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Characterization of the Lone Extracytoplasmic Function Sigma Factor, σS, and its Role in the Staphylococcus aureus Virulence and Stress Responses

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Characterization of the Lone Extracytoplasmic Function Sigma Factor, σ^S, and its Role in the *Staphylococcus aureus* Virulence and Stress Responses

by

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DEDICATION

I would like to dedicate this dissertation to a number of people who have guided and supported me through this process both professionally and personally. First to my PI Dr. Shaw, thank you for your continued encouragement and for pushing me towards achievements that I did not know were possible. Without your help and guidance, the study of a stealth protein would have gotten the best of me. To my committee members Dr. Riordan, Dr. Turos and Dr. Dao, thank you for all of your time and understanding. Dr. Riordan your knowledge and positive outlook have proven invaluable tools for the completion of my degree and for that I am grateful. Dr. Turos you were always positive and supportive about my research and have taught me the value of incorporating chemical structures in all areas of science, knowledge that I will take with me in my future endeavors. Dr. Dao, you provided me with my first impression of the graduate school here at the University of South Florida and if it were not such an inspiring occurrence I may not have made it to where I am today. To my fellow lab mates, both current and former, this project has extended itself across many lab benches and blown many minds and without your help I may still be there trying to find σ^S. I would like to extend a special thank you to Ronan for his ideas and hard work, which made a number of important experiments possible. To the two most amazing lab partners, Stacey and Frances, I have truly considered you sisters for quite some time and I cannot thank you

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ABSTRACT

Previously our laboratory had identified a novel component of the *Staphylococcus aureus* regulatory network, an extracytoplasmic function σ factor, σ^S, involved in stress response and disease causation. Here we present additional characterization of σ^S, demonstrating a role for it in protection against DNA damage, cell wall disruption and interaction with components of the innate immune system. Promoter mapping reveals the existence of four unique *sigS* start sites, one of which appears to be subject to auto-regulation. Transcriptional profiling revealed that *sigS* expression remains low in a number of *S. aureus* wild-types, but is upregulated in the highly mutated strain RN4220. Further analysis demonstrates *sigS* expression is inducible upon exposure to a variety of chemical stressors that elicit DNA damage, including methyl methanesulfonate (MMS) and ciprofloxacin, as well as those that disrupt cell wall stability, such as ampicillin and oxacillin. *Ex vivo* transcriptional analysis reveals that significant expression of *sigS* can be induced upon phagocytosis by RAW 264.7 murine macrophage-like cells. Regulation of σ^S appears to be unique, as the downstream encoded protein, SACOL1828, seemingly acts as a positive activator, rather than as an expected anti-sigma factor. Using a global transposon screen we have elucidated additional genes implicated in the regulation of *sigS*, including those involved in cell wall stability, cellular detoxification, virulence and DNA base excision repair. Phenotypically, σ^S mutants display sensitivity to a broad

range of DNA damaging agents, such as ultraviolet light, MMS and ethidium bromide. These effects are seemingly mediated via regulation of the purine biosynthesis pathway, as microarray, proteomic and qRT-PCR analysis of σ^S mutants reveal decreased transcription of all genes involved. Enzymatic profiling of PurA involved in adenine biosynthesis, demonstrates decreased activity in the σ^S mutant. Finally, we provide further evidence for the role of σ^S in *S. aureus* pathogenesis, revealing that *sigS* mutants display decreased ability to cause localized infections and are impaired in their interactions with components of the human innate immune system. Collectively, our data argues for the important, and perhaps novel, role of σ^S in the stress and virulence responses of *S. aureus*.

CHAPTER 1:

INTRODUCTION

Staphylococcus aureus. *Staphylococcus aureus* is a non-spore forming, non-motile, coccus, belonging to the low G+C species of Gram-positive organisms (Woese, 1987). The Staphylococcal cell wall has a thick peptidoglycan layer and is cross-linked via a pentaglycine bridge, making it innately resistant to lysozyme, but susceptible to lysostaphin (Schindler and Schuhardt, 1964). Cellular division of this organism occurs in multiple planes leading to the formation of its characteristic grape-like morphology, giving rise to the name staphylococci, which is derived from the Greek word staphyle, meaning bunch of grapes. The word aureus, Latin for golden, refers to the characteristic golden color of *S. aureus* cells, which is a result the carotenoid pigment, staphyloxanthin. This pigment acts to protect *S. aureus* from reactive oxygen species through its antioxidant properties (Clauditz *et al.*, 2006; Liu *et al.*, 2005). A facultative anaerobe, *S. aureus* exists throughout the environment in the soil, air and water. Additionally, isolates can be found colonizing surfaces in hospitals, day care centers, schools, military barracks, locker rooms, weight rooms and even synthetic turf; thereby, creating a substantial disinfection and public health concern (Begier *et al.*, 2004; Hewlett *et al.*, 2009; Montgomery *et al.*, 2010; Morrison-Rodriguez *et al.*, 2010; Oller *et al.*, 2010). Colonization of both inanimate and animate objects can lead to the development of

biofilms, which act as a protective matrix comprised of proteins, DNA and polysaccharides. These biofilms result in long-term contamination and persistence, and the transfer of *S. aureus* to those coming in contact with colonized surfaces (Hall-Stoodley *et al.*, 2004). Further to this, disinfection can be difficult, as a number of commonly used cleaning agents, such as sodium dichloroisocyanurate, hydrogen peroxide and peracetic acid, are ineffective at completely removing *S. aureus* biofilms from surfaces such as glass and stainless steel (Marques *et al.*, 2007). *S. aureus* also exists as a commensal of humans, where it colonizes the nares, axillae, vagina, pharynx and skin (Casewell and Hill, 1986; Noble *et al.*, 1967). Approximately 30% of healthy adults are colonized with *S. aureus* as part of the normal flora of their anterior nares; while transient carriage can occur in as much as 50% of the population, allowing ample opportunity for bacterial dissemination (Kuehnert *et al.*, 2006).

***Staphylococcus aureus* Pathogenesis.** Beyond its ability to exist as a commensal, *S. aureus* is also an opportunistic pathogen capable of causing a wide range of diseases from localized skin and soft tissue infections to life threatening septicemia. First identified in 1880 by the surgeon Sir Alexander Ogston in pus from a surgical abscess, this bacteria has become the most common cause of both hospital- and community-acquired infections, and is believed to be the leading cause of death by a single infectious agent in the United States (Emori and Gaynes, 1993; Klevens *et al.*, 2007; Ogston, 1882). Greater than one-half of all skin infections presented to emergency departments in 2004 was caused by *S. aureus* (Moran *et al.*, 2006). In 2005, *S. aureus* infections resulted in approximately 478,000 hospitalizations in the United States, with greater than 50% of

these attributable to methicillin resistant *S. aureus* (MRSA) strains (Klein *et al.*, 2007). Further to this, the incidence of MRSA nosocomial infections has risen from 2% of staphylococcal infections in 1974, to an overwhelming 64% by 2003 (Klevens *et al.*, 2006a). In 2005, 94,000 people were diagnosed with a first invasive MRSA infection; of these, 20% died even with antibiotic treatments available (Klevens *et al.*, 2007). In that same year, 14 million outpatient visits in the United States were attributed to skin and soft tissue infections (SSTIs) caused by *S. aureus* (Hersh *et al*., 2008). These localized SSTIs, which can take the form of boils, furuncles and abscesses are typical of the primary onset of *S. aureus* disease (Shenoy *et al.*, 2010). *S. aureus* can then enter the bloodstream through dissemination from localized infections or via damaged skin surfaces, where it can cause severe life threatening conditions such as endocarditis and bacteremia, which have mortality rates of 48% or higher, even with aggressive treatment (Bone, 1994; Mortara and Bayer, 1993; Mylotte *et al.*, 1987; Sanabria *et al.*, 1990; Sandre and Shafran, 1996). Upon dissemination into the body, *S. aureus* has the unique ability to cause a variety of metastatic infections, including sinusitis, tonsillitis, otitis, osteomyelitis, pyelonephritis and pneumonia. It also is a leading cause of toxinoses, diseases that are mediated specifically by toxins, such as food poisoning, toxic shock syndrome (TSS) and scalded-skin syndrome. As suggested earlier, *S. aureus* also has the ability to form biofilms, which can develop readily on indwelling devices, such as catheters and pacemakers, ultimately providing a source of infection for other areas of the body. These protective bacterial matrices can be up to 1000 times less susceptible to antibiotics and disinfection, allowing for persistent and recurrent infections (Akagi *et al.*, 2008; Brook, 2009; Chambers *et al.*, 1983; Goundan *et al.*, 2010; Kempker *et al.*, 2009;

Libman and Arbeit, 1984; Musher *et al.*, 1994; Sheehy *et al.*, 2010). *S. aureus* is also a zoonotic agent, as it has the ability to infect animals as well as humans, including horses, cattle, dogs, cats and pigs (Baptiste *et al.*, 2005; de Neeling *et al.*, 2007; Huijsdens *et al.*, 2006; Khanna *et al.*, 2008; Weese *et al.*, 2005; Weese *et al.*, 2006). Of note, it is considered the most common cause of chronic mastitis in U.S. dairy herds. Indeed a bio-economic model from 2009 estimates the annual incidence of infection is as high as 88%, with an estimated net cost of $300 million annually (Halasa *et al.*, 2009). Additional reports demonstrate serious disease in humans resulting from infection by livestock-associated strains, such as ST398. For example, a recent study demonstrated infection of an otherwise healthy 14 year old girl that resulted in lethal necrotizing pneumonia and death within 6 days, underscoring the potential for fatal transmission from animals to humans (Rasigade *et al.*, 2010).

