (15h) and sedimented via centrifugation. Pellets were resuspended in TSM buffer (100mM Tris-HCl, 500 mM sucrose, 10mM $MgCl_2$) and incubated in the presence of 100 µg of lysostaphin for 60

minutes at 37°C. Supernatants were collected and precipitated with 10% trichloroacetic acid, followed by centrifugation to recover precipitates. These were then washed thrice with 100% ice-cold ethanol, before being air dried. Triplicate samples of secreted and surface proteins for each strain were resuspended in urea buffer, with 15 µg/ml loaded and run on 12% SDS-PAGE gels. At least 2 lanes were left between each sample to prevent loading contamination, with wild-type and mutant strains run on separate gels. Following this, secreted protein gels were cut into 11 approximately equal fractions, whilst surface proteins gels were separated into 10 approximately equal fractions. These were then washed with ACN to remove SDS and bromophenol blue, before being dried using a SpeedVac centrifuge. Gel pieces were rehydrated with 100µl of 45 mM DTT and incubated at 55°C for 30 minutes. The supernatant was removed and replaced with 100 mM iodoacetamide and incubated in the dark at room temperature for 30 minutes. Following this, the buffer was removed and washed thrice with 50% ACN/50 mM ABC with agitation for 15 minutes. Gel pieces were dried again using a SpeedVac centrifuge. Promega Trypsin (12.5 ng/µl) was dissolved in Promega trypsin buffer, and enough trypsin solution was used to cover the gel pieces, before incubation at 37°C for 12-16h. The supernatant was removed and retained, and the reaction stopped with 5% glacial acetic acid. The gel pieces were covered with 100 µl of 50:50 ACN:water containing 0.1% formic acid and vortexed for 15 minutes. Supernatants were again removed and added to the previously collected supernatants. Samples were dried using a SpeedVac centrifuge, and resuspended in 1 ml of 0.1% formic acid in water. Samples were then desalted and analyzed using a linear ion trap-LTQ instrument mass spectrometer (LTQ

XL, Thermo) operated with Xcalibur (v2.0.7) data acquisition software, as described by us previously (Rivera et al., 2011).

Results

Verification of extracellular protease depletion in the LAC-protease null strain. An extracellular protease-null strain of *S. aureus* has previously been generated and described in USA300 LAC (Karlsson & Arvidson et al., 2002). As it forms the basis of the work described herein, we first set out to confirm the lack of secreted proteases in this strain using a targeted proteomics method. Targeted mass-spectrometry uses the inclusion of mass to charge ratios in order to guide MS sequencing to a pre-determined subset of peptides, and is more accurate than other methods that overlook certain proteins. The Aur, SspA, SspB, ScpA and SplA (used as a representative for the *spl* operon) sequences were analyzed using the Protein Prospector-MS digest program to determine the peptides that would result after trypsin digest. A peptide that is unique to each protease was chosen, and its mass to charge ratio included in a designed MS method. Overnight secretome samples (15h) from the LAC wild-type and LAC-protease null mutant were prepared and analyzed using an LTQ-MS. Spectral counts of proteins identified were compared, with the LAC wild-type producing robust counts for SspA (48), ScpA (49), SspB (50), Aur (47) and SplA (8). As expected, when the LAC-protease null mutant secretome was probed, no extracellular proteases were detected. This finding was also verified via gelatin zymogram, with the LAC wild-type producing several activity bands, whilst the mutant displayed none (Figure 29). To ensure that no additional, unintended mutations (specifically *agr*) had occurred in the LAC-protease null strain, we also performed real-time PCR analysis with primers specific to RNAII, RNAIII and *hla*, with the latter serving as a representative *agr* regulated virulence determinant. We found that, at both 5h and 15h of growth, there was no observable different between the 2 strains in

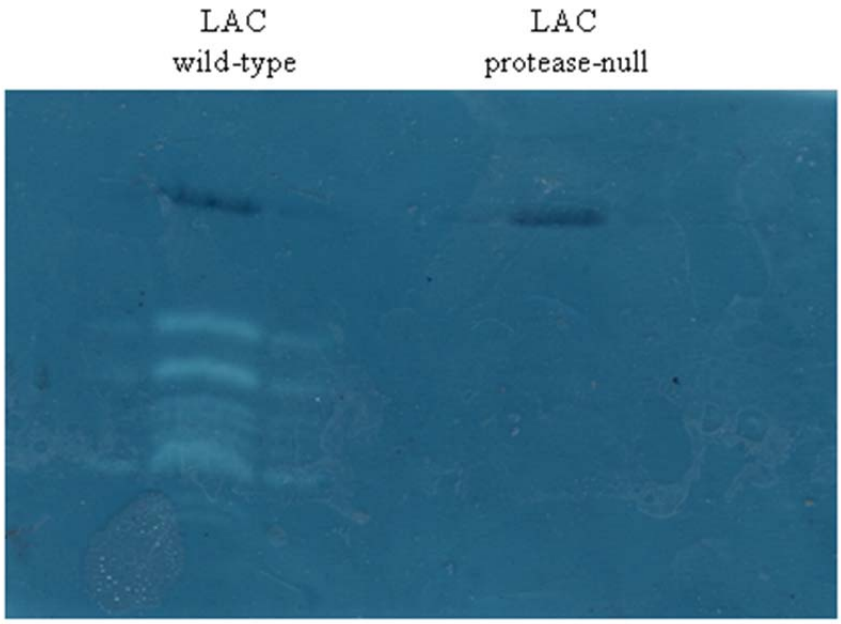

Figure 29. Gelatin zymography of the LAC wild-type and its protease-null mutant. The secretomes of the LAC wild-type and protease-null mutant were collected (15 h) and protease activity visualized on a zymogram gel containing gelatin.

the levels of these transcripts (Figure 30). As such, these analyses verify that an exoprotease-null strain of LAC was created, that seemingly bears no apparent additional mutations that impact the synthesis of virulence determinants.

The protease-null strain has fitness defects during growth in peptide based media and pig serum. We first sought to assess whether loss of extracellular proteases affected viability of the USA300 LAC strain. As such, growth profiling was performed in complex media (TSB) over a 96h period, with no difference observed compared to the parental strain (Figure 31A). This is perhaps to be expected, as, for example, *agr* mutants of *S. aureus* are aproteolytic, yet are undoubtedly viable. Following this, we next tested the ability of the protease null strain to grow in peptide based media. This was performed as many bacterial species possess a PrtP homolog, which is a surface exposed protease that functions to generate oligopeptides from polypeptides for nutrition (Liu et al., 2010). Such a protein is lacking in *S. aureus* (our unpublished data), and therefore we reasoned that the extracellular proteases perhaps fulfill such a function for the generation of oligopeptides in the absence of a PrtP protein in *S. aureus*. As such, we grew the LAC parental strain and its protease null mutant in 10% milk broth (Figure 31B), which is routinely used to evaluate peptide based nutrition (Borezee-Durant et al., 2009), as it contains few free amino acids, and abundant peptides. During the first 24 hours of profiling, no difference in viability for the LAC wild-type and its mutant strain was observed. Interestingly, after this time, both strains began to lose viability, with the protease null mutant proving significantly less able to resist starvation in peptide based media compared to the parent. Specifically, by day 2, the protease mutant produced a 2.3

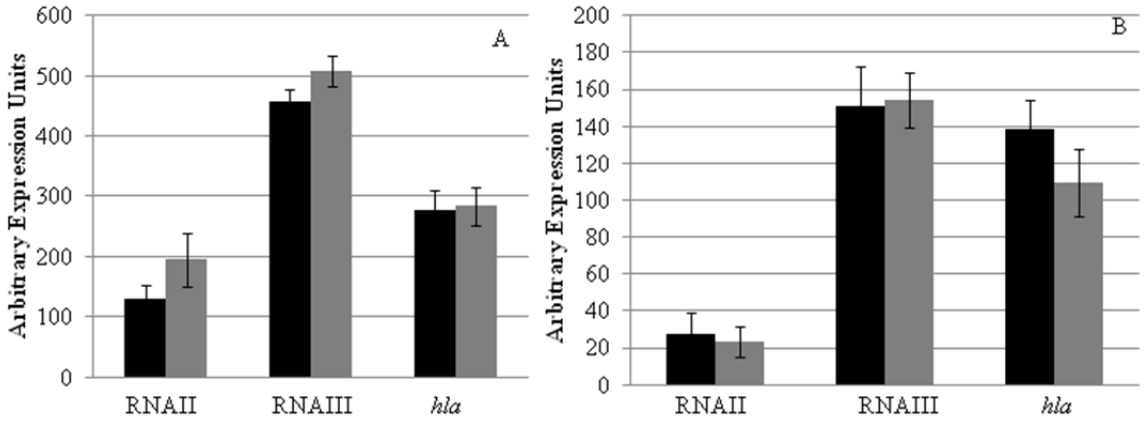

Figure 30. Exoprotease deletion does not lead to observable difference in *agr* or *hla* transcript levels. qRT-PCR was performed with primers specific to RNAII, RNAIII and *hla* in the LAC wild-type (black) and protease-null (grey) strains. These analyses were performed at (**A**) 5 hours and (**B**) 15 hours of growth. The data presented are from at least three independent cultures; error bars are shown as +/- SEM.

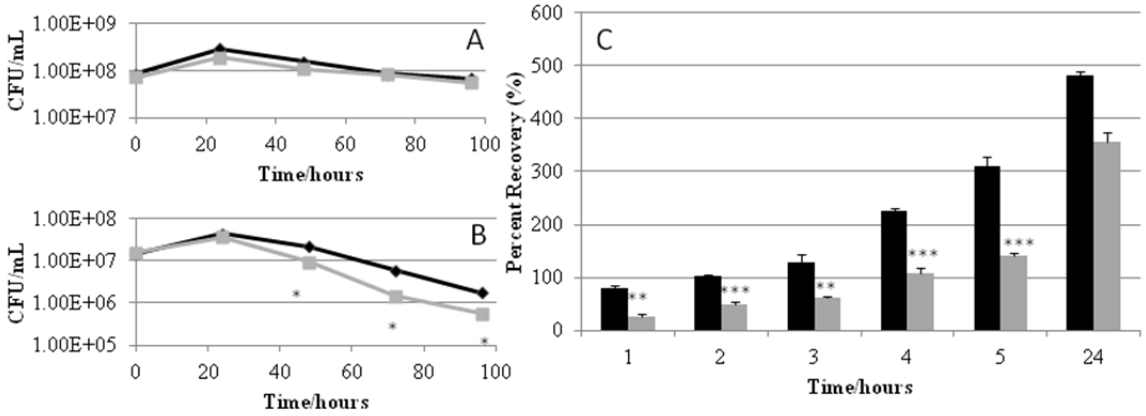

Figure 31. Secreted proteases aid in survival during growth in peptide based media and pig serum. The average CFU/mL of the LAC wild-type (black) and LAC-protease null mutant (grey) were compared over 4 days in: **A)** TSB and **B)** 10% milk broth. Data represents three replicates showing less than 10% variability. A Student's T-Test was used with a 5% confidence level to determine statistical significance, * = p□ 0.05. **C)** The average CFU/mL of the LAC wild-type (black) and LAC-ESPN mutant (grey) were compared at the times indicated during growth in pig serum. Data is expressed as percent recovery of the inoculum, and represents three independent experiments. A Student's T-Test was used with a 5% confidence level to determine statistical significance, ** = p□ 0.01, *** = p□ 0.001; error bars are shown as +/- SEM.

fold decrease in average CFU/mL, which was exacerbated at day 3 with a 4-fold decrease in survival. By day 4, a 3.1-fold decrease in mutant viability was determined.

We next set out to assess the fitness of the wild-type and protease mutant during growth in a more pathogenically relevant medium, in this case pig serum. Accordingly, exponentially growing cells of the parent and mutant were inoculated into pig serum, and the survivability of triplicate cultures determined (Figure 31C). When growth was analyzed during early time points, a large decrease in the viability of the protease null strain was observed. Specifically, after 1h a 3.2-fold decrease in mutant cell viability was observed (24.6% of the inoculum), compared to the parent strain (80.9% of the inoculum). Viability stabilized after this time, yet still produced a consistent 2-fold reduction in the mutant strain compared to the parent from hours 2-5. After 24h of incubation, whilst both strains had robust growth (Wt = 480.8% recovery of inoculum, protease-null strain = 355.6%), there was still a decrease in final loads of the mutant strain. This suggests that secreted proteases may have a role in nutrition acquisition in *S. aureus*, particularly during times of stress and starvation.

Secreted proteases play a role in resistance to antimicrobial peptides. Antimicrobial peptides (AMPs) are small molecules that have a key role in the innate immune system, displaying broad spectrum antibacterial activity. Previously it was shown that the *S. aureus* secreted protease, aureolysin, is able to cleave the human cathelicidin, LL-37 (Sieprawska-Lupa et al., 2004). To determine if secreted proteases facilitate resistance to only this AMP, or other such peptides, sensitivity profiling of the mutant was performed (Figure 32). When LD_{50} values were compared for LL-37 we observed a 2.7-fold

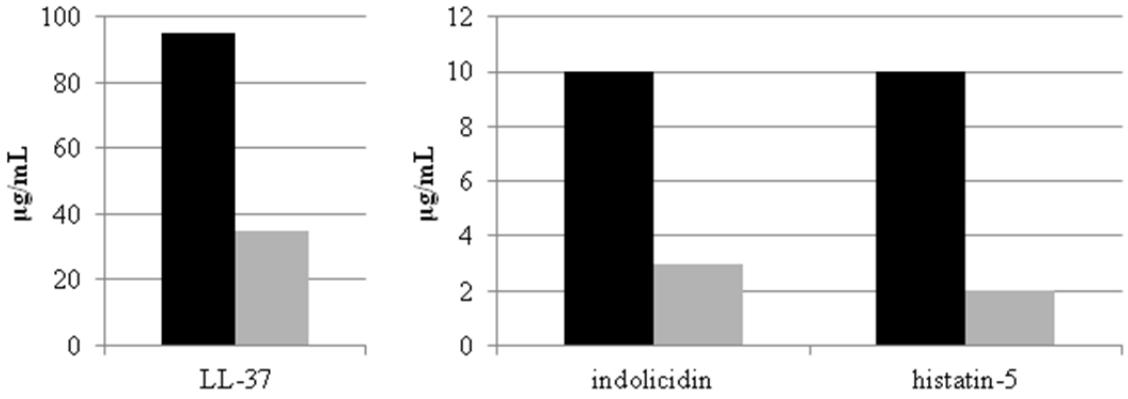

Figure 32. Secreted proteases play a role in the resistance of *S. aureus* to antimicrobial peptides. The LAC wild-type (black) and protease null mutant (grey) were cultured overnight with either LL-37, indolicidin or histatin-5. The LD_{50} for each was determined by OD_{600} evaluation of cultures grown with AMPs compared to those without. Data presented is from at least three independent replicates showing identical values.

increase in sensitivity for the protease null strain (35 μg/mL) when compared to the parent (95 μg/mL). Similarly increased sensitivity was observed when testing was performed with indolicidin, with the wild-type strain producing a LD_{50} of 10 μg/mL, whilst the mutant was 3.33-fold lower at only 3 μg/mL. Finally, when histatin-5 was used, the LAC wild-type LD_{50} was 10 μg/mL, yet the protease null strain displayed a 5-fold increase in sensitivity, with an LD_{50} of 2 μg/mL. These findings suggest that secreted proteases contribute to the ability of *S. aureus* to resist the toxic effects of AMPs.

Extracellular proteases are required for survival during interaction with the innate immune system. The *in vitro* AMP sensitivity assays suggest that extracellular proteases are important for survival during interaction with elements of the immune system. Accordingly, we next profiled survivability of the mutant strain in the presence of the innate immune system, including its cellular components. Accordingly, exponentially growing LAC wild-type and protease null mutant cells were separately cultured in whole human blood for 4 hours, and their viability determined (Figure 33). Analysis revealed that the LAC wild-type had bacterial loads that decreased only marginally from the initial inoculum (82.1% recovered). In contrast, the protease null mutant displayed significantly impaired survival, returning only 12.6% of the inoculum. This results in a 6.5-fold reduction in survivability of the mutant strain when compared to the parent. Ultimately this data corroborates our serum and AMP findings, and suggests that secreted proteases protect *S. aureus* during interaction with the host immune system.

Secreted proteases contribute to CA-MRSA wound formation. There are conflicting results regarding the role of extracellular proteases in *S. aureus* virulence (Shaw et al.,

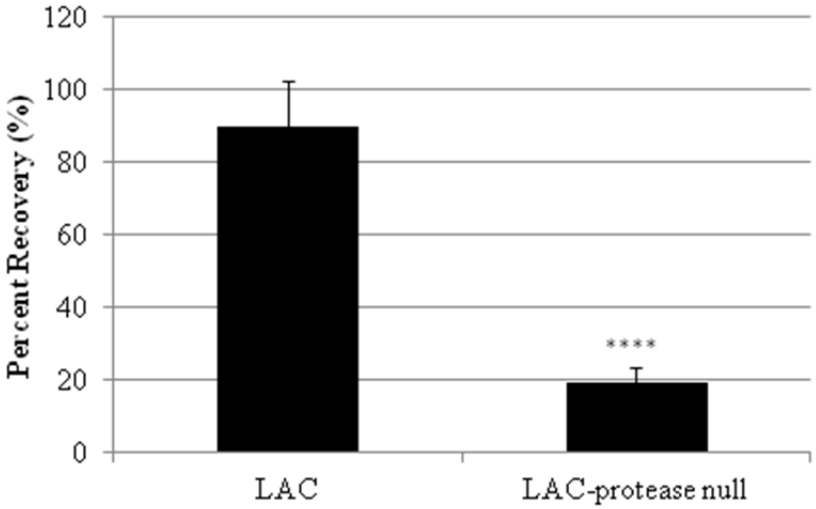

Figure 33. Secreted proteases are protective during interaction with the human innate immune system. The LAC wild-type and protease null strain were separately cultured in whole human blood for 4 hours, before their viability was determined. Data is expressed as percent recovery of the inoculum, and represents three separate blood samples and nine individual replicates. Error bars are shown +/- SEM; statistical significance was calculated using a Student's t test with 5% confidence level, **** = P□0.0005

2004; Coulter et al., 1998; Kubica et al., 2008; Burlak et al., 2007; Rice et al., 2001; Calander et al., 2004; Hossain et al., 2006; Diep et al., 2004). As such, we set out to determine if the 10 major secreted proteases collectively contribute to localized infection using a murine model of skin abscess. Accordingly, 10 mice were subcutaneously inoculated with either the LAC wild-type or protease null mutant, and the infection was allowed to proceed for 6 days. Following this time, all mice were euthanized, any abscesses harvested, and the bacterial loads per abscess determined (Figure 34). We observed that the protease null mutant had significantly reduced bacterial loads per abscess when compared to the wild-type. Specifically, the average wild-type CFU/abscess was 57.7% of the inoculum, whilst for the mutant it was 28.1%, representing a 2.0-fold decrease in bacterial burden.

Extracellular proteases play a key role during systemic CA-MRSA infections. In addition to presenting as skin and soft tissue infections, CA-MRSA is also a major cause of bacteremia and systemic disease. As such, we next set out to determine if extracellular protease deletion impacts systemic CA-MRSA infections. Accordingly, 30 mice each were inoculated via tail vein injection with 1 X 10^8 cells of either the LAC wild-type or protease null mutant. The infection was allowed to proceed for 6 days, or until mice reached a pre-moribund state (used as a measure of mortality). Mice were then euthanized and the brain, liver, kidneys, heart, lungs and spleens collected. Any mouse sacrificed before day 6 was recorded for mortality, but their organs were not analyzed for bacterial burden. Following recovery, each organ was homogenized, serial diluted and plated to determine bacterial load (Figure 35). Of the 30 LAC wild-type animals, 2 died prior to day 6, yielding a mortality rate of 3.3%. In contrast, 7 out of the 30 inoculated

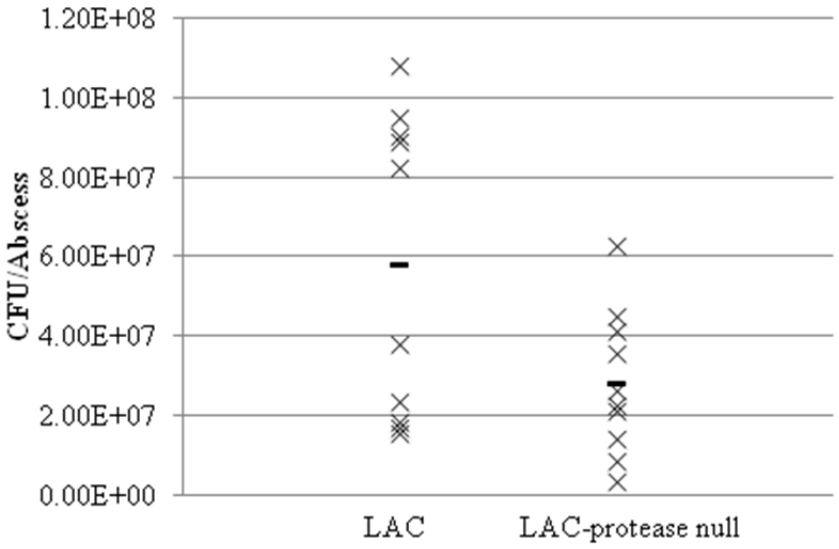

Figure 34. Secreted proteases contribute to CA-MRSA abscess formation. The LAC wild-type and protease null mutant were used to subcutaneously inoculated the scapula of 10 SKH-1 mice each, at 1 x 10^8 cells. After 6 days abscesses were harvested and bacterial loads determined via homogenization and serial dilution. The average cfu/abscess returned is indicated by horizontal bars. Statistical significance was calculated using a Student's t test with a 5% confidence level ($p < 0.05$).

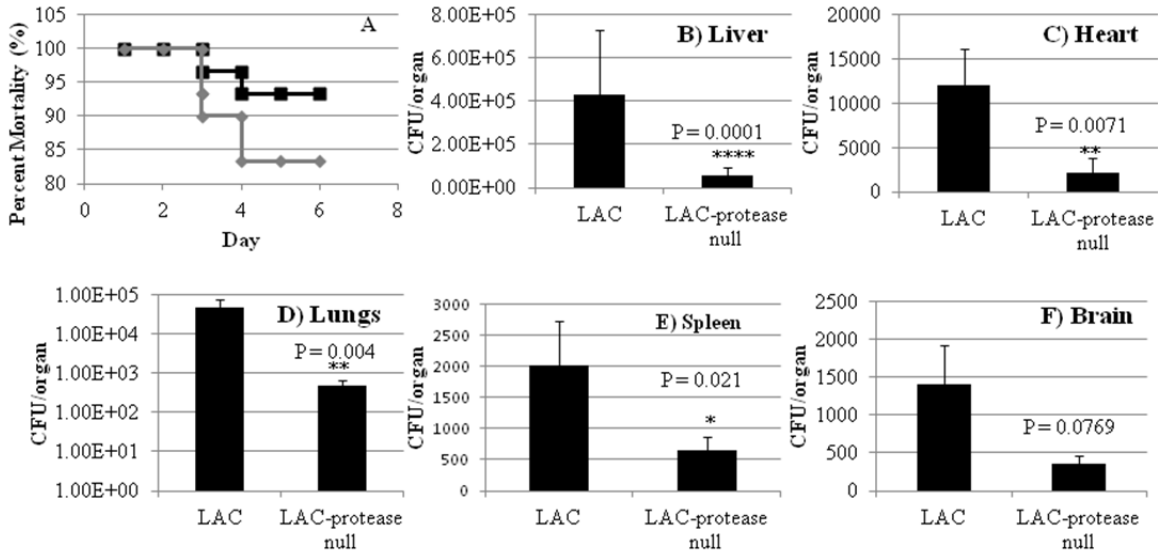

Figure 35. Secreted proteases have contrasting roles in the morbidity and mortality of systemic CA-MRSA infections. The LAC wild-type and mutant were inoculated via tail vein injection into 30 mice each, at 1 x 10^8 cells. Mortality for the LAC wild-type (black) and LAC-protease null mutant (grey) infected mice was measured over 6 days (**A**), and analyzed using a Log Rank and Chi Squared Test with 1-degree of freedom ($p = 0.079$). After 6 days, all surviving mice were euthanized, and their organs harvested for bacterial load determination (**A-F**). Average CFU/organ of the wild-type and mutant strain were compared; error bars are shown as +/- SEM. A Mann-Whitney test was used to determine statistical significance. *= p□ 0.05, ** = p□0.01, **** = p□0.0001.

with the LAC-protease null mutant died, giving a 23.3% mortality rate. This represents a 7-fold increase in mortality for the protease null strain compared to the parental strain. Conversely, when analyzing bacterial burden per organ between the two groups, we observed significant decreases for the mutant strain in the liver, lungs, heart and spleen. Specifically, the largest fold change, 98.7, was observed in the lungs, with the LAC wild-type returning an average CFU/lungs of 4.74×10^4 and the protease null mutant returning 4.80×10^2. Additionally, we observed a 7.4 fold decrease in bacterial load in the liver of inoculated mice, with the wild-type producing an average CFU/liver of 4.32×10^5 and the mutant 5.79×10^4. The heart produced a 5.7 fold decrease in mutant bacterial cells, with LAC yielding 1.2×10^4 and the protease mutant yielding 2.1×10^3. With regards to the spleen, we observed 2.01×10^3 CFU/spleen for the parental strain and 6.6×10^2 for the mutant, which is a 3.0 fold decrease. Finally, in the brain we observed a 3.8 fold decrease in the average CFU/brain for mutant infected mice, however this was found to be outside the range of significance, although clearly trending towards it ($p = 0.079$). Interestingly, there was no significant difference between the two strains in their ability to infect the kidneys of inoculated mice. The LAC wild-type returned 5.57×10^7 CFU/organ whilst the protease null mutant returned 5.87×10^7. As such it appears that secreted proteases play a major role in the survival of *S. aureus* during *in vivo* infection.

Secreted proteases are primary effectors of virulence determinant stability. It has previously been proposed (McGavin et al., 1997; McAleese et al., 2007; Lindsay & Foster 1999; Travis et al., 1995), and demonstrated by our group to a limited extent (Gonzalez et al., 2012), that secreted proteases regulate the stability of self-derived toxins. To determine if this is in fact the case, we assessed the impact of secreted

proteases on virulence determinant stability using proteomic techniques. Accordingly, stationary phase secretomes (15h) of the LAC wild-type and protease null mutant were collected in triplicate and separated via SDS-PAGE. Each gel was then cut into 11 identical fractions to facilitate processing. The data from all 11 fractions was pooled, and collectively analyzed for alterations in protein abundance using spectral counts (Table 5). In total, 19 known secreted proteins had increased abundance upon deletion of extracellular proteases. The highest fold change was seen for phenol soluble modulin alpha 4, with a 4.2-fold increase in the mutant. Other well known virulence determinants were also found to be more abundant in the protease-null strain, including 2 Lipases (Geh = 3.8-fold, SAUSA300_2603 = 2.5-fold), components of the γ-hemolysin (HlgA = 2.4-fold, HlgC = 2.1-fold), α-toxin (2.0-fold), leukotoxin LukE (2-fold) and enterotoxin Q (1.5-fold). Our analysis also revealed increased abundance of certain proteins in the wild-type strain, including all 10 secreted proteases, as expected, along with catalase (9-fold), the CamS pheromone (2-fold), and 2 putative lipoproteins (SAUSA300_2403 = 2-fold, SAUSA300_0411 = 3-fold).

A consideration with this pooled analysis is that a protein may be processed by secreted proteases into potentially inactive fragments, as a result of endoproteolysis. As the protein is not completely degraded to free amino acids, each of these fragments will be detected by MS-analysis; therefore one may not observe any fold-change in spectral counts, yet cleavage has rendered the protein non-functional. As such, protein abundance was next compared within individual fractions from the SDS-PAGE gels. Spectral counts for a given protein were only analyzed for the single fraction that would contain its full-length protein, based on predicted molecular weight. (Figure 36, Table 6). The 19

Table 5. Collective alterations in protein abundance for secreted proteins upon deletion of extracellular proteases in *S. aureus* USA 300.

Protein	Gene	Accession Number	WT[a,c]	M[b,c]	Fold Change
Phenol-soluble modulin alpha 4 peptide	*psmα4*		4	17	4.25
Triacylglycerol lipase	*geh*	SAUSA300_0320	276	1062	3.84
Autolysin	*alt*	SAUSA300_1921	117	303	2.58
Triacylglycerol lipase	*lip*	SAUSA300_2603	168	425	2.52
Gamma-hemolysin component A	*hlgA*	SAUSA300_2365	29	72	2.48
Gamma-hemolysin component C	*hlgC*	SAUSA300_2366	17	37	2.17
Alpha-hemolysin	*hla*	SAUSA300_1058	226	462	2.04
Staphylococcal superantigen-like protein 7	*ssl7*	SAUSA300_0401	2	6	2
Extracellular matrix-binding protein	*ebh*	SAUSA300_1327	2	4	2
Extracellular matrix protein-binding protein	*emp*	SAUSA300_0774	5	10	2
Leukocidin	*lukA*	SAUSA300_1975	88	176	2
Leukotoxin	*lukE*	SAUSA300_1769	8	16	2
Leukocidin	*lukB*	SAUSA300_1974	83	157	1.89
Putative staphylococcal enterotoxin		SAUSA300_0370	7	13	1.85
Ear protein	*ear*	SAUSA300_0815	8	14	1.75
Phenol-soluble modulin beta 1 peptide	*psmβ1*	SAUSA300_1067	5	8	1.6
Staphylococcal enterotoxin Q	*seq*	SAUSA300_0801	12	19	1.58
Immunoglobulin-binding protein	*sbi*	SAUSA300_2364	17	26	1.52
Phenol-soluble modulin alpha 3 peptide	*psmα3*		2	3	1.5
Putative lipoprotein		SAUSA300_2403	2	0	-2
Sex pheromone	*camS*	SAUSA300_1884	2	0	-2
Uncharacterized lipoprotein		SAUSA300_0411	3	1	-3
Catalase	*katA*	SAUSA300_1232	9	0	-9
Serine protease	*splA*	SAUSA300_1758	14	0	-14
Serine protease	*splD*	SAUSA300_1755	15	0	-15
Zinc metalloproteinase aureolysin	*aur*	SAUSA300_2572	15	0	-15

Table 5 continued

Serine protease	*splE*	SAUSA300_1754	18	0	-18
Serine protease	*splC*	SAUSA300_1756	18	0	-18
Serine protease	*splF*	SAUSA300_1753	27	0	-27
V8 protease	*sspA*	SAUSA300_0951	43	1	-43
Serine protease	*splB*	SAUSA300_1757	46	0	-46
Cysteine protease	*sspB*	SAUSA300_0950	123	0	-123
Staphopain A	*scpA*	SAUSA300_1890	143	1	-143

a-LAC wild-type
b- LAC protease-null mutant
c- total spectral counts identified for each protein

Table 6. Individual fraction analysis of alterations in protein stability for secreted proteins upon deletion of extracellular proteases in *S. aureus* USA 300.

Protein	Gene	Accession Number	Size	Fold Change
Immunoglobulin-binding protein	*sbi*	SAUSA300_2364	50 kDa	9
Alpha-hemolysin	*hla*	SAUSA300_1058	36 kDa	8.88
Phenol-soluble modulin alpha 4 peptide	*psmα4*		2 kDa	5
Leukocidin *lukB*		SAUSA300_1974	39 kDa	4.66
Triacylglycerol lipase	*lip*	SAUSA300_2603	77 kDa	4.6
Gamma-hemolysin component A	*hlgA*	SAUSA300_2365	35 kDa	4
Staphylococcal complement inhibitor	*scn*	SAUSA300_1919	13 kDa	3.66
Leukotoxin	*lukE*	SAUSA300_1769	35 kDa	3.66
Gamma-hemolysin component C	*hlgC*	SAUSA300_2366	36 kDa	3.33
Staphylococcal superantigen-like protein 7	*ssl7*	SAUSA300_0401	26 kDa	3
Extracellular matrix protein-binding protein	*emp*	SAUSA300_0774	38 kDa	3
Ear protein	*ear*	SAUSA300_0815	20 kDa	2.66
Triacylglycerol lipase precursor	*geh*	SAUSA300_0320	76 kDa	2.55
Panton-Valentine leukocidin	*lukS*	SAUSA300_1382	35 kDa	2.45
Autolysin	*alt*		137 kDa	2.3
Secretory antigen	*ssaA*	SAUSA300_2249	29 kDa	2
Extracellular matrix-binding protein	*ebh*	SAUSA300_1327	1123 kDa	2
Staphylococcal enterotoxin Q	*seq*	SAUSA300_0801	28 kDa	2
Leukocidin *lukA*		SAUSA300_1975	40 kDa	2
Staphylococcal enterotoxin K	*sek*	SAUSA300_0800	28 kDa	2
Phenol-soluble modulin alpha 3 peptide	*psmα3*		3 kDa	2
Phenol-soluble modulin beta 1 peptide	*psmβ1*	SAUSA300_1067	4 kDa	1.75

Table 6 continued

Putative staphylococcal enterotoxin		SAUSA300_0370	23 kDa	1.66
Panton-Valentine leukocidin,	*lukF*	SAUSA300_1381	37 kDa	1.5

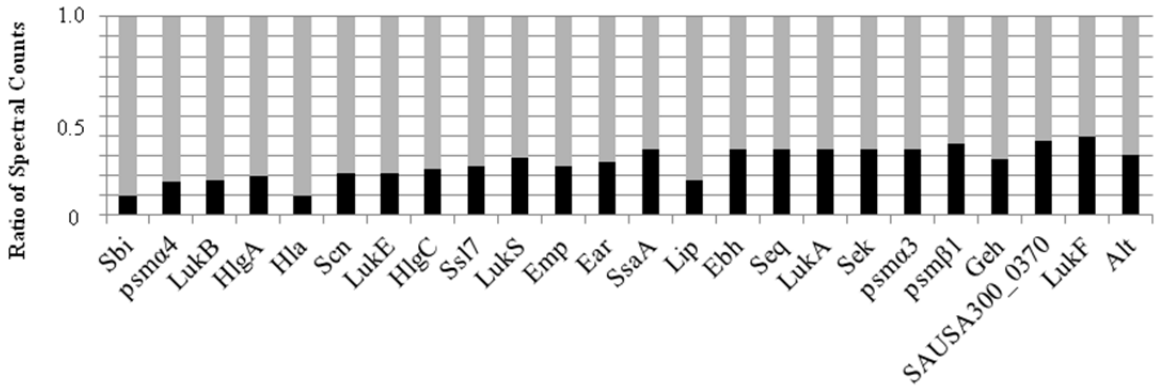

Figure 36. Extracellular proteases modulate the stability of a wealth of known secreted virulence factors. The secretome of LAC and its protease-null strain were collected and fractionated via SDS-PAGE. Known, full length secreted proteins were analyzed and their spectral counts determined. Shown are the ratios of abundance for each secreted protein in the wild-type (black) compared to the mutant (grey).

proteins identified from the pooled sample all had decreased abundance in the wild-type strain in their relative molecular weight fractions; in many cases to even greater degrees than in our collective analysis. For example, we observed increased abundance for Sbi (9-fold), alpha-toxin (8.8-fold), phenol soluble modulin alpha 4 (5-fold), γ-hemolysin (HlgA = 4-fold) and LukE (3.6-fold) upon deletion of extracellular proteases using this method. Furthermore, an additional 5 proteins that did not produce a significant fold change in the pooled samples were identified as being altered in protein stability in the mutant strain. These include the two components of the Panton-Valentine leukocidin (LukS = 2.4-fold, LukF = 1.5-fold), the staphylococcal complement inhibitor (3.6-fold), enterotoxin K (2-fold), and secretory antigen SsaA (2-fold).