Antibiotic Resistance in *S. aureus*. In 1928, Sir Alexander Fleming discovered the antimicrobial effects of penicillin, which were harnessed for mass use in 1941 (1944). Penicillin is active against Gram-positive bacteria, and functions via a β-lactam ring, which targets bacterial transpeptidase enzymes (also called penicillin-binding proteins, PBP), competitively inhibiting them, and blocking synthesis of the cell wall (Batchelor *et al.*, 1959; Blumberg and Strominger, 1972). The introduction of penicillin allowed once fatal infections to be cured; however, by 1943, 4% of *S. aureus* strains isolated from the wounds of soldiers serving in the Middle East were resistant to this drug (1944). Twelve years later, a staggering 80% of *S. aureus* isolates from London hospitals had acquired resistance to penicillin via plasmid-encoded penicillinases. These enzymes act by

cleaving the β-lactam ring, rendering this antibiotic completely ineffective (Kirby, 1944; Ridley *et al.*, 1970). In 1959, the next generation of β-lactam antibiotics was developed. Methicillin, a semi-synthetic derivative of penicillin, is insensitive to penicillinases due to the presence of a large side chain, creating steric hindrance, and therefore blocking, the β-lactam ring. Within two years, however, methicillin resistant isolates of *S. aureus* were reported (Eriksen, 1961). In these cases, the isolates had acquired the *mecA* gene, which encodes a low-affinity penicillin-binding protein (PBP2'), and, unlike the original PBPs, is not targeted by methicillin (Fuda *et al.*, 2005). The acquisition of this element allowed *S. aureus* to resist not only methicillin, but the entire β-lactam class of antibiotics, including penicillins, cephalosporins and carbapenems (Schaberg, 1994). More recently, the drug of choice for combating severe methicillin-resistant *S. aureus* (MRSA) infections has been the glycopeptide vancomycin. Therapeutically, it has been infrequently used, unless for life-threatening, drug-resistant Gram-positive infections that have failed to improve by treatment with other less toxic antibiotics, as it has the potential to cause severe kidney disease in patients and is poorly absorbed, making it most effective via multiple intravenous dosing (Farber and Moellering, 1983; Griffith, 1981; Levine, 2006; Moellering, 2006). Like the penicillins, it too acts by inhibiting cell wall synthesis; however, it does so by binding the D-Ala-D-Ala motif of the peptidoglycan precursors, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), blocking transpeptidation (Evers *et al.*, 1996). Currently, three classes of vancomycin resistant *S. aureus* exist based on their susceptibilities: vancomycin-intermediate *S. aureus* (VISA), heterogeneous vancomycin-intermediate *S. aureus* (hVISA) and vancomycin-resistant *S. aureus* (VRSA). The first VISA strain was isolated in Japan in 1996 and has

subsequently spread to a number of countries, including the United States where 1 year later, in 1997; two cases were reported (Hiramatsu *et al.*, 1997; Smith *et al.*, 1999). These isolates are also termed glycopeptide-intermediate *S. aureus* (GISA), as they have increased resistance to the entire glycopeptide class of antibiotics. Phenotypically, they display thickening of the cell wall, altered peptidoglycan cross-linking, modified penicillin-binding protein expression and slower growth rates (Garnier *et al.*, 2006; Howden *et al.*, 2004; Maor *et al.*, 2007; Maor *et al.*, 2009). It is hypothesized that this thicker cell wall provides a barrier, preventing the diffusion of vancomycin into the cell (Howden *et al.*, 2010). Those isolates considered to be hVISA have developed due to the selective pressure that exposure to vancomycin presents. During this time a subpopulation of cells that are considered intermediary-resistant begin to outgrow those that are susceptible and continue to do so in the presence of vancomycin leading to a uniform population of VISA clones (Hiramatsu, 2001). Approximately 30 years after the introduction of vancomycin, enterococci were reported that had high-level resistance to glycopeptides (Handwerger *et al.*, 1992). In 1992, *in vitro* and *in vivo* data demonstrated the ability of vancomycin resistance genes from *Enterococcus faecalis* to be acquired by *S. aureus* via horizontal gene transfer; however, it was not until 2002 in Michigan that the phenomenon was observed in a clinical setting (Chang *et al.*, 2003; Noble *et al.*, 1992). These fully resistant strains had acquired the Tn*1546*, transposon-encoded *vanA* gene, making them no longer susceptible to this drug (Clark *et al.*, 2005; Courvalin, 2006; Evers *et al.*, 1996). To date, 11 MRSA strains have been reported that are vancomycin resistant (VRSA) in the United States (Finks *et al.*, 2009; Sievert *et al.*, 2008). The ability of *S. aureus* to rapidly acquire resistance is true for a number of antibiotics

including the quinolones and aminoglycosides (Dornbusch *et al.*, 1990; Hooper, 2002; Wildemauwe *et al.*, 1996). The threat to public health posed by the virulence of *S. aureus* increases as treatment becomes difficult, owing to the continued emergence of multi-drug resistant strains. As the acquisition of resistance mechanisms continues, we are perhaps faced with a return to the pre antibiotic era, where the majority of *S. aureus* bacteremia infections were fatal (Archer, 1998; Emori and Gaynes, 1993; Klevens *et al.*, 2007).

Pathogenic Factors of *S. aureus.* The success of *S. aureus* as a pathogen is due, in part, to its plethora of secreted and surface-associated virulence determinants encoded within its genome. Collectively, these factors play a significant role in disease causation by allowing for adhesion, immune evasion and dissemination. A number of superantigens (SAs), cytotoxins and tissue-degrading enzymes are secreted by *S. aureus*. The SAs include enterotoxins A, B, C and D, which are responsible for food poisoning and toxic shock syndrome (TSS), as well as Exfoliatin A and B, which play a major role in scalded skin syndrome. The hemolysins and leukocidins include alpha- (Hla), beta- (Hlb), delta- (Hld), gamma-hemolysin (HlgA, HlgB and HlgC) as well as LukAB, LukED and PVL; all of which have cytotoxic effects. Alpha-toxin is a pore forming agent capable of targeting erythrocytes, mononuclear immune cells, epithelial/endothelial cells and platelets (Bhakdi and Tranum-Jensen, 1991). Unlike Hla, Hlb, also termed phospholipase C, is specific for sphingomyelin, a phospholipid located in the cell membranes of animals. It does not form pores, but induces hot-cold lysis by causing membrane invagination and lysis at cooler temperatures (Smyth *et al.*, 1975). Discovered

in 1947, Hld is small, heat-stable and active against a broad-range of cell types (Kantor *et al.*, 1972). Gamma-hemolysin, which lyses erythrocytes, and PVL, which damages neutrophils and macrophages, are both bicomponent toxins (Finck-Barbancon *et al.*, 1991; Sugawara *et al.*, 1997; Woodin, 1959, 1960). *S. aureus* also secretes a number of proteases, including the V8 serine protease (SspA), Staphopain A (ScpA), Staphopain B (SspB) and a metalloprotease, aureolysin. These enzymes are important for dissemination of infection, as well as the cleavage of a number of *S. aureus* virulence determinants including alpha-hemolysin, fibrinogen-binding protein, surface protein A and clumping factor (Chan and Foster, 1998; Drapeau, 1978; Karlsson *et al.*, 2001; Lindsay and Foster, 1999; McAleese *et al.*, 2001; McGavin *et al.*, 1997; Rice *et al.*, 2001; Shaw *et al.*, 2004). Other secreted enzymes include thermostable nuclease (*nuc*), which is important for dispersal from biofilms, and degradation of neutrophil extracellular traps (Berends *et al.*, 2010). Coagulase (Coa) and von Willebrand factor binding protein (vWbp) both promote clotting of plasma or blood via the activation of prothrombin, and subsequently the conversion of fibrinogen to fibrin (Cheng *et al.*, 2010).

S. aureus also relies on several cell surface components, also termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The MSCRAMMS are a group of adhesion proteins which aid in establishing initial infection through attachment of the bacteria to host tissues. *S. aureus* contains a number of these, including protein A (Spa), which binds immunoglobulins in order to evade the immune system (Dossett *et al.*, 1969). Additionally, fibronectin binding proteins A and B, as well as the collagen-binding protein (Cna) bind the respective host components that their name

suggests (Jonsson *et al.*, 1991; Menzies, 2003; Patti *et al.*, 1992; Patti *et al.*, 1994; Sinha *et al.*, 2000). Other surface proteins include capsular polysaccharides, such as the Cap5 and Cap8 proteins, which aid in immune system avoidance through inhibiting phagocytic engulfment (O'Riordan and Lee, 2004). Further adhesins in this group include the clumping factor proteins, ClfA and ClfB, which act as receptors for fibrinogen, and, as their names suggest, lead to clumping of bacterial cells (Hawiger *et al.*, 1982). All of these proteins bear an LPXTG motif, and are anchored to the cell surface via the enzyme sortase (Schneewind *et al.*, 1995; Spirig *et al.*, 2011).

The Epidemiology of *S. aureus* Infections. Classically, *S. aureus* diseases have been the result of hospital acquired-MRSA (HA-MRSA) isolates confined to nosocomial settings, and affecting those with immunosuppression, or indwelling medical devices (Thompson *et al.*, 1982). In the United States, the most common HA-MRSA isolates are CDC, pulsed field gel electrophoresis (PFGE) types USA100 and USA200, which typically carry a large SCC*mec*, type I, II or III (McDougal *et al.*, 2003). These HA-MRSA strains generally lead to chronic rather than acute infections and are not typically fatal; however, they display a high level of antibiotic resistance, making treatment of HA-MRSA infections exceptionally difficult (Naimi *et al.*, 2003). Recently, a distinct group of isolates have appeared, termed community acquired-MRSA (CA-MRSA). These strains are hyper-virulent, display multi-drug resistance and are responsible for severe infections in young, healthy individuals with no ties to health care settings (Naimi *et al.*, 2003; Popovich *et al.*, 2008). They are commonly CDC PFGE types USA300 and USA400, and often carry a smaller, and metabolically less expensive SCC*mec* types IV,

V or IV (Tenover and Goering, 2009; Tristan *et al.*, 2007). Core-genome associated toxins are common amongst HA-MRSA and CA-MRSA strains; however, the level and timing of their expression often varies enormously, depending on the isolate. This has significant impact on pathogenicity, as demonstrated by the increased virulence of CA-MRSA isolates (Tenover and Goering, 2009). Compared to major HA-MRSA strains, CA-MRSA isolates display increased production of a class of secreted staphylococcal peptides termed phenol-soluble modulins (PSMs) involved in the recruitment, activation and lysis of human neutrophils (Wang *et al.*, 2007). Mutation of these PSMs in CA-MRSA backgrounds leads to decreased virulence in murine models of sepsis (Wang *et al.*, 2007); thus increased production of these core-genome associated toxins is believed to play a role in the increased virulence of CA-MRSA clones. Additionally, it has been proposed that the enhanced virulence of these strains is a direct result of the acquisition of mobile genetic elements, such as PVL (Boyle-Vavra and Daum, 2007). PVL is a pore-forming toxin comprised of two components (LukS-PV and LukF-PV), and displays a preference for neutrophils (Woodin, 1960). Conflicting reports exist over the role of PVL in enhanced virulence, largely due to the specificity of this toxin to certain cell types (Loffler *et al.*, 2010). In addition to PVL, it is believed that the success of these isolates is due in part to the acquisition of the type I arginine catabolic mobile element (ACME). This genetic element, believed to be the result of horizontal transfer from *S. epidermidis*, contains both a gene encoding a secondary arginine deiminase system and an ABC transporter. While not contributing directly to the pathogenesis of CA-MRSA isolates, this element is believed to allow a selective advantage and facilitate colonization of the human skin and mucosal surfaces (Diep *et al.*, 2006; Diep *et al.*, 2008; Montgomery *et*

al., 2009). While CA-MRSA strains are currently more susceptible to antibiotics than HA-MRSA, resistance is on the rise, with community-associated strains believed to be replacing conventional hospital-associated strains in nosocomial settings (Klevens *et al.*, 2006b; Kourbatova *et al.*, 2005; Popovich *et al.*, 2008; Seybold *et al.*, 2006).