Following this, we also performed a similar analysis for surface proteins between the two strains. The rationale for this is that existing evidence in the literature suggests secreted proteases can also target surface exposed proteins in *S. aureus* (Karlsson et al., 2001; McAleese et al., 2001). As such, the cells used to generate secretomes were harvested, and their surface protein fraction isolated, before being processed in an identical SDS-PAGE based fashion. When performing a collective analysis of spectral counts for all fractions, we observed 10 proteins with a 1.5-fold or greater increase in the protease null mutant (Table 7). These include fibronectin-binding proteins A (4-fold) and B (3-fold), as well as fibrinogen binding protein (2.3-fold), clumping factor A (1.7-fold) and IsdA (1.6-fold). We also performed specific fraction analysis as detailed above to determine those proteins with altered protein stability upon deletion of the extracellular proteases (Figure 37, Table 8). Specific analysis revealed that, in addition to the 10 surface proteins identified from pooled studies, a further 7 had a fold change of 2 or higher in the protease

Table 7. Collective alterations in protein abundance for surface proteins upon deletion of extracellular proteases in *S. aureus* USA 300.

Protein	Gene	Accession Number	WT[a,c]	M[b,c]	Fold Change
Fibronectin-binding protein B	*fnbB*	SAUSA300_1052	1	4	4
Fibronectin-binding protein A	*fnbA*	SAUSA300_2441	1	3	3
Enolase	*eno*	SAUSA300_0760	45	129	2.86
Staphylokinase	*sak*	SAUSA300_1922	16	40	2.5
Fibrinogen-binding protein	*efb*	SAUSA300_1055	9	21	2.33
Cell wall surface anchor family protein	*sasG*	SAUSA300_2436	33	74	2.24
Transferrin receptor	*tpn*	SAUSA300_0721	1	2	2
Putative lipoprotein		SAUSA300_0372	24	44	1.83
Clumping factor A	*clfA*	SAUSA300_0772	11	19	1.72
Iron-regulated surface determinant protein A	*isdA*	SAUSA300_1029	10	16	1.6

a-LAC wild-type
b- LAC protease-null mutant
c- total spectral counts identified for each protein

Table 8. Individual fraction analysis of alterations in protein stability for surface proteins upon deletion of extracellular proteases in *S. aureus* USA 300.

Protein	Gene	Accession Number	Size	Fold Change
Clumping factor B	*clfB*	SAUSA300_2565	97kDa	8
Staphylokinase	*sak*	SAUSA300_1922	18 kDa	4.8
Fibrinogen-binding protein	*efb*	SAUSA300_1055	19 kDa	4.25
Fibronectin-binding protein B	*fnbB*	SAUSA300_1052	13 kDa	4
Enolase	*eno*	SAUSA300_0760	47 kDa	3.82
Iron-regulated surface determinant protein A	*isdA*	SAUSA300_1029	39 kDa	3.75
Fibronectin-binding protein A	*fnbA*	SAUSA300_2441	112 kDa	3
Cell wall surface anchor family protein *sasG*		SAUSA300_2436	49 kDa	2.93
Putative lipoprotein		SAUSA300_0372	21 kDa	2.5
Putative surface protein		SAUSA300_0883	16 kDa	2.47
Probable transglycosylase	*isaA*	SAUSA300_2436	24 kDa	2.36
Immunodominant staphylococcal antigen B	*isaB*	SAUSA300_2573	19 kDa	2.2
Clumping factor A	*clfA*	SAUSA300_0772	97 kDa	2.16
Immunoglobulin G binding protein A	*spa*	SAUSA300_0113	56 kDa	2.1
Elastin-binding protein	*ebpS*	SAUSA300_1370	53 kDa	2
Transferrin receptor	*tpn*	SAUSA300_0721		2
Staphylocoagulase	*coa*	SAUSA300_0224	69 kDa	2

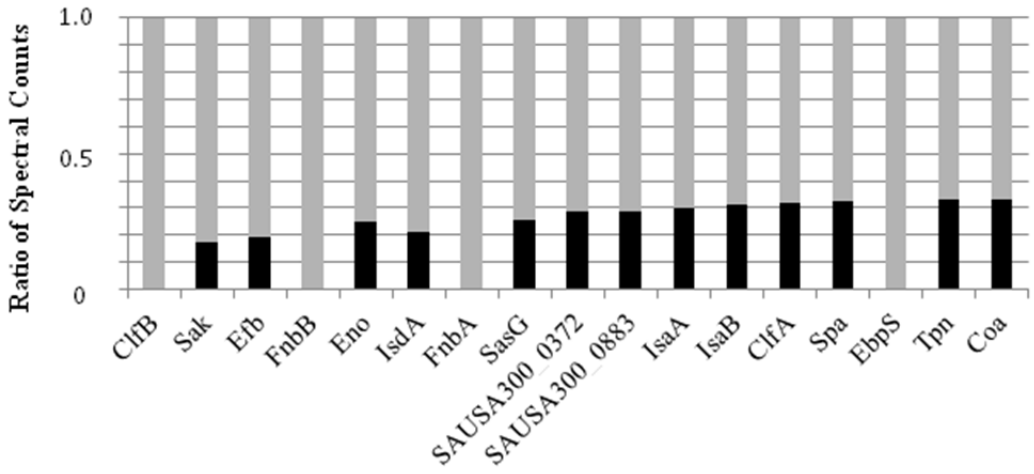

Figure 37. Extracellular proteases modulate the stability of a number of surface-associated virulence factors. The surfactome of LAC and its protease-null strain were collected and fractionated via SDS-PAGE. Known, full length surface proteins were analyzed and their spectral counts determined. Shown are the ratios of abundance for each secreted protein in the wild-type (black) compared to the mutant (grey).

mutant. These included immunodominant staphylococcal antigens A (2.3-fold) and B (2.2-fold), staphylocoagulase (2-fold), immunoglobulin G binding protein A (Spa = 2.1-fold), elastin-binding protein (2-fold) and a putative surface protein (SAUSA300_0883 = 2.4-fold). We again observed a general increase in protein abundance for the majority of proteins identified by fraction analysis when compared to the pooled data. The most striking of which was clumping factor B, which, upon fraction analysis, produced an 8-fold increase in abundance in the mutant.

Discussion

Recent reports suggest that MRSA is now the leading cause of infectious disease and death by a single agent in the United States (Coulter et al., 1998). This pathogenic success can largely be attributed to the meteoric increase in CA-MRSA infections in the last decade (Burlak et al., 2007; Calander et al., 2008); Calander et al., 2004). Although several CA-MRSA lineages exist, USA300 is now thought to account for more than 50% of all MRSA infections in some regions (Johnson et al., 2007; Moran et al., 2006). The reason for the surprising success of USA300 is somewhat unclear; however work by a number of groups suggests it may be attributable to the differential expression of core genomic elements (Li et al., 2009), including the PSMs, hemolysins, extracellular proteases and enterotoxins (Diep et al., 2008; Li et al., 2009; Adem et al., 2005; Kobayashi & DeLeo, 2009; Montgomery et al., 2008; Wang et al., 2007). A number of studies performed by ourselves and others have focused on determining the contribution of extracellular proteases to disease causation (Reed et al., 2001; Shaw et al., 2004; Coulter et al., 1998; Rice et al., 2011; Calander et al., 2004; Karlsson & Arvidson, 2002; Sifri et al., 2008; Prajsnar et al., 2008). Thus far these data have proved contradictory, perhaps as a result of using strains that have varying proteolytic capabilities. Some backgrounds used (e.g. RN6390) have limited clinical significance, whilst other studies have focused only on individual protease mutations. This latter point is of particular importance as it has been shown in other highly proteolytic bacteria that significant levels of functional redundancy exist (Travis et al., 1995). For these reasons, and to explore the role of this class of enzymes in CA-MRSA pathogenesis, we have focused in this study on a total exoprotease null mutant of USA300 LAC.

Interestingly, whilst we show that the mutant strain is viable in complex media, we did observe a survival defect during extended culturing in peptide based media. Previous reports have shown that *S. aureus* cells can only import octapeptides or smaller for nutrition based purposes (Hiron et al., 2007). Therefore, it is possible that the extracellular proteases of *S. aureus* cleave large oligopeptides into smaller fragments for growth and nutrition. Interestingly, the defect was only apparent after 24h of growth. Thus it is possible that at earlier time points, the major nutrient source is limited free amino acids, and oligopeptides with less than 8 residues. Once these more readily usable nutrients have been exhausted, the focus would then shift to breaking down oligopeptides with greater than 9 residues. In this scenario, any strain lacking extracellular proteases would be limited in their ability to generate smaller, importable peptides for nutrition, explaining the growth defect observed. This contention is supported by the observation that, unlike a variety of other organisms, *S. aureus* lacks a PrtP homolog, which serves to breakdown large oligopeptides for import and nutrition (Siezen et al., 1999). We also observed fitness defects in the protease null strain during growth in the more pathogenically relevant pig serum. This is perhaps a result of an inability to degrade proteins for nutritive purposes. Additionally, others have shown that nutrition defects become emphasized during growth in serum (Hammer & Skaar, 2011). Alternatively, given that serum contains complement, AMPs and other non-cellular components of the immune system, it is possible the growth defects observed stem from a role for extracellular proteases during interaction with innate immunity, as a decrease in survival of the protease-null mutant is observed at early time points. This is support by the recent

finding that aureolysin facilitates resistance of *S. aureus* to killing by complement as a result of C3 cleavage (Laarman et al., 2011).

This contention is further corroborated by our observation that the protease mutant strain is more sensitive to antimicrobial peptides (AMPs). It has previously been shown that aureolysin cleaves the cathelicidin AMP, LL-37 (Sieprawska-Lupa et al., 2004). Herein we show that cleavage of such agents by extracellular proteases is not confined to LL-37 alone, as we observed increased sensitivity to both indolicidin and histatin-5. During infection, AMPs bind to bacterial cells through cationic interactions at the cell surface, and exhibit antibacterial activity through several mechanisms (Nishi et al., 2004). Therefore, in *S. aureus*, secreted proteases appear to aid in immune evasion by preemptively degrading these agents of innate immunity. We further investigated this assertion by evaluating survival in whole human blood. In addition to complement and AMPs, whole human blood also contains cellular components of immunity. When such profiling was performed we also saw major survival defects in the mutant strain. The immune components of serum and blood are relatively similar, and thus the decreases in survivability observed may result from a lack of AMPs and complement system degradation in the mutant strain. However, in serum at 4h, the mutant produced a 2-fold decrease, while in blood a 4.72-fold decrease was observed at the same time. This suggests that secreted proteases are not only used for protection against the innate immune system, but have a larger role in evasion of cell mediated immunity. This finding is support by the previous work from our group, showing that a functional aureolysin is required to survive phagocytosis by human macrophages (Kubica et al., 2008). Additionally, others have shown that Staphopain B can cleave both CD11b and CD31,

which block the phagocytosis of *S. aureus* by neutrophils and monocytes (Smagur et al., 2009a; Smagur et al., 2009b). Further to this, a number of the secreted proteases have been shown to cleave components of human blood, such as pro-thrombin and pro-uPA, in order to facilitate survival and proliferation (Beaufort et al., 2008; Wegrzynowicz et al., 1980). Collectively, findings from the literature, along with our data presented herein, strongly argue that extracellular proteases play a protective and beneficial role during *S. aureus* infection.

We also observed significant influence of the secreted proteases on *in vivo* pathogenesis. This was first studied for localized infection, using a murine model of abscess formation. Over the course of a 6 day period we observed an approximately 2-fold decrease in virulence for the mutant strain. This decrease is perhaps not as large as would be expected; previous work has shown that 8325-4 *sspA* and *sspB* mutants are more than 3-fold impaired in virulence when using this same model (Shaw et al., 2004). The reason why a complete protease null mutant would display less attenuation than single protease mutants is likely explained by the proteomics works conducted in this study. We observe that, upon deletion of the secreted proteases, the stability of a large number of key virulence factors increases significantly. As such, a protease-null strain actually accumulates more toxins, rather than less. This is particularly important in this model, as key mediators of skin infections, such as α-toxin and the PSMs, are more abundant upon protease inactivation. Therefore, the observed 2-fold reduction is actually quite surprising, given the increased prevalence of wound enhancing toxins. As such, any observed decrease at all upon protease deletion is a major finding, and suggests that the

loss of these enzymes, despite being subverted by other wound-impacting toxins, influences the ability of *S. aureus* to cause localized disease.

When using systemic infection models, we observed further, and profound alterations in virulence that were far in excess of those seen for localized infections. Specifically, the mutant displayed significantly decreased bacterial loads in the lungs (98.79-fold), liver (7.45-fold), heart (5.72) and spleen (3.04-fold). The specific explanations for these findings, are likely complex and multifactorial. However, it has previously been shown that secreted proteases can cleave human α1-proteinase inhibitor (Potempa et al., 1986), α1-antichymotrypsin, the heavy chains of all human immunoglobulin classes (Prokesova et al., 1992), elastin (Potempa et al., 1988), fibrinogen, fibronectin, high molecular weight kininingen and plasminogen (Imamura et al., 2005; Massimi et al., 2002). Cleavage of each of these host proteins aids in tissue invasion, and perhaps explain the decrease in mutant cells in these organs. Further to this, we demonstrate in our proteomics studies that protease null mutant cells have increased decoration of their cell walls, as higher levels of surface proteins were observed upon deletion of proteolytic enzymes. Indeed our preliminary studies have shown that the protease null-strain is significantly more adhesive to surfaces coated with human proteins, including elastin (our unpublished observations). This, coupled with our findings that the protease mutant strain is less resistant to AMPs, and has decreased survival in both serum and whole-blood, might begin to explain this phenotype. As such, one would hypothesize that, upon inoculation into the blood, the protease null strain would immediately display decreased fitness for survival, increased attachment, decreased dissemination and invasion, and an inability to cleave key host proteins for survival, evasion and nutrition.

In stark contrast to this, we in fact observe increased virulence of the protease-null mutant when using mortality as a measure of infection. This finding is of primary importance, and speaks strongly to a major role for the secreted proteases in controlling, and tightly regulating the infectious process. Herein we show major increases in virulence determinant stability upon inactivation of extracellular proteases. Specifically we show that α-toxin, γ-hemolysin, PSMs, LukE, LukAB, PVL and others are all more abundant in protease-null strain. Importantly, each of these factors have been linked to increased virulence and mortality during *S. aureus* infection (Wang et al., 2007; Prajsnar et al., 2008; Bubeck Wardenberg & Schneewind, 2008; Bubeck Wardenberg & Schneewind, 2007; Menestrina et al., 2003; Morinaga et al., 2003; Dumont et al., 2011; Labandeira-Rey et al., 2007; Diep et al., 2007; Loffler et al., 2010 ; Tseng et al., 2009). Thus, despite the loss of extracellular proteases and their affects on the host, other major virulence factors are more prevalent and stable. We propose that, in the absence of proteolytic activity, these other virulence factors exist unchecked, and therefore provide the potential for the aggressive progression of infection observed. Indeed, the overproduction of PVL has frequently been linked to the rapid and increased mortality associated with necrotizing pneumonia (Garnier et al., 2006; Morgan, 2005). Indeed, such a scenario is supported by reports on the role of the cysteine protease, SpeB, from Group A Streptococci (GAS). When levels of this enzyme rise in GAS, the stability and abundance of other virulence factors is reduced, leading to impairments in invasion and virulence (Kansal et al., 2000; Kansal et al., 2003; Chatellier et al., 2000).

Therefore, this presents a scenario where, in addition to their own, independent virulence affecting roles, the secreted proteases exist as key check point enzymes to control the

severity and intensity of infection via modulating the stability of other toxins and virulence determinants. Indeed, one could suggest that the regulatory control of secreted proteases by the *agr* system is no accident, but in fact evolutionary design. In such a scenario, any enhancement of *agr* activity, such as that seen in CA-MRSA strains, would lead to massive toxin production, and death of the host organism. As an opportunistic pathogen, such rapid killing of the host by *S. aureus* is counterintuitive to survival. Therefore, by tying the production of other toxins to extracellular protease expression and activity, one is presented with an inbuilt mechanism to self regulate the overtly harmful, and often lethal effects of secreted toxins on the host, thus tempering and controlling the pathogenic process.

In summary, we demonstrate that the extracellular proteases of *S. aureus* play a variety of key roles in the virulence process. Specifically, they aid in protection against the innate immune system, at both cell-dependent and independent levels. They also strongly impact the progression of both localized and systemic CA-MRSA infections. Finally, and perhaps most importantly, they are key mediators of secreted and cell wall associated virulence determinant stability. Collectively our findings provide a unique insight into the progression of CA-MRSA infections, and the role of secreted proteolytic enzymes.

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Chapter 5: Final Discussion

Final Discussion

NsaRS is a two-component system which we show herein responds to several cell-envelope-targeting drugs. This is not surprising as it is an intramembrane-sensing histidine kinase (IM-HK), which as a family have very small external sensing domain, and most likely do not sense environmental signals, but damage to the cell superstructure (Mascher et al., 2006). NsaRS belongs to the group of IM-HKs that exert their effects by utilizing an adjacent transporter; NsaRS is located upstream of an ABC transporter, NsaAB, which we show in this work to be controlled by this TCS. A microarray on the *nsaS* mutant determined that NsaRS regulates 245 genes under standard conditions. The gene with the largest alteration of expression in the *nsaS* mutant was another ABC transporter, SACOL2525/2526, which homology analysis suggests may transport peptides or lantibiotics.

Our analysis shows that NsaRS and SACOL2525/2526 are expressed during the exponential phase of growth, which declines into stationary phase (Kolar et al., 2011; Chapter 3 of this dissertation). The expression of SACOL2525/2526 seems to be strongly regulated by NsaRS, which is in contrast to NsaAB, which is not temporally regulated, and is seemingly not expressed under standard conditions. Therefore, it is likely that NsaRS utilizes SACOL2525/2526 under standard conditions to exert its effects; with the

expression and role of NsaAB being limited during these times of stasis. In this scenario, NsaAB is only deployed by the cell, in an NsaRS dependent manner, to circumvent damage by cell-wall antibiotics, and as such is only expressed during antibiotic exposure, as is situation for other antibiotic transporters (Muthaiyan et al., 2008; Ouyang et al., 2010; Meehl et al., 2007). This makes SACOL2525/2526 most unusual for a putative drug transporter, in that it appears to have a role within the cell during standard growth conditions, without the input of stress signals.