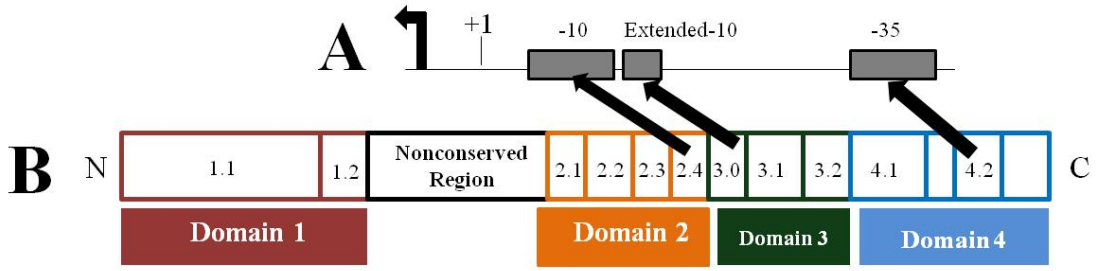
The Genetic Regulation of Virulence Determinant Expression. *S. aureus* regulates virulence determinants in a highly complex, multifactorial manner via a regulatory network made up of a number of modulators, including, two-component systems, DNA-binding proteins, regulatory RNAs and σ factors. Central to the virulence of *S. aureus* is the quorum sensing, two-component system (TCS) *agr* (Janzon *et al.*, 1986; Ji *et al.*, 1995). The *agr* locus is expressed as growth progresses from exponential to stationary phase (Janzon *et al.*, 1986; Ji *et al.*, 1995). During this time, there is a shift in gene expression profiles from surface proteins, to secreted proteases and toxins, in an effort to circumvent nutrient depletion (Janzon *et al.*, 1986; Ji *et al.*, 1995). The accessory gene regulator (*agr*) consists of a locus containing divergent promoters, P2 and P3. The P2 promoter transcribes a TCS (AgrCA), a transmembrane protein (AgrB) and an autoinducing ligand (AIP). AIP is encoded by *agrD*, which is processed and secreted into a cyclic octopeptide. AgrB acts to cleave the carboxy-terminal region of AIP, with additional modification required by type I signal peptidase, SpsB, which acts to remove the N-terminal leader sequence (Kavanaugh *et al.*, 2007). Once a threshold of extracellular AIP is reached, AgrC activates AgrA, which in turn upregulates transcription from the P2 and P3 promoters. The P3 transcript is RNAIII, which acts as the effector molecule, by serving as a regulatory RNA. Other important TCSs include

SaeRS, which regulates a number of extracellular proteins in response to both environmental stimuli and *agr* activity (Giraudo *et al.*, 2003; Goerke *et al.*, 2005; Novick and Jiang, 2003b). Additionally, ArlRS directly regulates certain accessory genes, autolysins, the *sarA*-encoded transcription factor, as well as negatively impacting *agr* expression (Liang *et al.*, 2005).

Further important global regulators include the Sar (staphylococcal accessory regulation) family of homologs. These transcription factors are helix-turn-helix DNA binding proteins and include SarA, R, S, T, U, V, Rot, TcaR and MgrA (Cheung *et al.*, 2004; Cheung *et al.*, 2008; Ingavale *et al.*, 2003; Luong *et al.*, 2003; Manna *et al.*, 2004; Schmidt *et al.*, 2001). These elements have the ability to interact not only with their specific target genes, but also with each other, and other regulators such as TCS and σ factors, leading to a highly complex regulatory network. For example, SarA affects a broad array of genes, including virulence determinants such as *hla*, *spa*, fibronectin-binding protein (*fnb*) and enterotoxin C (*sec*); however, it also regulates genes through an *agr*-dependant manner (Cheung *et al.*, 1995; Chien *et al.*, 1999). SarA is negatively regulated by SarR, and itself negatively regulates SarT (Bronner *et al.*, 2000; Manna and Cheung, 2001). Furthermore, transcription of the *sarA* gene occurs partially through a σ^B-dependent promoter (Deora *et al.*, 1997a).

Primary Bacterial σ Factors. All characterized bacterial systems utilize RNA polymerase (RNAP) to transcribe genes. Core-RNAP is composed of 5 subunits: two identical α subunits, as well as β, β' and ω subunits (Gruber and Gross, 2003; Ishihama,

2000; Paget and Helmann, 2003). Firmicutes also encode a δ subunit that is nonessential, and its role in *S. aureus* is still unclear; however, it has been shown to be important for virulence in *S. agalactiae* (Jones *et al.*, 2003; Pero *et al.*, 1975; Spiegelman *et al.*, 1978). An essential component of RNAP is σ factors, which bind to core-RNAP, creating the holoenzyme, and guide the complex to specific target promoters for transcription (Gruber and Gross, 2003; Ishihama, 2000; Paget and Helmann, 2003). All Eubacterial systems contain an essential housekeeping or primary σ factor, known as σ^{70} or σ^{A}, which directs general transcription in the cell (Burgess and Anthony, 2001; Helmann, 2002; Murakami and Darst, 2003). Binding of the σ factor occurs at amino acids 260-309 of the β' subunit of core-RNAP (Burgess and Anthony, 2001), with weaker binding also mediated by several alternate sites on the β and β' subunits (Anthony and Burgess, 2002; Arthur and Burgess, 1998; Arthur *et al.*, 2000; Gruber *et al.*, 2001; Katayama *et al.*, 2000; Luo *et al.*, 1996). During transcription initiation, the holoenzyme, directed by the σ factor, binds to DNA forming the closed complex. As the DNA is unwound and the initiation site exposed, the DNA/holoenzyme structure forms the open complex, allowing elongation to proceed. After transcription initiation, the σ factor is released from the RNAP/DNA complex, and can be recycled to bind additional core-RNAP complexes to again direct transcription (Craig *et al.*, 1998; Mulligan *et al.*, 1985; Saecker *et al.*, 2002; Sen *et al.*, 2000). The σ^{70} transcription factors contain 4 general protein domains as displayed in Figure 1 (Lonetto *et al.*, 1992; Rodrigue *et al.*, 2006; Wosten, 1998). Region 1 acts to prevent promiscuous binding of the σ factor to DNA without RNAP, and is not conserved among all σ factors (Helmann and Chamberlin, 1988). Region 2, or specifically, 2.1 and 2.2, are required for binding to core-RNAP; while region 2.3 facilitates DNA promoter



C

GROUP	DESCRIPTION	STRUCTURE
1	Housekeeping; Essential	N C
2	Similar to Group 1; Nonessential	N C
3	Niche Specific	N C
4	Extracytoplasmic Function	N C

Figure 1. The σ^{70} Family Protein Domain Organization. (A) A schematic of a typical promoter is shown, where +1 and the bent arrow represent the transcriptional start site and direction of transcription, respectively. The -35, extended -10 and -10 sequences are represented as grey boxes. These regions are recognized by regions 4.2, 3.0 and 2.4 respectively of the sigma factor protein. (B) A detailed depiction of the 4 different regions and subregions that comprise a typical Group 1 (σ^{70}) sigma factor are displayed. (C) Each group of sigma factors have unique characteristics, one of which is the different regions that they possess. Depicted here are the four σ^{70}-like groups and the representative regions they possess. Figure adapted from Rodrigue *et al*., 2006 and Gruber *et al*., 2003.

melting at the -10 promoter site, and region 2.4 recognizes the -10 consensus sequence, TATAAT, which consists of weak A-T bonds, facilitating DNA melting (Aiyar *et al.*, 1994; Gruber and Gross, 2003; Joo *et al.*, 1997; Sharp *et al.*, 1999; Shuler *et al.*, 1995; Tatti and Moran, 1995). The third region of σ^{70} factors interacts with DNA upstream of the -10 region, at extended -10 sequences, which act as alternate promoters and contain a TG motif located at approximately -15/-14 (Bashyam and Hasnain, 2004; Helmann and Chamberlin, 1988; Helmann, 1995; McCracken and Timms, 1999; Moran *et al.*, 1982; Sabelnikov *et al.*, 1995). Finally, region 4 contains a helix-turn-helix motif, which interacts with the TTGACA consensus at the -35 sequence of the promoter (Campbell *et al.*, 2002; Helmann, 1995; Murakami *et al.*, 2002; Travers, 1987).

Alternative σ Factors. In addition to primary σ factors, bacteria contain alternative σ factors, which are non-essential, recognize distinct promoter sequences and are further classified based on structure and function (Burgess and Anthony, 2001; Helmann, 2002; Murakami and Darst, 2003). All eubacterial σ factors are broken down into 5 distinct groups based on homology, the first of which comprises the housekeeping σ^{70} factors described above (Figure 1) (Burgess and Anthony, 2001; Helmann, 2002; Murakami and Darst, 2003). The remaining groups represent alternative σ factors, which are used to respond to an ever changing environment to rapidly initiate changes in transcription of specific subsets of genes. Group 2 of the alternative σ factors are structurally very similar to group 1 and contain regions 1-4; however they are nonessential (Gruber and Gross, 2003; Paget and Helmann, 2003). Group 3 consists of σ factors involved in niche specific regulation and can be further divided based on their regulation of flagellar, heat

shock or sporulation genes. Structurally, members of this group typically lack region 1 (Gruber and Gross, 2003; Paget and Helmann, 2003). Group 4 consists of the ECF or extracytoplasmic function σ factors, and is so named due to an initial observation that this group responds to periplasmic stress involving transport, secretion and environmental stimuli. These proteins structurally contain only the essential σ factor domains, 2 and 4 (Helmann, 2002; Paget and Helmann, 2003). Finally, group 5 contains the σ^{54} family of σ factors which are the most uncommon, and are highly divergent both structurally and functionally. Furthermore, they are believed to have evolved from DNA-binding proteins. They recognize a unique class of promoter elements and require an activator protein for transcription initiation (Beynon *et al.*, 1983; Buck *et al.*, 2000; Merrick, 1983; Studholme *et al.*, 2000). As a result of their ability to rapidly shift gene expression profiles in response to changing environments, bacteria typically encode a number of alternative σ factors.

The Extracytoplasmic Function σ Factors. Group 4 of the alternative σ factors is comprised of the extracytoplasmic function (ECF) σ factors and has become important for the study of pathogenic organisms as there are increasing reports that demonstrate a role for them in virulence (Ando *et al.*, 2003; Bashyam and Hasnain, 2004; Hahn *et al.*, 2005; Le Jeune *et al.*, 2010b; Llamas *et al.*, 2009; Schmitt *et al.*, 1994; Sun *et al.*, 2004). ECF σ factors represent a very distinct subfamily that displays a large degree of diversity within their own group (Helmann, 2002). As stated earlier, these σ factors contain only regions 2 and 4 of σ^{70}, and are characterized by a highly conserved AAC motif in their target promoter –35 region, and a clustering of CGT in the target -10 region. As the

name suggests, a general characteristic of this group is that they respond to periplasmic stress involving transport, secretion and environmental stimuli (Helmann, 2002). They are often the most numerate of the σ factors, for example *Streptomyces coelicolor* encodes 51 ECF σ factors, whilst *Pseudomonas aeruginosa* and *Caulobacter crescentus* contain 19 and 13 respectively (Helmann, 2002). Classically, they are co-transcribed with a downstream gene that acts as a negative regulator, and they often recognize their own promoter in a positive feedback loop (Helmann, 2002). The negative regulator, or anti-σ factor, is typically membrane bound, with an extracellular receiving domain and intracellular inhibitory domain that acts to sequester free σ factor and inhibit its activity (Helmann, 2002). When a signal is received, the anti-σ factor is cleaved by a cascade of proteolysis achieved through membrane proteases. Specifically, the stress signal is received by a site-1 membrane protease, which leads to initial cleavage of the anti-σ factor. This protease activity then triggers cleavage by a site-2 membrane protease, releasing the σ factor; thereby, allowing it to bind core-RNAP and upregulate gene expression (Figure 2) (Helmann, 2002; Hughes and Mathee, 1998; Raivio and Silhavy, 2001; Staron *et al.*, 2009; Yoshimura *et al.*, 2004). This level of post-translational regulation allows for the rapid shift in gene expression that is necessary to survive harsh, ever-changing external conditions.

ECF σ factor groups. The characteristics described above are typical for a number of ECF σ factors; however, this sub-family continues to expand as further factors are identified. As such, the large diversity that exists for these proteins continues to be magnified; leading to a recent subdivision of the ECF σ factors into 43 subgroups

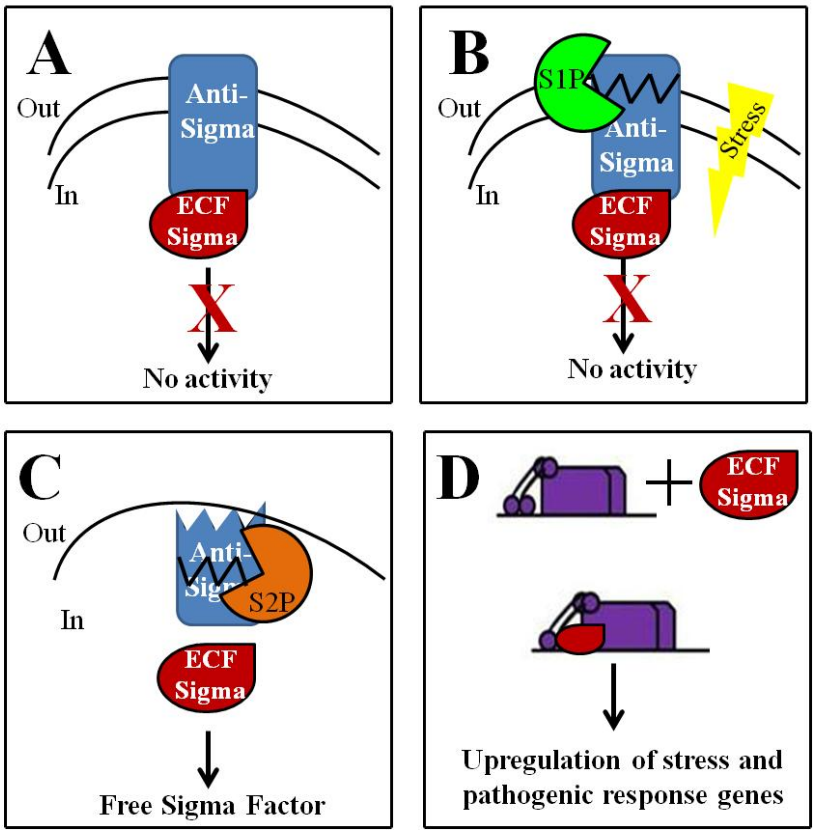


Figure 2. A Generalized Mechanism of the Classical ECF Sigma Factor Post-Translational Regulation. This figure depicts the proteolytic cascade that ensues after induction via environmental stress. (**A**) In the absence of stress, the ECF-sigma factor is bound and held inactive by the anti-sigma factor located in the cell membrane. (**B**) Once the bacteria encounters stress, the membrane associated site 1 protease (S1P) receives a signal and subsequently cleaves the anti-σ factor. (**C**) This proteolytic action then triggers the site 2 protease (S2P) to cleave the anti-sigma factor at an additional site, thereby freeing the σ factor. (**D**) The sigma factor can now bind core-RNA polymerase and direct it to upregulate a specific subset of genes to circumvent the stress.

(ECF01-ECF43) based on phylogeny (Staron *et al.*, 2009). Groups ECF01-ECF04 comprises the RpoE-like σ factors, which are characterized by RpoE of *Escherichia coli*. RpoE upregulates genes involved in the heat shock response, and is triggered by misfolded proteins in the periplasm or outer membrane (Figure 3) (Dartigalongue *et al.*, 2001). The anti-σ factor, RseA, encoded by the second gene in the *rpoE* four-gene-operon, contains one transmembrane domain (TMD), and an N-terminus that is localized in the cytoplasm for σ factor binding (Missiakas *et al.*, 1997). The remaining genes in the operon, *rseB* and *rseC* encode for a negative regulator of RpoE, located in the periplasmic space, and an inner membrane protein that is a positive regulator of RpoE, respectively (Missiakas *et al.*, 1997). The proteolytic cascade that acts to release RpoE begins by the site 1 protease, DegS, being activated by misfolded outer membrane porins such as OmpA or OmpC (see Figure 3). DegS cleaves the periplasmic domain of RseA between Val^{148} and Ser^{149} (Alba *et al.*, 2002; Kanehara *et al.*, 2002; Walsh *et al.*, 2003). This process initiates the site 2 protease (S2P), RseP, which cleaves the anti-σ factor in its transmembrane domain (Ala^{108}-Cys^{109}) (Akiyama *et al.*, 2004). RpoE is released by degradation of the cytoplasmic domain of RseA, via ClpXP or other ATP-dependent proteases (Chaba *et al.*, 2007). Other σ factors in these groups include σ^W from *Bacillus subtilis*, which is involved in the response to alkaline shock (Wiegert *et al.*, 2001), and AlgU from *Pseudomonas aeruginosa* that mediates the mucoid phenotype as a result of regulation of alginate biosynthesis genes (Jones *et al.*, 2010; Staron *et al.*, 2009).

Groups ECF05-ECF10 make up the FecI-like σ factors, the majority of which are involved in iron acquisition (Staron *et al.*, 2009). As displayed in Figure 4, FecI is an

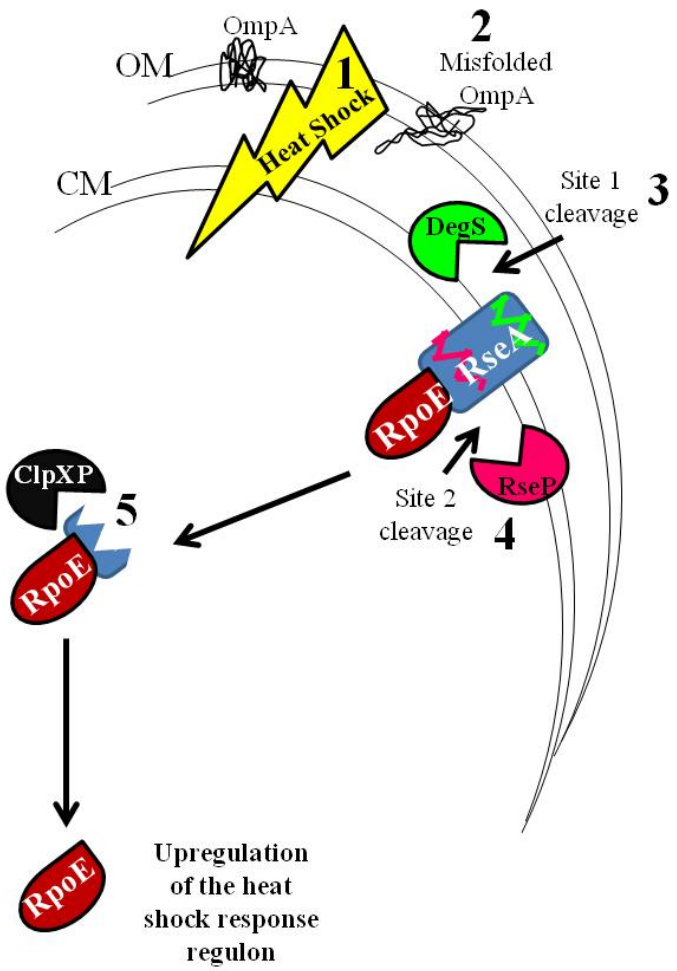


Figure 3. The Mechanism of RpoE Activation in *E. coli*. This figure depicts the regulatory cascade by which RpoE from *E. coli* is activated. **(1)** Exposure to high temperatures can lead to **(2)** misfolded outer membrane porins such as OmpA, which **(3)** triggers DegS to cleave the periplasmic domain of the anti-sigma factor RseA. **(4)** The site 2 protease, RseP, then cleaves the anti-sigma factor in the transmembrane domain. **(5)** Final cleavage is achieved via ClpXP and other cytoplasmic ATP-dependant proteases