Each of these 3 elements have been shown to respond to certain cell-envelope antibiotics, with some overlap in the compounds noted (Kolar et al., 2011; Hiron et al., 2011) when one compares the transcription of SACOL2525/2526, *nsaRS* and *nsaAB* in response to cell-envelope-targeting compounds (Figure 38). The expression profiles of *nsaRS* and SACOL2525/2526 are very similar, with 7 of the 10 drugs tested (phosphomycin, ampicillin, penicillin G, D-cycloserine, CCCP, daptomycin and oxacillin) producing an increase in expression for both elements; whilst only 3 drugs (nisin, gramicidin and chloropromazine) show opposing effects. In contrast, *nsaAB* shares only 4 drugs in common with NsaRS: CCCP, daptomycin and nisin produce an increase in transcription, and chloropromazine decreasing expression of both systems. Six drugs (phosphomycin, ampicillin, penicillin G, D-cycloserine, oxacillin and gramicidin) produce contrasting effects between NsaRS and NsaAB. None of the 3 systems respond to every drug tested, suggesting some specificity exists, however the overall expression pattern of NsaRS is more similar to SACOL2525/2526 than NsaAB. This further suggests that NsaRS relies more heavily on SACOL2525/2526 to exert its effects than it does NsaAB, at least in the conditions tested herein. Interestingly, NsaAB does not respond to any of the drugs tested

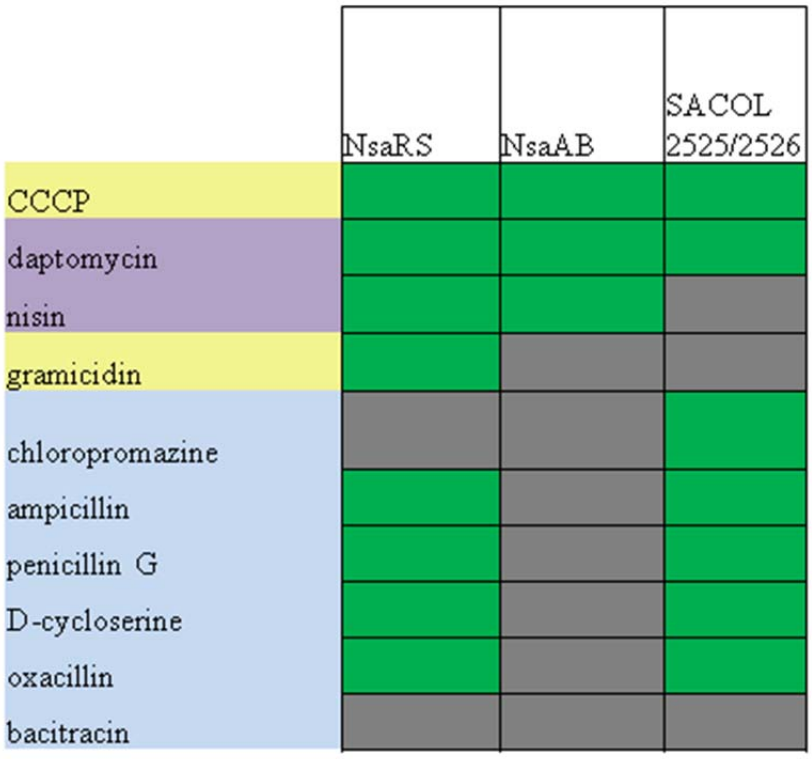

Figure 38. Transcription of *nsaRS*, *nsaAB* and SACOL2525/2526 in response to cell-envelope-targeting drugs. qRT-PCR was performed on either *nsaRS*, *nsaAB* or SACOL2525/2526 in the presence of sub-inhibitory concentrations of cell-wall-targeting (blue) or cell-membrane-targeting (yellow) drugs, along with those that target both (purple). Green boxes signifies an increase in transcription and grey boxes show no change in expression to the corresponding antibiotic.

that target the peptidoglycan synthesis pathway, as only drugs that target the cell membrane increased transcription of this element. In contrast, SACOL2525/2526 responded to all peptidoglycan synthesis targeting agents tested, with the exception of bacitracin, which none of the 3 systems appear to respond to. It has been shown by others that NsaRS and NsaAB are important in bacitracin resistance (Hiron., et al 2011). The inconsistency between this observation and our work may be the result of the concentration used, as these systems seemingly respond to only low levels of bacitracin, whilst in our study, the highest concentration of antibiotic that did not impair growth was utilized. Therefore, SACOL2525/2526 may respond to antibiotics that target peptidoglycan synthesis at high and possibly low concentrations, whereas NsaAB may only respond to cell membrane targeting agents, and low concentrations of certain cell-wall targeting drugs. This may suggest a broader spectrum of response by SACOL2525/2526 than NsaAB. The drugs that produced an opposing effect for SACOL2525/2526 and NsaRS target the membrane, although there are exceptions, as CCCP and daptomycin impact the membrane but also increased the expression of both systems. This is perhaps explained by the fact that daptomycin has also been shown to affect cell-wall biosynthesis pathway, in addition to the membrane (Canepari et al., 1990; Silverman et al., 2003; Cotroneo et al., 2008). When compared to other drugs that dissipate the proton potential, daptomycin takes 10 times longer to exert the same effects, however death still occurs, suggesting that an alteration in the proton potential through targeting of the membrane may not be the leading mode of action (Muthaiyan et al., 2007). Also, CCCP affects ATP levels within the cell, and a downstream affect of this is likely felt on the peptidoglycan synthesis pathway (Dimroth & Cook, 2004). Therefore,

NsaRS may deploy NsaAB in specific situations, such as in response to cell-membrane-targeting drugs (Figure 39). In contrast, NsaRS likely utilizes SACOL2525/2526 in response to drugs that target the peptidoglycan synthesis pathway.

NsaRS has been shown to be important in both bacitracin and nisin resistance by utilizing either SACOL2525/2526 or NsaAB (Kolar et al., 2011; Blake et al., 2011; Hiron et al., 2011). Both NsaRS and SACOL2525/2526 contribute to bacitracin resistance, as *nsaS* and SACOL2525/2526 mutants produced lower spontaneous mutation frequencies in the presence of this compound. Furthermore, Hiron et al. showed that NsaRS, NsaAB, and a second transporter VraDE work in concert to aid *S. aureus* in bacitracin resistance. Specifically, NsaRS utilizes NsaAB for sensing and signaling of bacitracin stress, with VraDE serving as the detoxification module that causes export of intracellular bacitracin. Since SACOL2525/2526 does not appear to be a detoxification module as it is expressed under standard conditions, it may provide a similar role to NsaAB in sensing and signaling. While it is currently unknown how NsaAB aids in sensing cell-wall stress, SACOL2525/2526 may do this by changing the fluidity of the membrane. If SACOL2525/2526 alters the cell-envelope, or specifically the fluidity of the membrane, other IM-HKs may be able to sense this as they respond to alterations at, or in, the membrane, and not an external signal. In addition, each histidine kinase contains a membrane spanning domain, and alterations in the fluidity of the membrane may change the conformational shape of the kinase, resulting in phosphorylation or de-phosphorylation. Therefore, SACOL2525/2526 may respond to cell-wall stress, and as a result, increases the fluidity of the membrane, which may in turn stimulate GraRS, NsaRS or VraRS. We show that SACOL2525/2526 contributes to the resistance against a

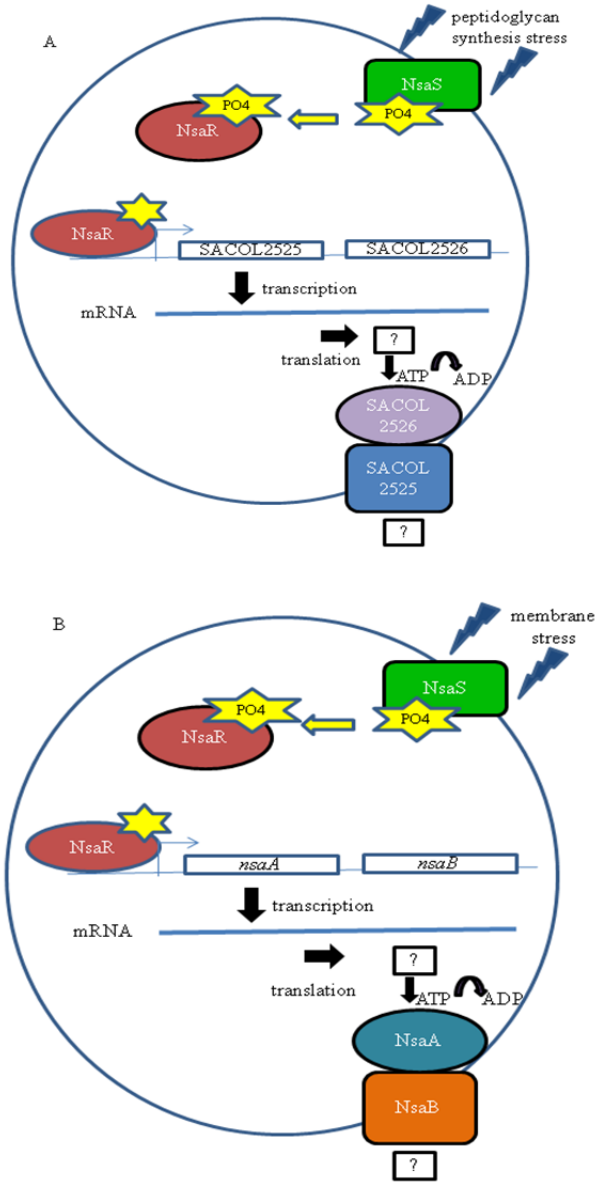

Figure 39. NsaRS regulates NsaAB and SACOL2525/2526 in response to different cell envelope stress. A). NsaAB is not expressed under standard conditions but has been shown to have increased expression in the presence of certain cell-membrane-targeting drugs, as a result of NsaRS action. In this model, NsaRS only use NsaAB in response to certain cell-membrane-targeting drugs. **B)**. SACOL2525/2526 has increased expression in the presence of drugs that targets peptidoglycan synthesis that is at least partly dependent on NsaRS action. NsaRS may utilize SACOL2525/2526 in response to drugs that target the peptidoglycan synthesis pathway.

number of drugs, and may respond to cell wall stress and signal to NsaRS, either by direct interaction; or by altering NsaRS activity by changing membrane fluidity, which would then regulate an exporter for removal of the antibiotic. One might speculate that the efflux pump utilized in the presence of bacitracin may be VraDE, with other transporters involved in this system yet to be identified. Since NsaAB only responds to a few cell-envelope targeting drugs, SACOL2525/2526 may respond to a broader spectrum of peptidoglycan stress, contributing to resistance against many antibiotics. It could also be possible that SACOL2525/2526 senses alterations in the peptidoglycan layer that are not associated with damage from an antibiotic, but instead are centered around cell division. Additionally, NsaRS was identified by Blake et al. as being important in nisin resistance; herein we show that NsaRS upregulates *nsaAB* in the presence of this compound. Therefore, a situation akin to the 6 component resistance mechanism for bacitracin may also occur is response to nisin, where NsaRS uses NsaAB in signaling nisin stress, and regulates an unknown transporter for the detoxification of nisin. Since NsaAB is not expressed under standard conditions it may not contribute to the regular functions of the cell, as SACOL2525/2526 does with membrane fluidity; NsaAB may therefore directly interact with NsaRS to aid in signaling. Some IM-HKs are associated with a membrane protein, which affects the phosphorylation cascade (Jordan et al., 2006). However, it has not been investigated whether the IM-HKs associated with an adjacent transporter may function in a similar fashion; instead of the transporter pumping a substrate in or out, it may have dual roles and also function as a sensor. This direct interaction may alter the phosphorylation state of the histidine kinase and ultimately the response regulator. This further suggests that NsaRS is important in specific antibiotic

resistance, and may utilize either SACOL2525/2526 or NsaAB for sensing and adaption to various compounds.

Although both NsaRS and GraRS positively regulate SACOL2525/2526 under standard conditions, only NsaRS contributes to the increase in transcription in response to cell-wall-targeting chemicals. In a *nsaS* mutant, in the presence of drugs shown to increase transcription, expression of SACOL2525/2526 decreased. Expression levels do not, however, return to those observed in unstimulated cells, suggesting that while NsaRS does positively regulate SACOL2525/2526 in response to cell-envelope-targeting drugs, it is not the only regulator. NsaRS is also responsible, at least in part, for the increased transcription of NsaAB in response to nisin. Again, the transcription of NsaAB decreased in the *nsaS* mutant when exposed to nisin, but did not to levels observed in unstimulated cells. Therefore, NsaRS does regulate NsaAB in response to nisin, but again, other factors are clearly at work. This is consistent with the literature, as *S. aureus* possesses multiple regulators that respond to the same stress. For example, glycopeptide resistance has been shown to be influenced by several regulators, including VraRS, GraRS, WalKR, the carbon catabolite control protein (CcpA), regulators of multidrug transporters (MgrA), the staphylococcal accessory regulator A (SarA), and the alternative sigma factor (σ^B) (Kuroda et al., 2007; Seidl et al., 2006; Meehl et al., 2007; Chen et al., 2006; Truong-Bolduc et al., 2005; Ballal & Manna, 2009; Piriz-Duran et al., 1996; Meier et al., 2007; Bischoff et al., 2004). Several other regulators have been identified for SACOL2525/2526 by the utilization of microarrays, including MgrA, SarA, Rot, ArlRS and σ^B (Bischoff et al., 2004; Cassat et al., 2006; Said-Salim et al., 2003; Liang et al., 2005). MgrA is a DNA-binding protein that has been shown to contribute to antibiotic

resistance by regulating several efflux pumps, including NorB and Tet38, along with an additional 50 genes implicated in transport (Truong-Bolduc et al., 2005). MgrA aids in antibiotic resistance by regulating efflux pumps that extrude drugs out of the cell, and transporters that import nutrients, which may give the cell energy to combat the encountered stress (Loung et al., 2006). Therefore, it is not surprising that MgrA has been shown to regulate SACOL2525/2526, and further suggests that this transporter may be important in antibiotic resistance. SACOL2525/2526 may also contribute to resistance by altering membrane fluidity, which has been shown to change the activity of transporters present in the membrane by increasing or decreasing their efficiency (Gustot et al., 2010). In this situation, the cell would not need to alter the transcription of each transporter, but instead change the expression of SACOL2525/2526 to produce similar effects, leading to a more rapid modification in activity of other transporters. In addition, an increase in membrane fluidity may stimulate other regulators, including the IM-HKs GraRS and NsaRS, which each regulate around 50 transporters independently (Kolar et al., 2011; Herbert et al., 2007). Thus far, only NsaRS has been demonstrated to regulate NsaAB, with other regulators yet to be identified (Hiron et al., 2011; Kolar et al., 2011). One could speculate that since GraRS has been shown to regulate genes important in nisin resistance, it may also regulate NsaAB (Meehl et al., 2007; Li et al., 2007). Therefore, in response to certain cell-envelope-targeting drugs, NsaRS, along with other factors, manipulate the transcription of these 2 ABC transporters independently, and utilizes them to exert effects in coping with stress.

Of the 245 genes modulated by NsaRS, only 34 are negatively regulated, and include those involved in capsule production (*capGOIHED*); the virulence genes β-hemolysin,

hyaluronate lyase, fibronectin binding protein A; and 6 of the 10 major secreted proteases. Secreted proteases have been shown to be inactive until they are secreted, and are processed outside of the cell, with the exception of the Spl proteins (Rice et al., 2001 and Shaw et al., 2004; Reed et al, 2001). Since extracellular proteases are only active outside the cell, it has been suggested that their targets include: 1) other secreted proteins, including virulence factors; 2) cell surface associated proteins; or 3) host proteins. These former 2 facts would allow for the regulation of externalized *S. aureus* proteins, and may suggest a post-translational modification mechanism utilized by *S. aureus* to respond and adapt to a changing environment. Indeed, such a theory was proposed more than ten years ago (Lindsay & Foster, 1999), however only a few studies have investigated this phenomenon (Karlsson et al., 2001;McAleese et al., 2001; McGavin et al., 1997; Zielinska et al., 2011). Whilst a number of reports on secreted proteases are contained within the literature, their findings are contradictory, and the role of these enzymes in protein modification and pathogenesis is currently unclear. In order to determine if the proposed protein modification/regulation theory has validity, a strain was constructed lacking all 10 major secreted proteases to study this phenomenon.

Using proteomics, we show that the secreted proteases do indeed play a major role in the modification of *S. aureus* surface-associated and secreted proteins, as suggested by Lindsay & Foster (Lindsay & Foster, 1999). Analysis of the wild-type strain revealed a decrease in abundance of 24 known proteins in the absence of secreted proteases, including 2 lipases, components of the γ-hemolysin, α-toxin, leukotoxin (LukE), enterotoxin Q, Panton-Valentine leukocidin, the staphylococcal complement inhibitor, enterotoxin K, and secretory antigen SsaA. A similar analysis was also performed for

surface proteins revealing 17 proteins that are cleaved by secreted proteases. These include fibronectin-binding proteins A and B, fibrinogen binding protein, clumping factor A, IsdA, immunodominant staphylococcal antigens A and B, staphylocoagulase, immunoglobulin G binding protein A, elastin-binding protein and a putative surface protein (SAUSA300_0883). This data suggests that secreted proteases act as post-translational regulators of several important secreted and surface-associated proteins.