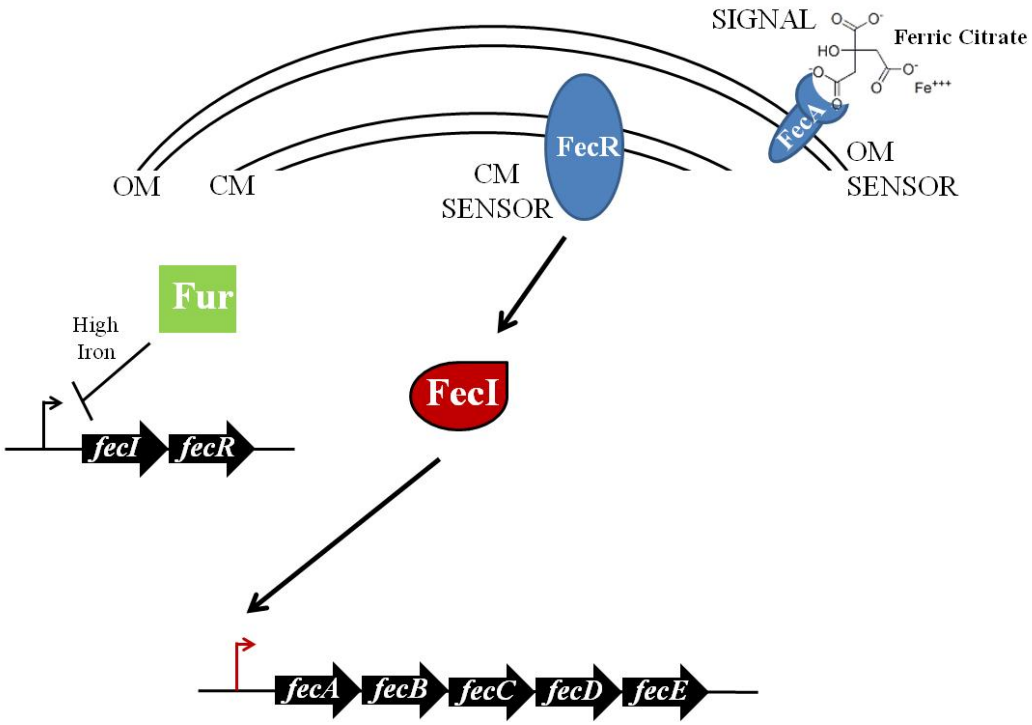


Figure 4. The Mechanism of FecI Regulation in *E. coli*. This figure depicts the post-translational activation of FecI in *E. coli*. Regulation begins when a signal (Ferric Citrate) is received by an outer membrane sensor, FecA. This interaction then leads to a conformational change that signals to FecR to activate FecI, which can upregulate the ferric citrate transport system. During times of high iron levels, transcription of *fecIR* is repressed by Fur.

ECF σ factor from *E. coli* that responds to ferric citrate, and subsequently upregulates the ferric citrate transport system genes *fecABCDE*. This is accomplished through a regulatory cascade that begins with the binding of ferric citrate to an outer membrane receptor, FecA. This binding leads to a conformational change that signals to FecR, a cytoplasmic membrane protein, to activate FecI in the cytoplasm through binding of the N-terminus of FecR to region 4 of FecI (Enz *et al.*, 2000; Mahren *et al.*, 2002; Stiefel *et al.*, 2001). Transcription of *fecIR* is activated by iron deficiency through derepression by the iron responsive regulator Fur (Braun *et al.*, 2003). Additional σ factors of this group include HasI from *Serratia marcescens* responsible for haem acquisition, PbrA from *Pseudomonas fluorescens* and PvdS from *P. aeruginosa*, both of which are involved in the response to iron starvation (Leoni *et al.*, 2000; Sexton *et al.*, 1995; Staron *et al.*, 2009).

Groups ECF11-ECF15 are largely distinct subfamilies, yet a number of them respond to oxidative stress, and, more intriguingly, differ significantly from the classical definition of ECF σ factors, in that they are all cytoplasmic sensing regulators. Examples from these groups include RpoE from *Rhodobacter sphaeroides*, which is inhibited by binding to a soluble zinc-dependant anti-σ factor, ChrR. This inhibition is reversed upon activation by a redox sensing region in the C-terminal domain of ChrR in response to singlet oxygen ($^1O2$) (Campbell *et al.*, 2007; Dufour *et al.*, 2008). Additionally, Ecf of *Neisseria gonorrhoeae* is involved in response to oxidative damage through regulation of *msrAB*, which encodes a methionine sulfoxide reductase (Gunesekere *et al.*, 2006). Further to this, σ^R from *S. coelicolor*, like the majority of σ factors in these groups,

responds to oxidative stress, specifically to the thiol-specific oxidant, diamide. Regulation of σ^R is accomplished by a thiol-disulfide redox sensing anti-σ factor, RsrA. Disulfide stress, more specifically redox changes within the cell, leads to the formation of disulfide bonds within RsrA, leading to the release of bound σ^R (Paget *et al.*, 2001a).

The remaining classes, ECF16-ECF43, make up a diverse set of σ factors, each with unique features that distinguish them from other members of the group. Of note, these groups typically deviate from each other as well as the classical model of ECF σ factor biology. A specific example includes ECF32 which make up the HrpL-like ECF σ factors. This group completely lacks an anti-σ factor and is instead regulated at the transcriptional level through two-component systems (Merighi *et al.*, 2003; Nizan-Koren *et al.*, 2003). Other examples that differ from the classical model of function and regulation include, RpoE of *Xylella fastidiosa*, which is involved in the response to heat-shock, and, unlike other ECF σ factors, does not autoregulate itself, but negatively regulates its own activity through upregulation of its cognate anti-σ factor encoded immediate downstream of the σ factor (Figure 5). This is achieved through upregulation of an internal promoter located downstream of *rpoE* (da Silva *et al.*, 2007). An interesting mode of regulation that exists for σ^R of *Streptomyces coelicolor* is that the protein exists in two forms (Figure 6). A stable form of σ^R is constitutively expressed and has a half life upwards of 70 min. In the absence of stress, σ^R is bound to its anti-σ factor RsrA; however, during exposure to thiol-oxidative stresses, σ^R is released and allowed to upregulate gene expression. In a positive feedback loop, σ^R autoregulates transcription from its own promoter; however, an isoform from an upstream start codon is produced.

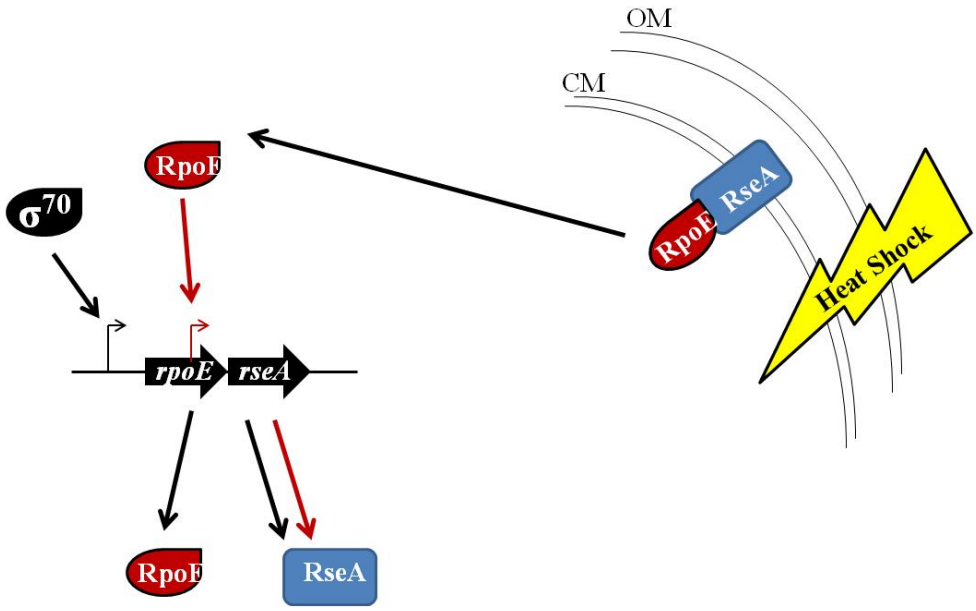


Figure 5. The Negative Feedback Loop of Regulation by RpoE in *Xylella fastidiosa*. In the absence of a stress, the *rpoE/rseA* operon is transcribed by the housekeeping sigma factor, σ^{70}. In the presence of high temperatures, RpoE is released from the anti-sigma factor and upregulates genes involved in protection against this stress. It also upregulates a promoter located internal to the *rpoE/rseA* operon (shown in red), creating a negative feedback loop as it increases the cellular concentration of its cognate anti-sigma factor.

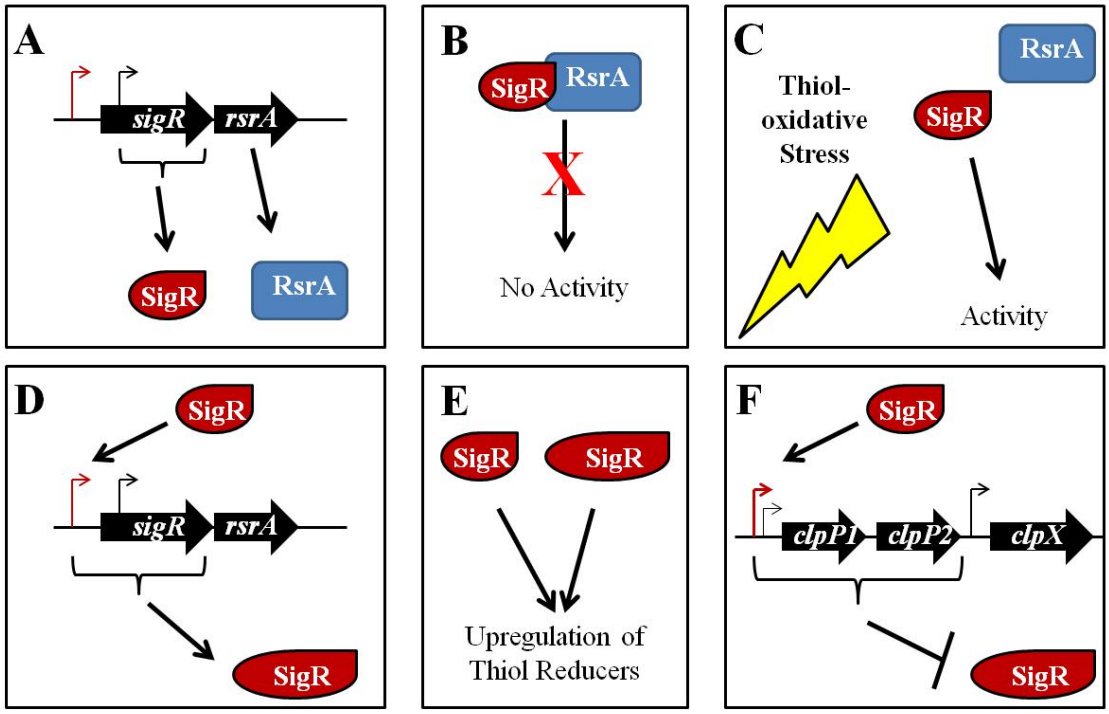


Figure 6. The Mechanism of SigR Regulation in *Streptomyces coelicolor*. (A) SigR and its anti-sigma factor are constitutively expressed under standard conditions. (B) In the absence of a stress, SigR is held inactive by its anti-sigma factor, RsrA. (C) In the presence of thiol-oxidative stress, SigR is released from RsrA. (D) SigR autoregulates its own transcript from an upstream promoter (shown in red) creating a larger isoform of itself. (E) Both forms of the sigma factor act to upregulate genes in the SigR regulon, including thiol reducers. (F) An additional set of genes in the SigR regulon are proteases. The longer, unstable form of SigR is more readily cleaved by these proteases; thereby creating a negative feedback loop.

This unstable form of σ^R contains a 55 amino acid N-terminal extension, which allows it to be targeted for degradation by ClpP1/P2 proteases, thereby shortening its half life to 10 min. Subsequently, a negative feedback loop is formed through upregulation of the protease transcript by σ^R (Kim *et al.*, 2009). Furthermore, σ^F of *Caulobacter crescentus* is involved in protection against oxidative stress during stationary phase growth, and works with a cognate anti-σ factor that contains 6 TMDs. This is yet another example that shows that while the classical model of 1 TMD for anti-σ factors is true for a majority of these proteins; wide variability exists, and is thus not a reliable tool for identifying possible anti-σ factors (Alvarez-Martinez *et al.*, 2006).

Sigma Factors of *S. aureus*. *S. aureus* is an exceedingly virulent and successful pathogen that manages to fine tune its gene expression with only 4 σ factors. The primary σ factor, σ^A, is the essential housekeeping σ factor and acts to transcribe genes for basic metabolic functions (Deora and Misra, 1995, 1996). As with the majority of Firmicutes, *S. aureus* possesses an alternative σ factor, σ^B, which is the general stress response regulator. In *S. aureus* it is involved in virulence and controls the expression of a variety of genes in response to challenging environmental conditions, such as exposure to alkaline and oxidative stress, and also heat shock (Deora *et al.*, 1997a; Horsburgh *et al.*, 2002; Kullik and Giachino, 1997; Pane-Farre *et al.*, 2006; Shaw *et al.*, 2006). This σ factor recognizes a unique consensus sequence of GTTT followed by a 12-17 nt spacer and then GGGTAT, and has a regulon of 198 genes that it up-regulates, and 53 that it down-regulates (Bischoff *et al.*, 2004; Gertz *et al.*, 2000). Included in its regulon are a number of genes involved in the defense against oxidative stress, intermediary

metabolism components and signaling pathway elements (Bischoff *et al.*, 2004). A number of genes in the σ^B regulon are directly up-regulated by it, such as *coa*, (Nicholas *et al.*, 1999) *sarA* (Bayer *et al.*, 1996) and *sarS* (Tegmark *et al.*, 2000); however, a significant number are controlled indirectly, as part of the global regulatory network of *S. aureus*. Phenotypically, mutation of *sigB* leads to decreased pigmentation, as staphyloxanthin, a carotenoid produced by *S. aureus*, is upregulated by this σ factor. The role of σ^B in the pathogenesis of *S. aureus* has previously been studied and it is believed to play a role in systemic infection, likely because it has a number of virulence factors under its control (Bischoff *et al.*, 2004; Jonsson *et al.*, 2004). The third σ factor, σ^H, is an alternative σ factor demonstrating homology to σ^H, of *Bacillus subtilis*. Overexpression of σ^H leads to upregulation of competence genes, and it has recently been demonstrated to have a role in the integration and excision of prophages (Morikawa *et al.*, 2003; Tao *et al.*, 2010). Finally, a discovery by our laboratory demonstrated the existence of a fourth σ factor, σ^S, belonging to the ECF-family (Shaw *et al.*, 2008).

Project Aims. Bacteria typically possess, on average, 6 ECF σ factors within their genomes; however, unlike many other organisms, σ^S is the lone ECF σ factor of *S. aureus* (Helmann, 2002; Staron *et al.*, 2009). Classically, ECF σ factors display maximal activity when cells are challenged by environmental stress, including disulfide, oxidative and cell wall destabilizing conditions, or exposure to antimicrobial agents. This inducibility alleviates the metabolic burden of being constitutively expressed when not required (Helmann, 2002; Staron *et al.*, 2009). The subsequent response leads to upregulation of a unique subset of genes to protect the cell. As σ^S is the only member of

the ECF family in *S. aureus*, we hypothesize that it likely plays an integral role in protecting this organism against a variety of stresses. Therefore in order to understand its role, our initial focus will be to investigate *sigS* regulation through promoter mapping, and analysis of its expression both under standard growth conditions and in the presence of chemical stressors. As previous work by our lab suggests a role for σ^S in the virulence response of *S. aureus* to systemic disease, we will also examine the inducibility of *sigS* during challenge by components of the innate immune system. ECF σ factors are often involved in complex regulatory cascades that include regulation at the transcriptional, translational and post-translational levels (Bastiat *et al.*, 2010; Brooks and Buchanan, 2008; Dona *et al.*, 2008; Mazurakova *et al.*, 2006; Staron *et al.*, 2009; White *et al.*, 2010; Zhang *et al.*, 2002). Therefore, we will elucidate the extant regulatory network for control of *sigS* expression through the use of a global transposon screen. Regulation of ECF σ factors is often controlled by a co-transcribed, downstream anti-σ factor. The *sigS* locus contains a candidate gene, SACOL1828, located 112 bp 3', and is conserved across all sequenced strains of the staphylococci. We hypothesize that SACOL1828 plays a role in the regulation of σ^S as an anti-σ factor, which we will investigate in this work.

In order to ascertain the role that this regulator plays in the cell, we will analyze the ability of the *sigS* mutant to survive under a number of stress conditions, including those that lead to induction of its expression based on our transcriptional analysis. As ECF σ factors bind core-RNAP and target it to specific promoters to upregulate genes in response to particular stresses, we will examine this function for σ^S via transcriptomic and proteomic analyses. Furthermore, there is mounting evidence to support the notion

that ECF σ factors often play a significant role in the virulence of pathogenic organisms including *Mycobacterium tuberculosis, Pseudomonas aeruginosa,* and *Enterococcus faecalis* (Ando *et al.*, 2003; Bashyam and Hasnain, 2004; Hahn *et al.*, 2005; Le Jeune *et al.*, 2010a; Llamas *et al.*, 2009; Schmitt *et al.*, 1994; Sun *et al.*, 2004). Our group has previously demonstrated a role for σ^S in the pathogenesis of *S. aureus* septic arthritis infection. As such, we will examine the ability of strains bearing *sigS* mutations to survive both during growth in whole human blood and post phagocytosis, which are important mechanisms for *S. aureus* to establish infection, and thus any defects observed might explain the avirulence of *sigS* mutants. Due to the ability of *S. aureus* to cause a wide range of infections, we will also analyze the ability of the *sigS* mutant to cause disease during localized infections using murine models of wound formation.

Collectively, the focus of this dissertation is to characterize σ^S**;** specifically the role it plays within the cell and how it contributes to *S. aureus* disease causation.

CHAPTER 2:

MATERIALS AND METHODS

Bacterial strains, Plasmids and Growth Conditions. All buffers were prepared using diH_20 and were stored at room temperature unless otherwise stated. Solutions that were used in sterile work or for *in vivo* DNA manipulations were sterilized by autoclaving. All of the methods in this chapter are as in (Sambrook *et al.*, 1989) unless otherwise stated. *S. aureus* and *E. coli* strains and plasmids are listed in Table 1. *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. Synchronized cultures for growth experiments were obtained by the following protocol. One ml of relevant overnight *S. aureus* cultures were used to inoculate fresh media, and allowed to grow for 3h. These exponentially growing cultures were used to seed new media at an OD_{600} of 0.05. Test cultures were then allowed to grow for the experimentally required time period. When required, antibiotics were added at the following concentrations: ampicillin 100 mg l^{-1} (*E. coli*), tetracycline 5 mg l^{-1} (*S. aureus*), erythromycin 5 mg l^{-1} (*S. aureus*), lincomycin 25 mg l^{-1} (*S. aureus*), and chloramphenicol 5 mg l^{-1} (*S. aureus*).