To determine the effect of this accumulation, or lack of degradation, the mutant and parental strain were evaluated in both localized and systemic models of infection. The protease null mutant produced a decrease in bacterial loads recovered from murine abscesses, suggesting a decrease in virulence of the protease null mutant strain compared to the wild-type. When using systemic infection models, we observed further alterations in virulence of the protease-null strain. Specifically, the mutant displayed significantly decreased bacterial loads in the lung, liver, heart and spleen, again suggesting attenuation of virulence for the mutant. Although there were lower bacterial loads, we did observe an increase in virulence for the protease-null mutant when using mortality as a measure of infection. Collectively, extracellular proteases contribute significantly to *in vivo* pathogenesis, which is an important finding in itself. Although these results seem contradictory, the observed results may be explained by the proteins secreted proteases cleave. The reasoning behind the observed increase in mortality is likely the result of increased stability for a large number of key virulence factors upon protease deletion. Specifically we show that α-toxin, γ-hemolysin, PSMs, LukE, LukAB, PVL and others are all more abundant in the protease-null strain. Importantly, each of these factors have been linked to increased virulence and mortality during *S. aureus* infection (Wang et a.,

2007; Prajnar et al., 2008; Bubeck Wardenburg & Schneewind, 2008; Bubeck Wardenburg et al., 2007; Menestrina et al., 2003; Morinaga et al., 2003; Dumont et al., 2011; Loffler et al., 2011; Tseng et al., 2009). We therefore that, in the absence of proteolytic activity, certain virulence factors remain unregulated at the post-translational level, and may lead to the aggressive progression of infection observed. The decrease in bacterial loads recovered from abscesses and organs may be explained by our proteomics studies, which show that protease null mutant cells have increased decoration of their cell walls, with higher levels of surface proteins observed. It has been shown that *S. aureus* sheds surface-associated proteins for increased dissemination via the action of these proteases. Therefore the protease null mutant likely has a dissemination defect, and cannot reach organs as efficiently as the wild-type. In addition, it has previously been shown that secreted proteases can cleave human α1-proteinase inhibitor (Potempa et al., 1986), α1-antichymotrypsin, the heavy chains of all human immunoglobulin classes (Prokesova et al., 1992), elastin (Potempa et al., 1988), fibrinogen, fibronectin, high molecular weight kininingen and plasminogen (Massimi et al., 2002; Imamura et al 2005); each of which contribute to invasion and pathogenesis. It has previously been shown that aureolysin cleaves the human cathelicidin AMP, LL-37, and we demonstrate here that the cleavage of AMPs is not specific to LL-37, but also includes indolicidin and histatin-5 (Sieprawska-Lupa et al., 2004). AMPs function as part of the host innate immune system, and this suggests that proteases aid *S. aureus* in immune evasion by degrading these elements. To further investigate the effects proteases on components of host immunity, survival in whole human blood and serum was evaluated for the protease-null strain. In both situations, the protease-null strain displayed decreased survivability

when compared to the wild-type. Serum contains complement and AMPs, while whole human blood also contains cellular components of immunity. The observed decreases in survivability may result from a lack of AMP and complement system degradation by the proteases. However, this also suggests that secreted proteases are not only used for protection against the innate immune system, but also impacts evasion of cell mediated immunity. This finding is support by previous work from a number of groups that show secreted proteases play a protective role during *S. aureus* infection, vis the cleavage of host factors (Smagur et al., 2009a; Smagur et al., 2009b; Wegrzynowicz et al., 1980). Therefore, the lack of proteolysis in the mutant strain of host factors including complement, AMPs and components of innate immune system may explain the observed decrease in bacterial loads found in the organs and abscesses. Our study suggests that extracellular proteases are not only virulence factors themselves, but also contribute to pathogenesis by cleavage of *S. aureus* surface-associated and secreted proteins. Collectively this provides insight into the role of secreted proteases on the virulence process by regulating the stability of *S. aureus* virulence factors and cleavage of host immune elements.

Future Directions

While some progress in understanding the role of NsaRS has been made, elucidating its mechanism of action within the cell-envelope stress response will lead to a clearer understanding of its function within the cell. We show that NsaRS regulates SACOL2525/2526 and NsaAB in response to certain cell-envelope targeting drugs, however it is not the only regulator, as transcription of these elements in the *nsaS* mutant

does not completely revert to wild-type levels. Therefore it is possible that NsaR may be able to utilize other phospho-donors in the absence of NsaS, which may account for the transcription of SACOL2525/2526 and NsaAB in the absence of NsaS. To determine if this is the case, NsaR could be purified, incubated with acetyl [P32] phosphate, and if phosphorylation occurs in the presence of this phospho-donor, it could be visualized by SDS-PAGE and Western blotting. In addition, the response regulator could be isolated from wild-type or *nsaS* mutant cells via an incorporated affinity tag after exposure to various growth conditions. The phosphorylation state could then be identified using mass-spectrometry to determine if the response regulator is phosphorylated by its cognate histidine kinase, or another phospho-donor under specific conditions. We have suggested that the alteration in membrane fluidity caused by SACOL2525/2526 may impact the phosphorylation status of IM-HKs, including NsaRS. To determine if this is the case, the phosphorylation state of NsaRS or GraRS could be determined in the SACOL2525/2526 mutant using a similar mass-spectrometry protocol. In addition, a *nsaAB* mutant could be constructed and the impact of this possible signal transporter on NsaRS and GraRS may be evaluated using the same method. We show that in the presence of nisin, NsaRS regulates NsaAB, yet the role of NsaAB in resistance to nisin and other antibiotics remains unknown. Therefore, characterization of a *nsaAB* mutant may reveal its contribution to membrane-targeting antibiotic resistance, as well as the function of NsaRS. The sensitivity of a NsaAB mutant to cell membrane damaging agents may reveal whether it acts as a specific signaling sensor to just bacitracin and nisin, or if its function is more broad in spectrum, and aids NsaRS in resistance to various antibiotics. Such sensitivity could be evaluated utilizing MIC assays, death curves, growth curves

and spontaneous mutation frequency assays. In addition, the mutant was shown to have increased capsule production and a more diffuse cell wall, which may impact the ability to disseminate in a host. By conducting systemic models of infection, the contribution of NsaRS in dissemination and tissue invasion may be elucidated, and provide new insights into *S. aureus* pathogenicity. In addition, analyzing *nsaS* mutant cell wall may provide a better understanding of the function of NsaRS and the genes it regulates. This can be accomplished by muropeptide analysis using liquid chromatography and mass spectrometry, and will not only determine any differences in muropeptide composition, but also O-acetylation of the peptidoglycan layer.

The membrane of the SACOL2525/2526 mutant seems to have decreased fluidity based on our findings herein. To determine the contribution of SACOL2525/2526 to membrane fluidity, the composition of SACOL2525/2526 mutant membrane should be compared to that of the wild-type. This could be determined by lipidomics, where lipids are extracted from the membranes of each strain, and, using mass spectrometery, the composition of fatty acids determined. One may expect the mutant membrane to contain more saturated fatty acids, as these do not contain double bonds and decrease membrane fluidity. This alteration in membrane fluidity may impact the proton potential of the cell, and hence may change the activity of transporters. An alteration of the proton potential may be the reason why a difference in survivability of mutant protoplasts in TSB was observed, which was reversed upon the addition of ions. To determine if this is an alteration in proton potential, the dye carbocyanine could be incubated with the wild-type or SACOL2525/2526 mutant cells, and allowed to cross the membrane, which is dependent on proton potential. This dye can then be measured using fluorescence, and will

determine if the proton potential is altered. In addition, if metabolomics was performed on the SACOL2525/2526 mutant and the metabolite profile compared to the wild-type, this may lead to the identification of the pathways that are being altered in the mutant strain. It is possible that genes in fatty acid synthesis, peptidoglycan synthesis and capsule production would displayed a change in expression, and this may give insight into the function of SACOL2525/2526; and also the substrate transported by this system. Since this substrate remains unknown, and SACOL2525/2526 is similar to other lantibiotic transporters, sensitivity of the mutant should be tested in the presence of other lantibiotics, including mersacidin and cinnamycin. Each of these should be evaluated as they belong to the type B class of lantibiotics, and in this study only nisin, which is a type A lantibiotic, was utilized. The SACOL2525/2526 mutant also possesses increased capsule production and may have decreased membrane fluidity, which may be important in the virulence process. Therefore, evaluation of survival and dissemination of the wild-type and SACOL2525/2526 mutant strains in a systemic model of infection may give insight into whether these attributes contribute to the success of *S. aureus* as a pathogen.

We have shown that secreted proteases are key virulence factors, and contribute to the pathogenicity of *S. aureus* in multiple ways. One significant aspect of our study was the identification of *S. aureus* surface-associated and secreted proteins that are modulated by cleavage via secreted proteases. These studies were performed by growing the cells in TSB, and it may be possible that when *S. aureus* is in a host, different secreted and surface-associated proteins are expressed. Therefore, by growing the cells in human blood or serum, followed by the same proteomics approached already performed herein, other protein targets of these enzymes may be identified. While this demonstrates two

types of proteins cleaved by proteases, surface-associated and secreted, another important factor to pathogenicity is the interaction of *S. aureus* with its host. If a complete list of host proteins cleaved by secreted proteases were produced, a better understanding of the *S. aureus*-host interaction would result, and possibly give insight into future therapeutics. Components of the host immune system that are cleaved by *S. aureus* secreted proteases may be identified by growing the wild-type and protease-null mutant cells in whole human blood, removing cellular components, and performing proteomics on the host proteins in a similar fashion detailed in this work. It would be important to identify not only substrates of the secreted proteases, but also the site of cleavage, as this may reveal the molecular mechanism of specificity and signaling. The cleavage sites of the secreted proteases may be identified using N-terminomics. This procedure tags the N-terminus of proteins, separates the peptides containing the tag, which can then be identified using mass spectrometry. While we demonstrate that a strain inactive in all 10 major secreted proteases displays an abundance of virulence factors from the lack of proteolytic activity, the contribution of individual proteases still remains unclear. As such, proteomics should be performed on single mutations to determine if each secreted protease has specificity for certain proteins. Finally, secreted proteases have been shown to be expressed at different levels between the USA lineage strains, and their contribution may vary depending on the isolate. Therefore, mutants lacking all 10 major secreted proteases could be constructed in different backgrounds to determine if they are utilized in the infection process globally, by performing systemic models of infections. However, each strain has a preference for the infection site, and therefore these newly constructed strains, along with the LAC protease null mutant, should be tested in a variety of

systemic infection models, including endocarditis model and osteomyelitis models. Further to this, the cytolytic effect of mutant strains on neutrophils from humans, mice, rabbits and monkeys should be evaluated to determine the effect of secreted proteases on host specificity and virulence between the lineages.

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Appendix 1

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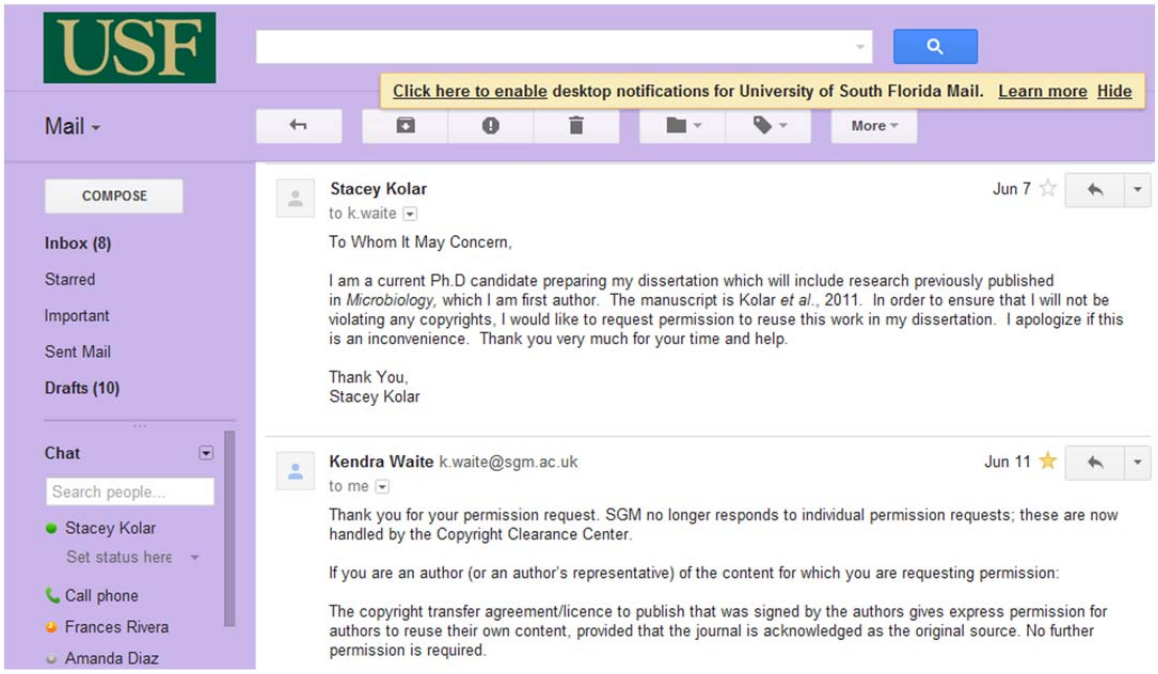

Appendix 1 continued

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NsaRS is a cell-envelope-stress-sensing two-component system of *Staphylococcus aureus*

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Staphylococcus aureus possesses 16 two-component systems (TCSs), two of which (GraRS and NsaRS) belong to the intramembrane-sensing histidine kinase (IM-HK) family, which is conserved within the firmicutes. NsaRS has recently been documented as being important for nisin resistance in *S. aureus*. In this study, we present a characterization of NsaRS and reveal that, as with other IM-HK TCSs, it responds to disruptions in the cell envelope. Analysis using a *lacZ* reporter–gene fusion demonstrated that *nsaRS* expression is upregulated by a variety of cell-envelope-damaging antibiotics, including phosphomycin, ampicillin, nisin, gramicidin, carbonyl cyanide *m*-chlorophenylhydrazone and penicillin G. Additionally, we reveal that NsaRS regulates a downstream transporter NsaAB during nisin-induced stress. *NsaS* mutants also display a 200-fold decreased ability to develop resistance to the cell-wall-targeting antibiotic bacitracin. Microarray analysis reveals that the transcription of 245 genes is altered in an *nsaS* mutant, with the vast majority being downregulated. Included within this list are genes involved in transport, drug resistance, cell envelope synthesis, transcriptional regulation, amino acid metabolism and virulence. Using inductively coupled plasma-MS we observed a decrease in intracellular divalent metal ions in an *nsaS* mutant when grown under low abundance conditions. Characterization of cells using electron microscopy reveals that *nsaS* mutants have alterations in cell envelope structure. Finally, a variety of virulence-related phenotypes are impaired in *nsaS* mutants, including biofilm formation, resistance to killing by human macrophages and survival in whole human blood. Thus, NsaRS is important in sensing cell damage in *S. aureus* and functions to reprogram gene expression to modify cell envelope architecture, facilitating adaptation and survival.

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INTRODUCTION

Staphylococcus aureus is a highly successful and dangerous human pathogen that is a leading agent of both nosocomial and community-associated infections. It has an extremely diverse pathogenesis, causing infection in a plethora of ecological niches within the host. Typically these proceed from a localized site of infection (e.g. wound infection), through to systemic dissemination (bacteraemia) leading to metastatic disease (e.g. osteomyelitis, endocarditis and septic arthritis). The pathogenic success of *S. aureus* is

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ICP-MS, inductively coupled plasma-MS; IM-HK, intramembrane-sensing histidine kinases; MU, 4-methyl umbelliferone; TCS, two-component system; TEM, transmission electron microscopy.

The dataset for the microarray carried out in this study has been deposited in the NCBI GEO database under accession number GSE27061.

Two supplementary tables are available with the online version of this paper.

largely the result of its arsenal of virulence determinants, which include haemolysins, toxins, adhesins and other exoproteins, such as proteases, staphylokinase and protein A (Lowy 1998; Novick, 2006). These damaging virulence factors are subject to multi-level and multi-factorial regulation, both temporally and spatially, in response to the environments encountered during growth. Accordingly, *S. aureus* has a diverse and wide-reaching network of regulatory elements that serve to modulate gene expression (Cheung *et al.*, 1992; Fournier *et al.*, 2001; Giraudo *et al.*, 1994; McNamara *et al.*, 2000; Novick *et al.*, 1995) and bring about infection. These include all of the classical regulators of microbial gene expression, including DNA-binding proteins, regulatory RNAs, alternative sigma factors and two-component systems (TCSs). This latter class is an elegant mechanism of modulating gene expression in response to external conditions. As the name suggests, they are composed of two elements: a membrane-associated histidine kinase and a cytoplasmically located response regulator. These systems function to sense change in the external environment and reprogram gene expression accordingly, to circumnavigate stress.

In *S. aureus*, there are 16 conserved two-component regulators encoded within the various genomes sequenced thus far. Of this number, there are those that control autolysis (ArlRS, LytRS), virulence (SaeRS, AgrCA) and cell wall synthesis/drug resistance (WalKR, GraRS, VraSR) (Brunskill & Bayles, 1996; Delaune *et al.*, 2011; Fournier *et al.*, 2001; Giraudo *et al.*, 1994; Kuroda *et al.*, 2003; Meehl *et al.*, 2007; Novick *et al.*, 1995). There are yet others that sense external iron (HssRS), nitrogen (NreBC) or oxygen (SrrRS) conditions (Schlag *et al.*, 2008; Torres *et al.*, 2007; Yarwood *et al.*, 2001). Recent phylogenetic analysis of bacterial TCSs revealed a conserved group of regulators termed intramembrane-sensing histidine kinases (IM-HKs), which are largely confined to the firmicutes (Joseph *et al.*, 2002; Mascher, 2006). These unique TCSs are characterized as having very small histidine kinase proteins (less than 400 aa) with limited extracellular spanning domains (no more than 25 aa). The IM-HKs can be further divided into two major classes, those that are encoded upstream of a two-component ABC-type transport system, and those that are genetically linked to a conserved membrane protein. The latter class is only found in the low G+C Gram-positive bacteria and bears a HPK7 subfamily protein with multiple membrane-spanning domains. The best studied example of this class of regulators is VraRS from *S. aureus*, which is involved in resistance to cell-wall-targeting agents, and has been shown to protect against cell damage by modulating components of the peptidoglycan biosynthesis pathway (Gardete *et al.*, 2006; Kuroda *et al.*, 2003).