Table 1. 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	Sambrook *et al.*, 1989
S. aureus		
RN4220	Restriction deficient transformation recipient	Lab Stocks
8325-4	Wild-Type Laboratory Strain *rsbU* mutant	Lab Stocks
RN6390	Wild-Type Laboratory Strain *rsbU* mutant	Lab Stocks
SH1000	Wild-Type Laboratory Strain *rsbU* functional	Horsburgh *et al.*, 2002
Newman	Wild-Type Laboratory Strain, human clinical isolate	Lab Stocks
USA300 LAC	USA300-LAC MRSA isolate cured of pUSA300-LAC-MRSA	Paul Fey UNMC
UAMS-1790	Sequenced USA300-HOU MRSA isolate cured of pUSA300-HOU-MRSA	Kolar *et al.*, 2010
FPR	Sequenced USA300-FPR MRSA isolate cured of pUSA300-FPR-MRSA	Carroll *et al.*, 2012
LES57	SH1000 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	Shaw *et al.*, 2008
HKM01	RN4220 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM02	8325-4 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM03	Newman pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM04	USA300 LAC pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM05	UAMS-1790 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM06	USA300 FPR pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM07	RN4220 pAZ106::*sigS-lacZ*$_{(P1-P3)}$ *sigS* $^+$	This study
HKM08	8325-4 pAZ106::*sigS-lacZ*$_{(P1-P3)}$ *sigS* $^+$	This study
HKM09	USA300 LAC pAZ106::*sigS-lacZ*$_{(P1-P3)}$ *sigS* $^+$	This study
HKM10	SH1000 *ecfX*::*tet ecfX* $^-$ pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
LES59	RN4220 *ecfX*::*tet ecfX* $^-$ pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	Shaw *et al.*, 2008
HKM11	8325-4 *ecfX*::*tet ecfX* $^-$ pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM12	SH1000 *agr*::*tet agr*$^+$ pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM13	SH1000 *sigB*::*tet sigB*$^+$ pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM14	RN6390 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$	This study
HKM15	SH1000 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$ NTG A	This study
HKM16	SH1000 pAZ106::*sigS-lacZ*$_{(P1-P4)}$ *sigS* $^+$ NTG B	This study
HKM17	8325-4 pSC-A::*tet*::*sigS-lacZ sigS* $^+$	This study
HKM18	8325-4 pSC-A::*tet*::*sigS-lacZ sigS* $^+$ pRN3208	This study
HKM19	SH1000 pSC-A::*tet*::*sigS-lacZ sigS* $^+$	This study
HKM20	USA300 LAC pSC-A::*tet*::*sigS-lacZ sigS* $^+$	This study
HKM21	8325-4 *sbcC*::Tn*551*	This study
HKM22	SH1000 *sbcC*::Tn*551*	This study
HKM23	USA300 LAC *sbcC*::Tn*551*	This study
HKM24	8325-4 *msa*::Tn*551*	This study
HKM25	SH1000 *msa*::Tn*551*	This study
HKM26	USA300 LAC *msa*::Tn*551*	This study
HKM27	8325-4 *msrA*::Tn*551*	This study
HKM28	SH1000 *msrA*::Tn*551*	This study
HKM29	USA300 LAC *msrA*::Tn*551*	This study
LES55	SH1000 *sigS*::*tet sigS* $^-$	Shaw *et al.*, 2008
HKM30	RN4220 *sigS*::*tet sigS* $^-$	This study
HKM31	8325-4 *sigS*::*tet sigS* $^-$	This study
HKM32	USA300 LAC *sigS*::*tet sigS* $^-$	This study
LES56	SH1000 *ecfX*::*tet ecfX* $^-$	Shaw *et al.*, 2008
LES58	RN4220 *ecfX*::*tet ecfX* $^-$	Shaw *et al.*, 2008
HKM33	USA300 LAC *sigS*::*tet* pMK4::*sigS* $^+$	This study
HKM34	USA300 LAC *ecfX*::*tet* pMK4::*ecfX* $^+$	This study
Plasmids		
pAZ106	Promoterless *lacZ erm* insertion vector	Kemp *et al*., 1991
pMK4	Shuttle vector	Sullivan *et al*., 1984
pSC-A	TA clone suicide vector	StrataClone
pRN3208	TS shuttle vector harboring Tn*551*	Kornblum *et al*.,1986
pDG1515	Tetracycline resistance antibiotic cassette in Bluescript KS(+) (Amp^r)	Guerout-Fleury *et al*., 1995
pLES202	pAZ106 containing a 2.2 kb *ecfX* fragment	Shaw *et al.*, 2008
pLES204	pLES202 containing a tetracycline cassette within ecfX	Shaw *et al.*, 2008
pHKM1	pAZ106 containing a 1.0 kb sigS fragment	This study
pHKM2	pMK4 containing a 2.1 kb *sigS* fragment	This study
pHKM3	pMK4 containing a 2.1 kb *ecfX* fragment	This study
pHKM4	pSC-A containing the *sigS-lacZ* fragment from pLES205	This study

Table 1. Continued.

Strain, plasmid or primer	Genotype or description	Reference or source
pHKM5	pSC-A containing a *tet* cassette and *sigS-lacZ* fragment from pLES205	This study
Primers (Restriction Sites are Underlined)		
OL281	ACT GGA TCC CAG TTG CAG ATG CAT CTC TCC	
OL285	ACT GGA TCC GAC CAT CAC GAT ACA TCA	
OL286	CTT CAC TGA CAA CTA TGC CG	
OL287	GCG ATT ACA TTC TAG AAG TTC C	
OL288	GGA ACT TCT AGA ATG TAA TCG C	
OL522	ATG TCT AGA GAG TAA TGC TAA CAT AGC	
OL523	ATG TCT AGA CCC AAA GTT GAT CCC TTA ACG	
OL909	ATG CTG CAG CAG GAC CCA ACG CTG CCC GAG	
OL1036	CCG CGC ACA TTT CCC CGA AA	
OL1184	AGC CGA CCT GAG AGG GTG A	
OL1185	TCT GGA CCG TGT CTC AGT TCC	
OL1275	ACC TTG AAG GAT ACA AGC AA	
OL1276	GGC ATT TAC GCT TAA CGG AC	
OL1366	ACG TGC ACC GAT ACA A	
OL1367	GGC TCA TCA ACT TCT AGC	
OL1568	CGA TTA CGC AAA TGA ATG	
OL1569	CAA GTA GTC ATT CTC CAA G	
OL1570	ATG GAA TTC TCA AGT AGT CAT TCT C	
OL1715	ATG CTG CAG CAA GTC TAT CTG GCG TAC	
OL1800	CGA AGC GCA TTT TCT CAA CA	
OL1801	TTT AAT CGC CCC TTA CCT GC	
OL1802	GAA GGT ATT AAA TAT GGC GGT ACA AG	
OL1803	ACA CTT TGC ATA CCG GCT G	
OL1804	CAA GGT GAT GGT ACT AGG GAA G	
OL1805	CTG TTG AAA ATG ACT ACC TGT TGG	
OL1806	CAG AGG TAA GAA AGG CTA CGA G	
OL1807	GCC CGT GGA ATG TTA AAT GC	
OL1808	CGA GGC ATG TAT AAC GGA GAC	
OL1809	ACG GTT ATC TAA TGC ACT CGG	
OL1843	GGG AGA CGA AGG AAA AGG AAA	
OL1844	AAA TTG AAT GGT ATG GCC TGC	
OL1845	CGG CAC ATT TAC CAG GTA TG	
OL1846	TTG CAA CCG GAA TAC CTC C	
OL1862	TCC AGG TAA TGA GGC AAT GAC	
OL1863	CGG CTG TTC TGG ACC TAT AAC	
OL1864	GGT CTG ATT TTC TCA CAA CGT G	
OL1865	CGG TGT TTT CGT TTC CCA AG	
OL1866	GTG GTA TGG GCT CTT TAG GTG	
OL1867	GTA ACG CAC CTT TAT AAG CCG	
OL1868	ACA CAG TAG GTA TTC GTG CAG	
OL1869	AGA CTT CCC AAT CGA TGC	

Alternative Growth Media. Where specified, chemically defined minimal media (CDM) and metal ion limiting media (CL), were prepared as described previously (Horsburgh *et al.*, 2001; Watson *et al.*, 1998). Porcine serum plates were created by adding filter sterilized porcine serum (Sigma) to preautoclaved and cooled 2% agar in di$H_2$0.

Construction of the σ^S Mutant Strains. All strains used in this study were created via φ11 mediated transduction from strains previously described (Table 1).

Construction of the *sigS-lacZ*$_{(P1\text{-}P4)}$ Fusion Strains. All strains used in this study were created via φ11 mediated transduction from strains previously described (Table 1).

Construction of the *sigS-lacZ*$_{(P1\text{-}P3)}$ Fusion Strains. The promoter region of *sigS* including just the three upstream promoters was amplified as a 1 kb PCR fragment using primer pair OL-281/OL-1570 (Table 1). The purified DNA fragment was digested with BamH1 and EcoRI and cloned into similarly digested pAZ106 (Kemp *et al.*, 1991). *S. aureus* RN4220 was transformed with the resulting plasmid, pHKM1, and integrants were confirmed by PCR analysis. A representative clone was then used to transduce *S. aureus* SH1000 using φ11, with clones again confirmed by PCR analysis. This created strain HKM07 (*sigS-lacZ*$_{(P1\text{-}P3)}$).

Construction of the *ecfX* Mutant Strains. A plasmid for the mutagenesis of *ecfX* was constructed by PCR amplification. Two approximately 1kb fragments were PCR

generated surrounding the *ecfX* coding region (1 located upstream, primer pairs OL-285/OL-288; and 1 located downstream, primer pairs OL-286/OL-287). Primers OL-287 and OL-288 are identical, but divergent to each other, and contain mismatching that converts the wild type sequence of TCTTAA (~100bp from the SACOL1828 Met) to a TCTAGA XbaI site. These fragments were purified and used together as the template for further PCR using primer pair OL-285/OL-286. The resultant 2.2 kb DNA fragment was digested with BamHI and SphI, and cloned into the suicide vector pAZ106 (Kemp *et al.*, 1991) to generate pLES202, using standard cloning techniques (Sambrook *et al.*, 1989).

The novel XbaI site in pLES202 were then used as a target sites for the insertion of a tetracycline resistance cassette, generated from pDG1515 (Guerout-Fleury *et al.*, 1995) using primer pair OL-522/OL-523. The XbaI digested cassette was cloned into pLES202, yielding pLES204. This was then used to transform electrocompetent *S. aureus* RN4220, according to the method of Schenk and Ladagga (Schenk and Laddaga, 1992), with clones selected for on the basis of tetracycline and erythromycin resistance. Integrants were confirmed by PCR analysis and used as donors for the transduction of *S. aureus* strain SH1000 using phage φ11. Transductants were selected for their resistance to tetracycline (indicating the presence of the cassette) and sensitivity to erythromycin (indicating loss of the plasmid and associated functional copy of *ecfX*, before being confirmed by PCR analysis. This created strains LES56.

Construction of σ^S Complement Strains. The complement construct generated contains approximately 1 kb of upstream DNA, and 710 bp of downstream DNA, relative

to the σ^S coding region. This was PCR amplified using primer pair OL281 and OL1715 (Table 1), and cloned into the Gram-positive shuttle vector pMK4 (Sullivan *et al.*, 1984), creating pHKM2. *S. aureus* RN4220 was transformed with this construct, with clones confirmed by PCR analysis, using a combination of gene and vector specific primers (OL-281/OL-1036). A representative clone was selected to transduce the USA300 LAC *sigS* mutant. Clones were again confirmed by PCR analysis, creating strain HKM33.

Construction of *ecfX* Complement Strains. The complement construct generated contains approximately 1.5 kb of upstream DNA through the *ecfX* coding region. This was PCR amplified using primer pair OL281 and OL1715 (Table 1), and cloned into the Gram-positive shuttle vector pMK4 (Sullivan *et al.*, 1984), creating pHKM3. *S. aureus* RN4220 was transformed with this construct, with clones confirmed by PCR analysis, using a combination of gene and vector specific primers (OL-281/OL-1036). A representative clone was selected to transduce the USA300 LAC *sigS* mutant. Clones were again confirmed by PCR analysis, creating strain HKM34.