Quite distinct from the VraRS family is the second, and by far the most numerate, class of IM-HKs, which are defined by an HPK3i subfamily protein, containing only two membrane-spanning domains (Mascher, 2006). This family of regulators functions by sensing cell envelope damage, and upregulating adaptation and survival pathways. This is mediated by a variety of processes, including remodelling of cell architecture, and detoxification via transport systems (Mascher, 2006). In *S. aureus* there are two members of this latter family, the relatively well-studied GraRS, and the recently named NsaRS (Blake *et al.*, 2011). GraRS has been described as being important in the resistance of *S. aureus* to antimicrobial peptides by a variety of mechanisms which remodel the cell wall (Herbert *et al.*, 2007; Li *et al.*, 2007). Reference to NsaRS has only recently appeared in the literature, where it was demonstrated to be important for nisin resistance in *S. aureus* (Blake *et al.*, 2011). In this study we present a characterization of the NsaRS system and reveal that, as with other IM-HK TCSs, it responds to disruption of the cell envelope and redirects gene expression to mediate resistance. Additionally, we demonstrate that NsaRS is required for full biofilm formation across a variety of *S. aureus* strains, and contributes to survival during challenge by components of the human innate immune system.

METHODS

Bacterial strains, plasmids and growth conditions. *S. aureus* and *Escherichia coli* strains, along with the plasmids used in this study, are listed in Table 1. The strains were grown as described previously (Shaw *et al.*, 2008). Cultures for growth experiments were obtained by using the following protocol. Overnight *S. aureus* cultures (1 ml) were used to inoculate fresh medium and allowed to grow for 3 h. These exponentially growing cultures were used to seed new medium at OD_{600} 0.05. Test cultures were allowed to grow for the time period required for the experiment. Where used, CLR medium consisted of CL (containing 400 μM magnesium) with the following metals at a final concentration of 0.2 μM: calcium chloride, copper sulfate, ferrous sulfate, manganese chloride, nickel sulfate and zinc sulfate. We deviated from published protocols (Horsburgh *et al.*, 2001a) by omitting molybdenum sulfate and including 0.2 μM cobalt sulfate.

Construction of an *nsaRS* reporter fusion strain. The putative promoter region of the *nsaRS* locus was amplified as a ~1.5 kb fragment (OL802/OL803) from *S. aureus* SH1000 genomic DNA. The reporter fusion was then constructed as described previously (Shaw *et al.*, 2004). Briefly, the PCR fragment was cloned into the Gram-positive suicide vector pAZ106 creating plasmid pSLK1. *S. aureus* RN4220 was transformed with the resulting plasmid, and clones were selected for using TSA containing erythromycin (plasmid-encoded). The resulting strains were confirmed by genomic DNA extraction and PCR analysis. Transformants were used to generate 80α phage lysates for transduction of *S. aureus* SH1000, with clones again confirmed by PCR analysis. This created strain SLK1.

Construction of the *nsaS* mutant strain. We generated an *nsaS* histidine kinase mutation to probe the role of this system as described previously (Shaw *et al.*, 2004). An 800 bp PCR fragment within the *nsaRS* locus was amplified (primers OL837/OL838) and cloned into the pAZ106 suicide vector, generating pSLK2, which was used to transform *S. aureus* RN4220 cells. A representative clone was used to generate a phage lysate for transduction of *S. aureus* SH1000, Newman and UAMS-1790 [USA300-HOU-MRSA (Highlander *et al.*, 2007) cured of pUSA300-HOU-MRSA, a gift from Dr Mark Smeltzer] using 80α. This created strains SLK2, SLK3 and SLK4, respectively. In this mutation, the forward primer (OL837) is located 191 nt downstream of the ATG of *nsaR*, whilst the reverse primer (OL838)

Table 1. Strains, plasmids and primers used in this study

Restriction sites are underlined. Primers are given in 5′–3′ orientation.

Strain, plasmid or primer	Genotype or description	Reference or source
Strains		
E. coli		
DH5α	*ϕ80 Δ(lacZ)M15 Δ(argF-lac)U169 endA1 recA1 hsdR17* (r_K^- m_K^-) *deoR thi-1 ?<?show=sr]supE44 gyrA96 relA1*	Lab stocks
S. aureus		
RN4220	Restriction-deficient transformation recipient	Lab stocks
8325-4	Wild-type laboratory strain *rsbU* mutant	Lab stocks
SH1000	Wild-type laboratory strain *rsbU* functional	Horsburgh *et al.* (2002)
Newman	Wild-type laboratory strain	Lab stocks
UAMS-1790	Sequenced USA300-HOU-MRSA isolate cured of pUSA300-HOU-MRSA	Highlander *et al.* (2007); from M. Smeltzer
SLK1	SH1000 *nsaRS–lacZ* fusion	This study
SLK2	SH1000 *nsaS* mutant	This study
SLK3	Newman *nsaS* mutant	This study
SLK4	USA300 *nsaS* mutant	This study
SLK5	SH1000 *nsaS* mutant complemented with pSLK3	This study
SLK6	Newman *nsaS* mutant complemented with pSLK3	This study
SLK7	USA300 *nsaS* mutant complemented with pSLK3	This study
Plasmids		
pAZ106	Promoterless *lacZ erm* insertion vector	Kemp *et al.* (1991)
pMK4	*cm* shuttle vector	Sullivan *et al.* (1984)
pSLK1	pAZ106 containing a 1.5 kb *nsaRS* promoter fragment	This study
pSLK2	pAZ106 containing an 800 bp mutagenic *nsaS* fragment	This study
pSLK3	pMK4 containing a 2.5 kb *nsaRS* complementation fragment	This study
Primers		
OL802	ATGGGATCCGTCACGTGTGCAGCATATGC	
OL803	ATGGAATTCCAGGCGCGTCATGTTAACAGC	
OL837	ATGGGATCCCATTGGTGTCAAGAAATCCGAA	
OL838	ATGGAATTCCAGTTGTGCTGCAGTAATGGGTG	
OL931	ATGGAATTCGTGCTGTCGTAGCATTCAAAC	
OL1036	CCGCGCACATTTCCCCGAAA	
OL1180	TGGTTACGCAAGGTGTTG	
OL1181	TCAACTGGTGAAGGACTG	
OL1222	GAAGCACAACATGGTGGT	
OL1223	TTGCTGCTACTCCACCA	
OL1224	AGATGAACTCGTCCA	
OL1225	GCACATCTGAAGGCG	
OL1226	CGTCATTGATGAGTGGTG	
OL1227	GGTACACTCCAAACATGC	
OL1333	CGGTGTTATTGTCGTTG	
OL1334	ACCATT0TAACGTTGGCA	
OL1335	TGCATGCCATGTTGCT	
OL1336	TTCACCAGCTCCAACT	

is located approximately one-third of the way into the *nsaS* coding region (~350 nt into the 942 nt gene). Accordingly, the recombination event results in a full-length *nsaR* along with approximately 350 nt of *nsaS* connected to the natural promoter. The remainder of the *nsaS* gene, including the histidine kinase region, is disrupted by the insertion event. To further verify the mutation, we conducted reverse-transcription studies, which confirmed, as predicted, that only ~350 bp of *nsaS* is transcribed with *nsaR*, and that no mRNA containing the last two-thirds of *nsaS* (including the histidine kinase domain) is produced (data not shown). To determine if mutation of *nsaS* affected *nsaR*, we conducted real-time PCR analysis for *nsaR* in both the SH1000 wild-type and its *nsaS* mutant. We observed no alteration in expression of *nsaR* between the two strains (data not shown), indicating that *nsaS* mutation produced no unintended effects, such as compensatory *nsaR* overexpression.

Construction of *nsaRS* complemented strains. In order to construct a complementation vector the entire *nsaRS* locus was amplified (OL802/OL931) as a 2.5 kb fragment. *In silico* analysis reveals an additional gene (SACOL2647) upstream of *nsaR* that likely forms the first coding unit of this operon. Accordingly, the forward primer is located 703 nt upstream of SACOL2647, and the reverse

primer terminates downstream of *nsaS*. This fragment was cloned into the Gram-positive shuttle vector pMK4, creating pSLK3. *S. aureus* RN4220 was transformed with this construct, and clones were confirmed by genomic DNA extraction and PCR analysis, using a combination of gene- and vector-specific primers (OL802/OL1036). A representative isolate was selected to generate an 80α phage lysate, to transduce the SH1000, Newman and UAMS-1790 *nsaS* mutants. Clones were again confirmed by PCR analysis, creating strains SLK5, SLK6 and SLK7, respectively.

Plate-based assay to determine alterations in transcription resulting from external stress. This assay was performed as described previously (Shaw *et al.*, 2008). Briefly, TSA plates were overlaid with TSB top agar (0.7 % w/v) containing X-Gal, and seeded with exponentially growing SH1000 *nsaRS–lacZ* reporter-fusion cells. Sterile filter discs were overlaid onto these plates (three per plate) which were then inoculated with 10 µl of the following stress inducing chemicals: 6 M HCl, 85 % phosphoric acid, 100 % TCA, 88 % formic acid, 0.2 M acetic acid, 6 M sulphuric acid, 6 M nitric acid, 6 M sodium hydroxide, 2 M NaCl, 1 M glucose, 95 % ethanol, 100 % methanol, 100 % 2-propanol, 10 % SDS, 10 % Triton X-100, 10 % Tween-20, 1 M *N*-lauroyl sarcosine, 30 % hydrogen peroxide, 1 M methyl viologen, 1 % menadione, 2 mg pyrogallol ml $^{-1}$, 1 M sodium nitroprusside, 1 M methyl-methane sulfonate, 5 mg penicillin G ml $^{-1}$, 5 mg vancomycin ml $^{-1}$, 2 mg phosphomycin ml $^{-1}$, 5 mg spectinomycin ml $^{-1}$, 100 mg ampicillin ml $^{-1}$, 5 mg nisin ml $^{-1}$, 5 mg gramicidin ml $^{-1}$, 5 mg tetracycline ml $^{-1}$, 50 mg kanamycin ml $^{-1}$, 50 mg neomycin ml $^{-1}$, 10 mg chloramphenicol ml $^{-1}$, 20 mg puromycin ml $^{-1}$, 2 mg bacitracin ml $^{-1}$, 1 mg oxacillin ml $^{-1}$, 1 mg cefotaxime ml $^{-1}$, 5 mg D-cycloserine ml $^{-1}$, 1 mg teicoplanin ml $^{-1}$, 5 mg polymyxin B ml $^{-1}$, 5 mg chlorpromazine ml $^{-1}$, 0.1 mg carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) ml $^{-1}$, 1 mg valinomycin ml $^{-1}$, 2 mg mupirocin ml $^{-1}$, 500 mM diamide, 12.8 mg berberine chloride ml $^{-1}$, 4.21 M peracetic acid, 0.1 M EDTA, 1 M DTT. Plates were incubated for 24 h at 37 °C and screened for a blue halo around the perimeter of the filter discs, indicating expression.

Kirby–Bauer sensitivity profiling of cell-wall-damaging agents. Overnight broth cultures of the SH1000 wild-type and its *nsaS* mutant were diluted 1 : 1000 and added to 5 ml top agar; this was mixed and used to overlay TSA plates. Lawns were allowed to dry for 15 min and sterile filter disks were added to each plate. Penicillin G, oxacillin, ampicillin, cefotaxime, D-cycloserine, phosphomycin, gramicidin, teicoplanin, bacitracin or polymyxin B (all 10 µl) was added to the filter disks from a 5 mg ml $^{-1}$ stock solution. Plates were incubated at 37 °C overnight and zones of inhibition were recorded.

β-Galactosidase assays. Levels of β-galactosidase activity were measured as described previously (Shaw *et al.*, 2006, 2007). Fluorescence was measured using a BioTek Synergy 2 plate reader and calibrated with standard concentrations of 4-methyl umbelliferone (MU). One unit of β-galactosidase activity was defined as the amount of enzyme that catalysed the production of 1 pmol MU min $^{-1}$ (OD_{600} unit) $^{-1}$. Assays were performed on duplicate samples and the mean value was determined. The results presented here are representative of three independent experiments that showed less than 10 % variability.

Transcriptomic studies. The SH1000 wild-type and *nsaS* mutant were grown for 3 h, which corresponds to a window of maximal *nsaRS* expression. After this time RNA was extracted, converted to fluorescently labelled cDNA and hybridized to *S. aureus* COL genome microarrays version 6.0, as described previously (Delgado *et al.*, 2008). Four hybridizations were performed for this study, including a biological replicate and a dye-swap experiment for each replicate to account for dye bias. Spots flagged as empty or bad were excluded and the raw data from each slide were normalized using the LOWESS method, with background correction. Data from the replicates were combined (using the median value) and a one-sample *t*-test was performed. A volcano plot was used with a fold change cut-off of $\geqslant 2$ and a *P*-value of <0.05 to filter the genes that were differentially expressed. ORF IDs in the list of differentially expressed genes were then mapped to *S. aureus* COL IDs where possible. The entire dataset for this study has been deposited in the NCBI GEO database under accession number GSE27061.

Real-time PCR. Quantitative real-time PCR analysis was conducted as described previously (Livak & Schmittgen, 2001; Riordan *et al.*, 2010) using primers specific for alanine dehydrogenase (OL1222/OL1223), Rex (OL1224/OL1225), NarI (OL1226/OL1227), L-lactate dehydrogenase (OL1180/OL1181), *nsaR* (OL1333/OL1334) and *nsaA* (OL1335/OL1336). The control primers were for the 16s rRNA gene, as described previously (Koprivnjak *et al.*, 2006).

Thin-section electron microscopy. Aliquots of bacteria grown in TSB or 10 % NaCl for 3 and 6 h were processed and fixed as described previously (Shaw *et al.*, 2005). Samples were observed and photographed in an FEI Morgagni 268D (FEI)transmission electron microscope with an Olympus SIS MegaView III (ResAlta Research Technologies) camera at the Integrative Biology Electron Microscopy Core Facility at the University of South Florida.

Negative staining electron microscopy. A method of fixing bacteria in osmium tetroxide prior to pelleting was employed to stabilize cell walls. Osmium does not cross-link any protein in culture medium to bacterial cells, and allows preservation of the cell wall structure throughout centrifugation. This permits rinsing to remove culture media proteins prior to preparing cells for negative stain preparation and transmission electron microscopy (TEM) analysis. This method imparts electron density similar to that of uranyl acetate or other similar negative stains commonly used to observe bacteria via electron microscopy. Aliquots of cells grown in TSB for 3 and 6 h were initially fixed in an equal volume of 2 % osmium tetroxide in distilled water for 1 h at 4 °C. Following fixation, cells were rinsed in distilled water and pelleted three times at 5000 r.p.m. for 10 min. Dilutions were performed to obtain approximately 2000–3000 cells per drop, before one drop of each sample was applied to a carbon–Formvar-coated copper grid. Grids were allowed to air dry and were visualized by electron microscopy.

Biofilm formation assay. Biofilm formation assays were performed as described previously (Beenken *et al.*, 2003). The absorbance of samples was read using a BioTek Synergy 2 plate reader.

Metal ion profiling using inductively coupled plasma (ICP)-MS. The SH1000 parental strain, its *nsaS* mutant and the complemented strain were grown in TSB and metal-limiting media for 4 h at 37 °C. Cells were harvested and their pellets were weighed and dried at 80 °C for 12 h. For hot acid digestion, filter pipette tips were rinsed with 2 % nitric acid in ultrapure water. Dried pellets were allowed to incubate for 1 h at room temperature in 500 µl ultrapure nitric acid OPTIMA. Each sample was then transferred to a borosilicate tube and placed in a silicone oil bath at 140–150 °C until ~200–250 µl each sample remained. To each sample, 250 µl of 30 % hydrogen peroxide ULTREX II was added and incubated at room temperature for 5–10 min. Tubes were placed back into the oil bath at 120 °C and the hydrogen peroxide was allowed to evaporate until ~200–250 µl remained. Tubes were removed from the water bath and ultrapure water was added to each sample up to 5 ml. For ICP-MS analysis, standards of the desired elements (1, 5, 10, 25, 100, 250 and 1000 p.p.b.) in 2 % nitric acid were made to create a standard curve. The ICP mass-spectrometer was tuned with 10 p.p.b. of elements $\geqslant$Li for optimization of lenses, and the internal standard used was 10 p.p.b. Indium. A blank (2 % nitric acid) standard curve and the

samples were loaded and processed using the Thermo PlasmaLab software. The amount of each metal was determined based on p.p.b. given and the dried cell weights. The data presented are derived from at least five independent cultures.

Macrophage culture and *S. aureus* intracellular survival assay. The Newman wild-type and its *nsaS* mutant were analysed using a human macrophage model of survival and clearance, as described previously (Kubica *et al.*, 2008). The data presented were generated from six independent experiments. The data were analysed using Student's *t*-test with a 5% confidence limit to determine statistical significance.

Whole human blood survival assay. Exponentially growing cultures of the SH1000 wild-type, *nsaS* mutant and the complemented strain were washed three times with PBS and resuspended in 1 ml whole human blood. The initial inoculum of each strain was also determined at this time by serial dilution and plating on TSA. Blood cultures were incubated at 37 °C with shaking for 3 h, before the c.f.u. ml $^{-1}$ of each strain was determined, again by serial dilution and plating. Data are presented as percentage survival of each strain compared with initial inocula. These experiments were performed with two separate blood samples and represent five independent replicates.

RESULTS

Transcriptional profiling of the *nsaRS* TCS

We initially set out to investigate the role of *nsaRS* by analysing its transcription in the laboratory strain SH1000. A reporter–gene fusion, using a promoterless *lacZ* cassette, was created and monitored for transcription during growth. Analysis revealed that maximal transcription of *nsaRS* occurs during the exponential and post-exponential phases of growth (2–5h) and declines into stationary phase (Fig. 1a). Given that TCSs function by sensing external stimuli, we sought to determine if the transcription of *nsaRS* could be modulated by the presence of chemical stressor compounds. As such, a plate-based assay was employed, as described by Cao *et al.* (2002) and Shaw *et al.* (2008), containing a variety of stress-inducing compounds (see Methods) to mimic natural external conditions. What we found was that a variety of cell-envelope-targeting antibiotics upregulated transcription of *nsaRS*, including ampicillin, phosphomycin, D-cycloserine, gramicidin, nisin, CCCP and penicillin G (data not shown). To quantitatively confirm these observations, we conducted real-time PCR on *S. aureus* SH1000 grown in either TSB or TSB containing subinhibitory concentrations of ampicillin, phosphomycin, penicillin G and nisin (Fig. 1b). When analysed during a window of peak *nsaRS* expression (3 h) we found that phosphomycin had the most dramatic effect, increasing expression by approximately fivefold compared with standard conditions. Further to this, analysis using nisin revealed a 3.3-fold upregulation, whilst studies with ampicillin revealed a twofold increase in transcription. Finally, subinhibitory concentrations of penicillin G revealed no alteration in *nsaRS* expression, perhaps suggesting that greater concentrations of this agent, as used in the plate-based assay, are required to induce expression of this TCS.

NsaRS regulates transcription of the downstream transporter NsaAB in response to nisin stress

As with other members of the IM-HK TCS family, NsaRS is encoded upstream of a transcriptionally distinct locus that specifies an ABC type transporter (termed NsaAB). The existing paradigm of this arrangement is that the transporter aids in detoxification of the cell in response to cell-envelope-damaging agents, and is commonly regulated by its cognate TCS (Staroń *et al.*, 2011). In order to ascertain if NsaRS regulates NsaAB, we conducted real-time PCR

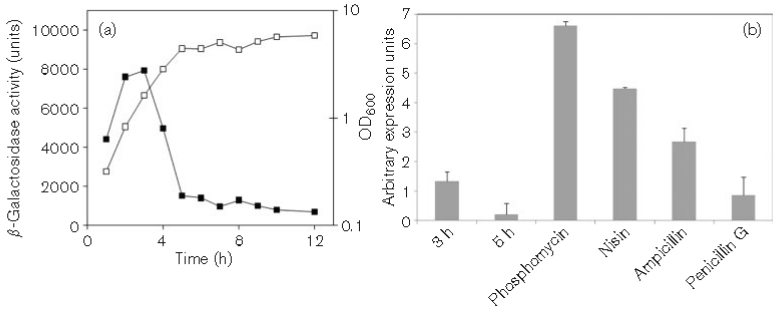

Fig. 1. Transcriptional profiling of *nsaRS*. (a) A reporter *lacZ* fusion was created for *nsaRS* in the SH1000 background, and analysed using 4-MU. Cultures were grown in TSB and OD_{600} (□) and β-galactosidase activity (■) was measured every hour throughout growth. Data shown are from at least three independent cultures that demonstrated less than 10% variability. (b) qRT-PCR was performed for *nsaRS* under standard conditions (3 and 5 h), and also following the addition of subinhibitory concentrations of cell-wall-damaging chemicals (3 h). The data presented are from at least three independent experiments; error bars, SEM.

analysis on *nsaAB* in both the SH1000 wild-type and its *nsaS* mutant during a window of high NsaRS expression (3 h). Interestingly, we saw no alteration in *nsaAB* transcription between these two strains under standard conditions (Fig. 2). Given that these ABC transporters commonly function as detoxification units, we repeated *nsaAB* qRT-PCR analysis with cells grown under conditions shown to induce *nsaRS* expression. Accordingly, SH1000 was grown for 3 h in the presence of subinhibitory concentrations of phosphomycin, ampicillin and nisin, and analysed for *nsaAB* expression. Interestingly, whilst phosphomycin and ampicillin did not affect transcription of *nsaAB*, nisin resulted in an approximately tenfold upregulation of the transporter. To determine if the nisin-dependent stimulation of *nsaAB* expression was mediated via NsaRS, we repeated these analyses with the *nsaS* mutant. Compared with the wild-type strain we observed a twofold reduction in *nsaAB* expression in the *nsaS* mutant when grown in a subinhibitory concentration of nisin. Interestingly, the expression levels of *nsaAB* were still increased fivefold above that of the wild-type grown under standard conditions. This suggests that NsaRS plays a major role in regulating *nsaAB* in response to nisin stress but that other factors may also contribute to this process. These findings are consistent with existing literature on similar systems, which reveals that there is often discrepancy between IM-HK TCS induction and downstream transporter regulation (Staroń *et al.*, 2011).

NsaRS has a significant role in the ability of *S. aureus* to develop resistance to bacitracin

In this study, we show that a number of cell-envelope-targeting antimicrobials upregulate *nsaRS* transcription. Additionally, others have shown the importance of NsaS in the development of resistance to nisin (Blake *et al.*, 2011). Finally, a previous study revealed that an *nsaS* mutant had a fourfold increase in sensitivity to bacitracin (Matsuo *et al.*, 2010). As such, we set out to assess the sensitivity of the *nsaS* mutant to a variety of cell-envelope-targeting antimicrobial compounds. Using a modified Kirby–Bauer disk-diffusion approach we were unable to identify any increase in sensitivity of the mutant compared with the wild-type strain (see Methods for list; data not shown). Further qualification of this observation by determining minimum bactericidal concentration (MBC) also revealed no alteration between SH1000 and its *nsaS* mutant (data not shown). We suggest that these findings are consistent with the existing literature, which demonstrates that mutants of IM-HKs are themselves typically not sensitive to the antibiotics they sense (Staroń *et al.*, 2011). Moreover, it is mutants of the transporters they regulate that demonstrate this type of sensitivity (Staroń *et al.*, 2011). This observation is somewhat surprising, however, given that an *nsaS* mutant was previously reported as being more sensitive to bacitracin (Matsuo *et al.*, 2010). When performing MIC analyses, we found that the *nsaS* mutant was not altered for bacitracin in the SH1000 and USA300 backgrounds. The disparity between our findings, and those of Matsuo *et al.* (2010) is probably explained by bacitracin MICs of the strains chosen; our study used SH1000 and USA300, whilst the previous study used MW2. SH1000 and USA300 have an MIC of 16 $\mu g\ ml^{-1}$ for bacitracin, whilst MW2 has an MIC of 64 $\mu g\ ml^{-1}$. The fact that Matsuo and colleagues reported an MIC of 16 $\mu g\ ml^{-1}$ for the *nsaS* mutant in MW2, which is that observed for our wild-types, suggests that intrinsic drug resistance mechanisms of MW2 may have some impact on this effect.

Given the recent documentation of the role of NsaS in mediating resistance to nisin, we next determined if the *nsaS* mutant was impaired in its ability to mediate resistance to other cell-wall-targeting compounds. As such, we analysed spontaneous mutation frequencies for D-cycloserine, phosphomycin, vancomycin, cefotaxime and bacitracin. When performing 13 replicates from five separate cultures, we were unable to find alterations in the spontaneous mutation frequency for D-cycloserine, phosphomycin, vancomycin or cefotaxime. We did, however, find a major discrepancy between the wild-type strain and *nsaS* mutant for bacitracin. Specifically, we obtained 886 mutants from a combined inocula of 5.77×10^{10} for

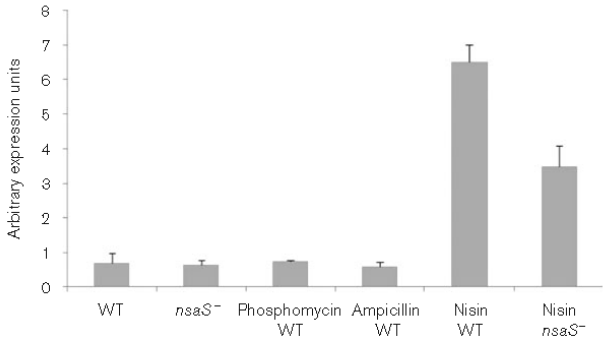

Fig. 2. Role of NsaRS in regulating the transporter *nsaAB*. Transcription of *nsaAB* was initially determined under standard conditions in SH1000 (WT) and its *nsaS* mutant grown for 3 h. These analyses were repeated using TSB containing subinhibitory concentrations of phosphomycin, ampicillin and nisin in SH1000 and its *nsaS* mutant (no change observed for phosphomycin and ampicillin, data not shown). The data presented are from at least three independent experiments; error bars, SEM.

SH1000 when plated on bacitracin agar plates containing 3× MIC. In contrast, we were only able to obtain three mutants from a combined inocula of 3.84×10^{10} for the *nsaS* mutant strain. This results in a spontaneous mutation frequency of 1.53×10^{-8} for SH1000 and 7.81×10^{-11} for the *nsaS* mutant, representing a 196-fold decrease in spontaneous mutation frequency for the mutant strain.

Profiling the NsaRS transcriptome

Given that TCSs function via modulation of gene expression, we set out to analyse those genes that fall either directly or indirectly under the control of NsaRS. As such, we analysed an *nsaS* histidine kinase mutant via microarray analysis. Cultures of both SH1000 and its *nsaS* mutant were grown in TSB for 3 h, which corresponds to a window of maximal *nsaRS* expression. Total RNA was converted to cDNA and analysed using *S. aureus* COL genome microarrays v. 6.0 as described previously (Delgado *et al.*, 2008). A total of 245 genes were found to be altered in transcription at a level of twofold or greater. Of these genes, 34 are negatively controlled by NsaRS (see Supplementary Table S1, available with the online version of this paper), whilst 211 are positively regulated (Supplementary Table S2), indicating that this TCS functions overwhelmingly as a positive regulator of gene expression. Of those genes found to be repressed, a large number were components that had been previously implicated in virulence, including β-haemolysin, hyaluronate lyase, fibronectin binding protein A, seven of the genes required for capsule formation and six of the ten extracellular proteases. This last category of enzymes was amongst the most upregulated in the *nsaS* mutant, with members of the *spl* operon transcribed at levels more than sixfold higher than in the parental strain. Perhaps unsurprisingly, of the 211 genes positively controlled by NsaRS, a large number specify proteins whose function is associated with the cell envelope. These include components involved in cell wall biosynthesis, surface proteins, cell-wall-anchoring enzymes and, most extensively, those classified as encoding transporters. Indeed, 55 such elements were identified of this latter class, encompassing those either previously shown or implicated in the transport of metal ions, amino acids, peptides, sugars and antimicrobial compounds. In addition to this, there were alterations in genes that function in the biosynthesis and metabolism of amino acids, including arginine, lysine, threonine, glutamate, aspartate, phenylalanine and methionine. Another major class of genes with altered expression included those involved in transcriptional regulation, with 14 such elements identified, including SarS and Rex. Other ontologies of function identified included metabolic enzymes, DNA-damage-repair-encoding genes and elements involved in nucleotide biosynthesis. Interestingly, there were also a large number of genes contained within the transcriptome that have previously been shown to function in anaerobic growth, including acetoin reductase, L-lactate dehydrogenase and D-lactate dehydrogenase (Pagels *et al.*, 2010).

Confirmation of transcriptome data was performed using qRT-PCR analysis for selected genes identified in the dataset. Specifically, this was performed for alanine dehydrogenase, Rex, *narI* and L-lactate dehydrogenase, revealing 12.56-, 2.69-, 3.83- and 2.12-fold changes, respectively, in the *nsaS* mutant compared with the wild-type, akin to that seen in the transcriptome.

ICP-MS profiling reveals that NsaRS is required for metal ion transport under limiting growth conditions

Transcriptomic analysis revealed a total of 55 transporters displaying decreased expression in the *nsaS* mutant. Of these elements, at least 20 appear to be specific to metal transportation, particularly with respect to divalent metal ions. Accordingly, we set out to analyse by ICP-MS the ability of the wild-type, *nsaS* mutant and complemented strains to import a variety of these ions. Quintuplet samples of each strain were grown in either TSB or metal-ion-limited media (CLR) supplemented with 400 μM magnesium sulfate and 0.2 μM calcium chloride, copper sulfate, ferrous sulfate, manganese chloride, nickel sulfate, zinc sulfate or cobalt sulfate. Samples were then analysed for the presence of the eight specific ions: Mg, Ca, Fe, Zn, Co, Ni, Cu, and Mn. We found that whilst little variation was observed during growth in replete media, the mutant strain displayed a marked decrease in its ability to import six of the eight metal ions tested when grown in limiting media (Fig. 3). Specifically, we determined a 2-fold decrease in cobalt, a 2.5-fold decrease in iron and calcium, a 3.5-fold decrease in copper and an approximately 5-fold decrease in both zinc and magnesium ions in the *nsaS* mutant strain. These findings suggest a role for NsaRS in metal ion import, particularly with respect to low-abundance growth conditions.

Electron microscopy reveals alterations in the cell envelope of *nsaS* mutant cells

Given that our data thus far suggest a role for the NsaRS TCS in sensing cell envelope stress, we performed TEM analysis of the wild-type, mutant and complemented strains. When cells were grown in TSB for 3 h, we observed that the *nsaS* mutant had a diffuse and less defined/smooth cell wall compared with the parental and complemented strains (Fig. 4). Additionally, negative stain analysis revealed markedly increased encapsulation of the *nsaS* mutant after 3 h of growth, which became even more striking at later time points (data shown from 6 h). This finding is perhaps explained by the observed greater than twofold increase in transcription of seven of the ten genes required for capsule biosynthesis in the *nsaS* mutant. Finally, we also observed decreased transcription of a number of osmoprotectants (*cudT* and *opuCA*) in the *nsaS* mutant, along with a number of solute importers and general permeases. Accordingly, we grew the strains in TSB containing 10% NaCl to assess whether the *nsaS* mutant

Appendix 1 continued

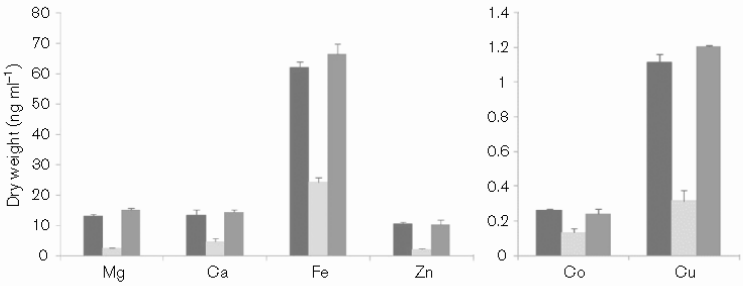

Fig. 3. The transport of divalent metal ions under low-abundance conditions requires a functional NsaRS. The SH1000 wild-type (black), its *nsaS* mutant (light grey) and the complemented strain (dark grey) were grown for 4 h in metal-ion-limiting media (CLR) containing 400 μM magnesium sulfate and 0.2 μM calcium chloride, copper sulfate, ferrous sulfate, manganese chloride, nickel sulfate, zinc sulfate and cobalt sulfate. Cells were harvested and analysed for the presence of the supplementary metal ions using ICP-MS. The data presented are from five independent cultures; error bars, SEM. Two graphs are presented because of the widely differing *y*-axis scales.

displayed impaired growth. Upon analysis we observed only minor defects in the *nsaS* mutant when compared with the wild-type strain (1.5-fold maximal decrease in viability at 6 h growth). However when these same cultures (6 h) were analysed by TEM we observed dramatic alterations in cellular appearance. Following the trend of TSB-grown cultures, mutant cells incubated with 10 % NaCl showed highly irregular and diffuse cell walls compared with similarly grown wild-type and complemented cells. Taken together, these data suggest that NsaRS influences cell envelope architecture, particularly during times of stress.

NsaRS contributes to biofilm formation in a variety of diverse *S. aureus* strains

NsaRS is an orthologue of GraRS in *S. aureus*, another IM-HK TCS involved in sensing disruption and instability of the cell wall (Li *et al.*, 2007; Kraus *et al.*, 2008). Previous work by other groups has revealed that GraRS mutants are impaired in their ability to form a biofilm (Boles *et al.*, 2010; Herbert *et al.*, 2007). In addition to this, we observed that a number of proteins required for biofilm formation display altered transcription in the *nsaS* mutant strain. Specifically, proteases are strongly upregulated in the *nsaS* mutant strain, which, as demonstrated by the recent work

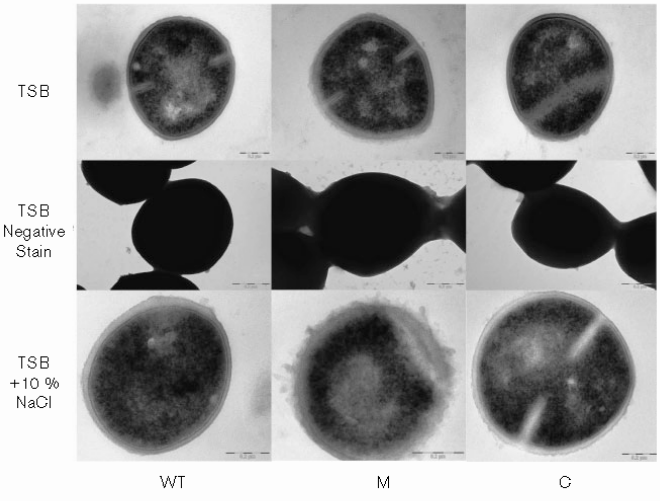

Fig. 4. NsaRS mediates remodelling of cell envelope architecture. SH1000, its *nsaS* mutant and the complemented strain were grown in TSB for thin section (3 h cultures) and negative stain (6 h cultures) analyses. Additionally, thin section images were captured for strains grown in TSB containing 10 % NaCl (6 h). WT, Wild-type; M, *nsaS* mutant; C, *nsaS* mutant strain complemented *in trans* within *nsaRS*. The images presented are representative of more than 10 frames from three independent cultures. Bars, 0.2 μm.

by ourselves and others, would be counterproductive to biofilm formation (Beenken *et al.*, 2010; Boles & Horswill, 2008; Tsang *et al.*, 2008). Additionally, the *nsaS* mutant has reduced expression of a number of anaerobiosis genes, which should also negatively impact biofilm formation (Beenken *et al.*, 2004). Finally, the synthesis of the surface proteins SasG and surface protein A, which have previously been shown to be required for biofilm formation, are also reduced in the mutant (Corrigan *et al.*, 2007; Merino *et al.*, 2009). Analysis of the SH1000 *nsaS* mutant for biofilm formation (Beenken *et al.*, 2003) revealed a 3.7-fold reduction for the mutant strain compared with the wild-type (Fig. 5). This phenotype was reversed when the *nsaRS* locus was introduced into the mutant strain *in trans*. Given the importance of biofilm formation to *S. aureus* disease progression, we assessed whether this phenotype was reproducible in other strains. Accordingly, we tested *nsaS* mutants in strain Newman and a community-acquired MRSA USA300 isolate. Again, in each case we were able to demonstrate a decrease in biofilm formation (Newman, 2.7-fold; USA300, 3.9-fold) for *nsaS* mutant strains, which was restored to wild-type levels upon complementation. Thus, it would appear that NsaRS contributes to biofilm formation across diverse and clonally distinct *S. aureus* strains.

NsaRS functions in protecting *S. aureus* cells during interaction with components of the innate immune system

In order to determine the impact of NsaRS on *S. aureus* virulence, we examined interaction of the *nsaS* mutant with components of the innate immune system. This was performed using a human macrophage model of survival and clearance previously described by us (Koziel *et al.*, 2009; Kubica *et al.*, 2008). As such, the Newman wild-type and *nsaS* mutant were challenged with human monocyte-derived macrophages (hMdMs) at an m.o.i. of 1 : 50, and monitored for up to 5 days. Our use of Newman in these studies rather than SH1000, as in other studies, stems from the observation that SH1000 produces variable and inconsistent results in this model, whilst our experience reveals that Newman generates far more reproducible data (Koziel *et al.*, 2009; Kubica *et al.*, 2008). As such, we observed a consistent and significant reduction in the number of *nsaS* mutant cells recovered from infected hMdMs over the course of the infection period (Fig. 6a). Specifically, we observed a 25-fold reduction in mutant cells after 24 h compared with the parental strain (P=0.060). Furthermore, by the second day post-infection we observed a further 70-fold reduction in *nsaS* mutant cells (P=0.036), and by the third day we were unable to recover any cells from *nsaS*-infected hMdM cells, whilst the parental strain still returned 100–1000 c.f.u. ml^{-1} at this time.

Further to this, we also used a whole human blood survival assay to examine the pathogenic fitness of the *nsaS* mutant. Accordingly, SH1000, its *nsaS* mutant and complemented strain were grown to exponential phase in TSB, before being inoculated into whole human blood. Cells were incubated for 3 h, and the survivability of each strain was determined and converted to per cent survival of the inoculum. An analysis with two different blood samples and five different replicates revealed that the wild-type and complemented strains not only were able to survive incubation in whole human blood but also continued to grow. Specifically, we were able to recover 324 ± 43.29 % (SEM) and 290 ± 31.29 % of the wild-type and complemented strain inocula, respectively, after 3 h incubation (Fig. 6b). In contrast, the *nsaS* mutant demonstrated a 2.4-fold decrease in bacterial load compared with the parental strain, returning only 136 ± 48.88 % of the inoculum upon incubation. Thus it would appear that the *nsaS* mutant strain is not only more rapidly cleared by hMdMs than the wild-type but also impaired in its ability to proliferate in human blood. As such, the NsaRS system seemingly has a significant role during interaction with components of the human innate immune system.

DISCUSSION

In this study, we present a characterization of the TCS NsaRS from *S. aureus*. This TCS belongs to the IM-HK

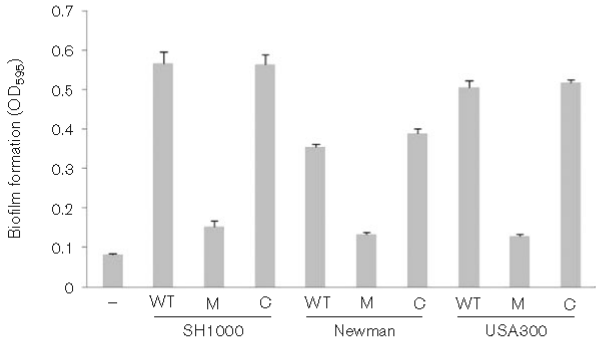

Fig. 5. The NsaRS TCS contributes to biofilm formation in *S. aureus*. *nsaS* mutants in the SH1000, Newman and USA300 background were assayed for biofilm formation. WT, Wild-type; M, *nsaS* mutant; C, *nsaS* mutant strain complemented *in trans* within *nsaRS*. The negative control (–) was the non-biofilm-forming strain 8325-4. Data presented are from at least five independent cultures. Error bars, SEM.

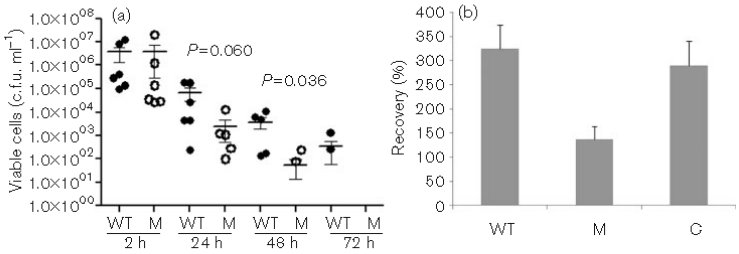

Fig. 6. NsaRS plays an important role during the interaction of *S. aureus* with components of the human innate immune system. (a) Phagocytosis assays were carried out for the Newman strain (●) and *nsaS* mutant (○) at an m.o.i. of 1 : 50 (hMdMs). After challenge, cultures were maintained for the desired time before infected cells were lysed by treatment with ice-cold water. Lysates were serially diluted to determine the presence of viable staphylococci remaining within cells. The data presented were generated from six independent experiments and analysed for statistical significance using Student's *t* test. (b) Per cent recovery of SH1000 (WT), its *nsaS* mutant (M) and complemented strain (C) in whole human blood. Data presented are from two separate blood samples and five individual replicates; error bars, SEM.

family of regulators which commonly modulate cell envelope stability by sensing disruption of the cell wall and membrane (Joseph *et al.*, 2002; Mascher, 2006; Staroń *et al.*, 2011). We present evidence here that NsaRS fulfils a similar function in *S. aureus*, responding to cell envelope damage mediated by a variety of antimicrobial agents. A recent study demonstrated that development of spontaneous resistance to nisin in *S. aureus* is primarily mediated by NsaS (Blake *et al.*, 2011). Nisin is a polycyclic antibacterial peptide produced by *Lactococcus lactis* and is particularly effective against other Gram-positive bacteria. It functions by binding to the carbohydrate–phosphate moiety of the cell wall biosynthesis component lipid II (de Kruijff *et al.*, 2008; Schneider & Sahl, 2010). By binding and sequestering lipid II, nisin blocks cell wall biosynthesis and can lead to delocalization of biosynthetic components and aberrant septum formation (Hasper *et al.*, 2006; Hyde *et al.*, 2006). Additionally, nisin is also believed to use this docking event with lipid II to engineer pore formation in the membranes of target cells. In this study we provide corroborative evidence for the observations of Blake *et al.* (2011) by demonstrating that nisin results in elevated *nsaRS* expression and upregulation of the downstream NsaAB transporter, which is, at least in part, dependent on NsaRS.

Interestingly, in this study we demonstrate that a number of other antimicrobial agents that target the cell wall also upregulate *nsaRS* expression. Specifically, both phosphomycin and D-cycloserine, which affect enzymes involved in intracellular peptidoglycan assembly, upregulate expression of *nsaRS*. The observation with regard to phosphomycin supports that of a previous study, which demonstrates that both *nsaR* and *nsaS* are upregulated in *S. aureus* following phosphomycin challenge (Petek *et al.*, 2010). Furthermore, microarray analysis in this study reveals that NsaRS is responsible for regulation of another TCS, *yhcSR*. Previous studies with cells depleted for *yhcSR* reveal elevated sensitivity to this antibiotic (Sun *et al.*, 2005), suggesting interplay between these two systems when sensing phosphomycin-mediated damage. Further to this, using a plate-based screening assay, we demonstrate that ampicillin and penicillin G, both of which are involved in extracellular peptidoglycan cross-linking, results in increased *nsaRS* expression. This information, in the context of our finding that *nsaS* mutants have an almost 200-fold decreased capacity for spontaneous bacitracin resistance, which blocks dephosphorylation and recycling of undecaprenyl-pyrophosphate, suggests a major role for NsaRS in sensing and responding to alterations in the cell wall. Indeed this latter finding corroborates a recent study which demonstrates that an NsaS mutant of MW2 has a fourfold increase in sensitivity to bacitracin (Matsuo *et al.*, 2010).

Thus it appears that NsaRS has a role in sensing and responding to perturbation of the cell wall biosynthesis pathway at each step in the cycle, from early, intracellular peptidoglycan subunit assembly (phosphomycin and D-cycloserine), to the transport and anchoring of these moieties via lipid II (nisin), followed by extracellular cross-linking of the peptidoglycan subunits (ampicillin and penicillin G), and finally re-entry of the bactoprenol carrier to continue the cycle (bacitracin). Interestingly, *nsaRS* does not appear to respond to every cell-wall-targeting antibiotic, as we observe no upregulation of *nsaRS* or sensitivity of the *nsaS* mutant to other drugs, including vancomycin. Additionally, we find no upregulation of *nsaRS* by, or sensitivity of the *nsaS* mutant to, a variety of antimicrobial peptides (data not shown). We do, however, show that certain cell-membrane-damaging agents, such as gramicidin, nisin and CCCP, upregulate expression of *nsaRS*. Furthermore, a previous study by Muthaiyan *et al.* (2008) reveals upregulation of *nsaR* in response to daptomycin. This indicates that this system responds to

perturbation of not only the cell wall but also the cell membrane. Thus, it appears that NsaRS has a general role in sensing and responding to disruption of the cell envelope in *S. aureus*. The fact that it does not respond to every cell-envelope-targeting compound is consistent with the literature from other IM-HK studies (Staroń *et al.*, 2011) and is perhaps explained in *S. aureus* by a number of other cell membrane or cell wall stress-responding TCSs, such as GraRS, VraRS and WalKR (Li *et al.*, 2007; Kraus *et al.*, 2008; Pietiäinen *et al.*, 2009; Delaune *et al.*, 2011).

In addition to findings focused on cell-envelope-targeting antibiotics, we also present electron microscopy data revealing that *nsaS* mutants have a more diffuse and less defined/smooth cell wall. This disparity from the parental and complemented strains is even further enhanced when cells are subject to salt stress, with *nsaS* mutant cells displaying striking and irregular cell wall appearances. This is perhaps explained by the observation that transcription of a number of osmoprotectants (*cudT* and *opuCA*) is repressed in the *nsaS* mutant, along with a number of solute importers and general permeases. These alterations in cell wall structure can perhaps be further explained by diminished expression in the *nsaS* mutant strain of genes previously shown to function in cell wall biosynthesis (e.g. *tagB*, *fmhA* or *scdA*). Additionally, we observed decreased expression of *glpQ* in the *nsaS* mutant, which is part of the glycerol utilization pathway (Nilsson *et al.*, 1994). Analysis of *S. aureus* cells that are deprived of their ability to utilize glycerol reveals major remodelling of the cell envelope and an accumulation of fatty acids in the cell resulting from a failure to incorporate them into the phospholipid membrane (Ray *et al.*, 1972). Pilot metabolic profiling conducted in our laboratory reveals that a large number of saturated fatty acids do indeed accumulate in *nsaS* mutant cells (S. L. Kolar and L. N. Shaw, unpublished observation).

Further electron microscopy studies using negative staining techniques reveal a significant alteration in encapsulation of the *nsaS* mutant compared with the wild-type and complemented strains. During growth in TSB and high salt concentrations (data not shown) we observed in the mutant strain a significantly increased association of cells in what appears to be an extracellular capsule. This is perhaps explained by microarray analysis, which reveals that *nsaS* mutant cells demonstrate a greater than twofold increase in transcription of seven of the ten capsule-encoding genes. In addition to this, we also noted a number of other genes in the transcriptome that have functions connected with cell envelope formation and architecture. These include membrane proteins, transporters, cell-wall-anchoring proteins and cell-wall-associated proteins. Thus, it is clear that, in addition to its role in sensing perturbation of the cell wall and membrane, NsaRS has a significant effect on remodelling cell envelope architecture during growth of *S. aureus*. This is perhaps not surprising given that many other IM-HK TCSs, including the NsaRS orthologue GraRS, have similar roles (Joseph *et al.*, 2002; Mascher, 2006; Li *et al.*, 2007).

In addition to this, and perhaps equally intriguing, is the number of elements identified in the transcriptome that have a role in transport. We identified that 55 such genes have a known or implied role in the import/export of sugars, metal ions, amino acids and antimicrobial compounds. Given this, it is perhaps not surprising that we demonstrate a role for NsaRS in the process of cellular import. When grown during metal-ion-limiting conditions, the levels of intracellular divalent metal ions in the *nsaS* mutant were significantly reduced. This is in contrast with our finding that the same strain grown in replete media has no such defect. As we have shown that there are alterations in the cell surface of the *nsaS* mutant, it is possible that these findings result from a decreased association of metal ions with the surface of mutant cells. However, this is perhaps unlikely given that under metal replete conditions we do not observe similar decreases in internal metal concentrations. Thus it would appear that NsaRS mediates internal metal ion pools, not during conditions of abundance but more specifically during times of scarcity. As such, *S. aureus* cells may utilize NsaRS as a modulator of low abundance metal ion import during times of starvation. It could be speculated that this would be of particular importance during life within the host, which is known to be an environment of famine for metal ion availability. Indeed, such a mechanism might perhaps contribute to the diminished virulence phenotype we observed during *ex vivo* testing with the *nsaS* mutant.

Of interest from the microarray studies was the observation that other TCSs are controlled by NsaRS. The positive regulation of SACOL0201/0202 by NsaRS is intriguing because, whilst this regulatory element has not formally been studied thus far, it has been suggested to play a role in the anaerobic response of *S. aureus* (Fuchs *et al.*, 2007). As such, the finding that a number of components involved in anaerobiosis have reduced expression in the *nsaS* mutant strain (such as *idhA*, *idh2*, acetoin reductase, *fdhD* and *ald1*) is reasonable, and could perhaps be mediated, at least in part, via control of SACOL0201/0202 by NsaRS. Further to this, we also identified decreased expression of the anaerobiosis repressor Rex in the *nsaS* mutant. This finding is somewhat at odds with our other data, as it could be predicted that a strain displaying reduced Rex expression would also demonstrate increased transcription of anaerobically involved loci (Fuchs *et al.*, 2007; Pagels *et al.*, 2010). However, it is tempting to speculate that SACOL0201/0202 and Rex have antagonizing roles in the process of controlling the anaerobic response, and that NsaRS mediates their interaction via regulatory control. It is also equally possible that NsaRS participates in upregulation of the anaerobic response directly, thus proving dominant to the inhibitory capacity of Rex.

We demonstrate that *nsaS* mutants are impaired in their ability to form biofilms across a variety of *S. aureus* strains. There are a number of regulatory elements that have been suggested as having roles in this process in *S. aureus*, including other TCSs and global regulatory loci such as *agr*,

sarA and σ^B (Beenken *et al.*, 2004). Recent work conducted by ourselves and others (Beenken *et al.*, 2010; Boles & Horswill, 2008; Tsang *et al.*, 2008) has focused not on these regulatory elements but rather on the physiological conditions that facilitate or prevent this aggregate lifestyle. As such, when the NsaRS regulon is analysed, it is perhaps no surprise that a mutant in this system would be impaired in forming and/or maintaining a biofilm. Specifically, the expression of a variety of extracellular proteases (including the V8 protease, staphopain B and a number of Spls), all of which have an important role in the breakdown of extracellular components required for biofilm formation, are increased in the *nsaS* mutant (Beenken *et al.*, 2010; Boles & Horswill, 2008; Tsang *et al.*, 2008). Further to this, the *nsaS* mutant has reduced expression of a number of anaerobiosis genes, which would again be counterproductive to this process (Beenken *et al.*, 2004). Finally, the *nsaS* mutant has diminished synthesis of the surface proteins SasG and surface protein A, which have been shown to be required for biofilm formation (Corrigan *et al.*, 2007; Merino *et al.*, 2009). Collectively, these observations probably explain the role of *nsaRS* in biofilm formation, a phenotype which is broadly maintained across a number of distinct *S. aureus* lineages.

Finally, we present data evaluating the role of *nsaRS* in the virulence of *S. aureus*. Using a human model of macrophage clearance and survival we observed a 25-fold reduction of mutant cells after 24 h, a 70-fold reduction after 2 days and a lack of mutant cells after this time. Thus it would appear the *nsaS* mutant is more rapidly cleared than the wild-type upon interaction with human macrophages. Additionally, when performing survival experiments using whole human blood, we observed a reduction in the capacity of the *nsaS* mutant to proliferate. Whilst the wild-type and complemented strains are able to survive and rapidly proliferate upon challenge, *nsaS* mutant growth seemingly stalls when faced with these hostile conditions. Human blood is replete with a variety of antimicrobial agents, including leukocytes, antimicrobial peptides/proteins and factors of the complement system (Levy, 2000). As such, whole blood survival assays corroborate the *ex vivo* macrophage survival and clearance data, and suggest an important role for NsaRS in mediating resistance of *S. aureus* to the human immune system. Additionally, transcriptome analysis reveals that the expression of a number of elements known to be involved in virulence (such as *essABC*, *sarS*, *spa*, *srtB*, *htrA*, *clpB* and *isaB*) is decreased in the *nsaS* mutant. Conversely a number of known virulence factors were also elevated in the transcriptome (such as the V8 protease, staphopain B, FnpB, Hlb, HysA). It is entirely possible that these increases in transcription present a situation akin to that of a *sarA* mutant, which overexpresses extracellular proteases and has attenuated virulence. Accordingly, this imbalance of virulence determinant expression would likely destabilize the pathogenic response of *nsaS* mutant cells and perhaps explains the phenotypes observed.

In summary, we present a characterization of the IM-HK family member and GraRS orthologue, NsaRS. NsaRS appears to have parallel roles to its better studied counterpart in a number of processes, including biofilm formation and impaired interaction with components of the innate immune system (Li *et al.*, 2007; Kraus *et al.*, 2008; Boles *et al.*, 2010; Cheung *et al.*, 2010). This is probably explained, in part, by the relatedness of these two systems, and the observation that *nsaR* has previously been shown to be subject to twofold repression by GraRS (Herbert *et al.*, 2007). Additionally, we demonstrate a number of unique NsaRS phenotypes, indicating its independent contribution to, and role in, sensing and adapting to cell-envelope-mediated stress. We also show that the NsaRS regulatory system appears to have an important role in the transport of metal ions, and is required for full biofilm formation across a number of genetically diverse *S. aureus* strains. Finally, the defects we present for the *nsaS* mutant when interacting with components of the innate immune system strongly argue for the importance of this TCS in the *in vivo* lifestyle of *S. aureus*.

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