Rapid Amplification of cDNA End (RACE). RACE was performed utilizing the TaKaRa 5'-Full RACE Core Set kit according to the manufacturer's instructions as described previously (Carroll *et al.*, 2012). Briefly, total cellular mRNA was extracted via the Qiagen RNeasy Kit by first growing the culture in conditions conducive to maximal expression of the gene of interest. Cultures were snap frozen by placing 2 mL into 1:1 ice-cold EtOH:acetone and stored in the $^{-}80^{o}$C overnight. Cells were then pelleted and lysed via bead beating and the supernatant was treated with 7 µl β-

mercaptethanol, 700 µl of RLT Buffer and 500 µl EtOH then collected onto an RNeasy mini spin column. The column was then treated with 350 µl RW1 buffer followed by on column DNase treatment with the Qiagen DNase 1 Kit. Following DNase treatment the sample was washed again the RW1 buffer and RPE buffer and finally eluted in 40 µl RNase-free H_20. The 1^{st} strand cDNA was synthesized from the mRNA via reverse transcriptase with a 5' end-phosphorylated primer. The RT reaction was carried out in a thermal cycler for optimal reaction conditions (10 min at 30^oC, 50 min at 50^oC, and 2 min at 80^oC). The hybrid RNA was then degraded by addition of 5X hybrid RNA degradation buffer and 1 µl of RNase H and incubated for 1 h at 30^oC. The cDNA was then precipitated by addition of 5 volumes of 96 % (v/v) EtOH and 1 volume of 3 M sodium acetate, thoroughly vortexed and placed in the -80^oC for 10 min. The cDNA was then collected via centrifugation at 13,000 rpm for 10 min, washed with 250 µl of 70 % (v/v) EtOH and allowed to dry at room temperature for 5 min. The cDNA was then ligated to form concatemers by addition of 8 µl of 5X RNA (ssDNA) ligation buffer, 20 µl of 40 % PEG and 1 µl of T4 RNA ligase and incubated at 15^oC for 18 h. Standard PCR (94^oC 3 min, 25 cycles of (94^oC 30 sec, 55^oC 30 sec, 72^oC 0.5-5 min)) was carried out utilizing the forward primer S1 and the reverse primer A1. The resulting PCR product was then used as a template for a second PCR reaction using the forward primer S2 with the reverse primer A2. The resulting product was introduced into pSC-A via the StrataClone PCR Cloning Kit (Stratagene) then were sent to Eurofins MWG Operon for sequencing using primer T7.

Transcriptional Analysis of the *lacZ* Reporter Gene Fusion Strain. Qualitative analysis of *sigS* activity was performed by plating the *lacZ* reporter gene fusion strains on tryptic soy agar (TSA) containing 25 µl of Ery at 5 µg ml^{-1} and 25 µl of 40 µg ml^{-1} X-GAL which were then incubated overnight at 37^{o}C and subsequently analyzed for the production of a blue color. Quantitative analysis was performed by regular sampling of a synchronized culture grown under standard conditions in TSB at 37^{o}C. Analysis was performed by removing 500 µl aliquots every hour for a period of 12 hours and again at hour 24. The cells were then recovered via centrifugation at 13,000 rpm for 10 min and finally stored at -80^{o}C until the assay could be performed. The assay was carried out by first allowing the samples to thaw before being resuspended in 500 µl of ABT buffer (100mM NaCl, 60mM K_2HPO_4, 40mM KH_2PO_4 and 0.1% v/v Triton-X-100) followed by addition of 100 µl of 4-MUG (4 mg ml^{-1}). The samples were inverted to mix and allowed to incubate at 25^{o}C for 60 min at which time the reaction was stopped by the addition of 500 µl of 0.4 M Na_2CO_3. Fluorescence was measured using a BioTek Synergy 2 plate reader and calibrated with standard concentrations of MU (4-methyl umbelliferone). One unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the production of 1 pmol MU min^{-1} OD_{600} $unit^{-1}$. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent experiments that showed less than 10 % variability. The β-galactosidase activity was then determined from a calibration curve using the following equation.

(((Fluorescence – Background fluorescence) – C) / M) x ((A / B) / (T x OD_{600} x V)) = β-galactosidase units ml^{-1} OD_{600}^{-1} min^{-1} (C = Value of intercept of calibration curve (Determined from fluorescence readings of known concentrations of 4MU), M = Gradient of the calibration curve (Determined from fluorescence readings of known concentrations of 4MU), A = Volume of the assay (1.1 ml total of 500 µl ABT, 500 µl 0.4 M Na_2CO_3 and 100 µl of 4-MUG), B = Volume in Microtitre well (0.225 ml), T = Reaction time (60 min), V = Volume of cells originally sampled (0.5 ml)).

Plate Based Assay to Determine Alterations in Transcription Resulting From External Stress. The *lacZ* reporter gene fusions were analyzed via disk diffusion techniques adapted to transcriptional profiling as described previously (Kolar *et al.*, 2011). This was done by placing a sterile filter disk (7 mm, 3MM Whatman Paper) on a TSA plate overlayed with TSA top agar (0.7% agar) inoculated with 5 µl of an overnight culture of the *sigS*-fusion strain and 100 µl of 40 µg ml^{-1} X-GAL. This was followed by the addition of 10 µl of the following stress 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% isopropanol, 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 ml^{-1} pyrogallol, 1 M sodium nitroprusside, 1 M ethyl methane sulfonate, 1 M methyl methane sulfonate, 5 mg ml^{-1} penicillin G, 5 mg ml^{-1} ciprofloxacin, 5 mg ml^{-1} nalidixic acid, 5 mg ml^{-1} cefotaxime, 5 mg ml^{-1} vancomycin, 2 mg ml^{-1} phosphomycin, 5 mg ml^{-1} spectinomycin, 100 mg ml^{-1} ampicillin, 100 mg ml^{-1} oxacillin, 5 mg ml^{-1} gramicidin, 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, 4.21 M peracetic acid, 0.1 M EDTA, 1 M DTT. Plates were incubated for 24 h at 37^{O}C and subsequently analyzed for the presence of a blue ring at the edge of the zone of inhibition, indicating β-galactosidase activity and therefore *sigS* transcription.

Quantitative Real-Time PCR. Quantitative Real-Time PCR (qRT-PCR) analysis was conducted by first collecting total cellular mRNA via the Qiagen RNeasy Kit as per the manufacturer's protocol. Briefly, cells were grown under conditions required to carry out the relative experiment. Cultures were snap frozen by placing 2 mL into 1:1 ice-cold EtOH:acetone and stored in the $^{-}$80^{o}C overnight. Cells were then pelleted, resuspended in 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and lysed via bead beating for 40 sec. Samples were then treated with 7 µl β-mercaptethanol, 700 µl of RLT Buffer and subjected to a second round of 40 sec lysing. Samples were centrifuged and 600 µl of supernatant was added to 900 µl EtOH then collected onto an RNeasy mini spin column. The column was then treated with 350 µl RW1 buffer followed by washes with 2 x 500 µl RPE buffer and finally eluted in 40 µl RNase-free H_20. Samples were subjected to DNase treatment with Ambion's TURBO DNA-free Kit as per the manufacturer's instructions. DNase treatment was carried out at 37^{o}C for 40 min followed by inactivation of the DNase as per the manufacturer's protocol. Synthesis of cDNA from the mRNA was carried out via reverse transcriptase with random primers utilizing the iScript cDNA Synthesis Kit as per the manufacturer's protocol. The RT reaction was carried out in a thermal cycler for optimal reaction conditions (5 min at

25°C, 30 min at 42°C, and 5 min at 85°C). Quantitative Real-Time PCR analysis was conducted using SYBR Green I PCR Master Mix at a ratio of (10 µl SYBR, 2 µl forward primer, 2 µl reverse primer, 4 µl diH20 and 2 µl cDNA (diluted to a total concentration of 50 ng μl^{-1}). Primers utilized were specific for $sigS_{(P1\text{-}P3)}$ located 47 bp upstream of P4 (OL1568/OL1569), $sigS_{(P1\text{-}P4)}$ located 357 bp downstream of the translation start site (OL1275/OL1276), *ecfX* (OL1366/OL1367), *purC* (OL1800/OL1801), *purA* (OL1843/OL1844), *purE* (OL1845/OL1846), *purD* (OL1862/OL1863), *purB* (OL1864/OL1865), *guaB* (OL1866/OL1867) and *guaA* (OL1868/OL1869), *mutM* (OL1802/OL1803), *ogt* (OL1804/OL1805), SACOL2339 (OL1806/OL1807), *uvrB* (OL1808/OL1809). Control primers were for the 16s rRNA gene (OL1184/OL1185), as described previously (Koprivnjak *et al.*, 2006). Values were calculated from three independent experiments, and the data was analyzed using a Student *t* test, where $p < 0.05$ was considered statistically significant.

Transcriptional Analysis During Growth in Porcine Serum. Synchronous cultures of the *sigS-lacZ* fusion strains were standardized to an OD_{600} of 0.5, pelleted and washed twice in PBS before being resuspended in 1 ml of filter sterilized porcine serum (Sigma). The suspension was then incubated at 37°C in a rotator device for a period of 1, 5 or 24 h. At the appropriate time point 1 ml samples were pelleted and stored in -20°C for future analysis. Concomitantly, the CFU per ml for each sample was determined via serial dilution and plating on TSA. Harvested bacterial cells were assayed for β-galactosidase production as described previously, with the following alterations. Arbitrary expression units were calculated as a measure of substrate cleavage (4-MUG) by β-galactosidase into

4MU, which was evaluated by measuring the fluorescence of each sample at 355 / 460 nm, 0.1 sec, divided by the CFU ml^{-1}. Samples collected from the initial inocula were analyzed for β-galactosidase activity, and used as a measure of baseline expression to identify changes in transcription, as described previously (O'Croinin and Dorman, 2007; Walthers *et al.*, 2007). The data presented was generated from 3 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Macrophage Cell Culture and *S. aureus* Intracellular Transcriptional Analysis. Assays were carried out using the RAW 264.7 murine leukaemic monocyte macrophage cell line (ATCC TIB-71) as described previously (Walthers *et al.*, 2007). Cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin solution (Sigma) until infection, at which time antibiotics were used as described below. RAW 264.7 cells were seeded into 6-well plates and allowed to grow to a density of 1.0×10^6 cells per well. These were then infected with *S. aureus* strains resuspended in cell culture medium at 1.0×10^8 CFU per well to give an MOI of 100. To synchronize infections and facilitate contact between bacteria and RAW 264.7 cells, plates were centrifuged at 1000 rpm for 10 min. Cells were subsequently incubated for 1 h at 37^oC in a humidified atmosphere containing 5% CO_2 to allow phagocytosis. After this time, wells were washed twice with PBS and any remaining non-phagocytosed bacteria were killed by the addition of media containing 30 μg ml^{-1} gentamicin, for 1 h. This was then replaced with fresh DMEM containing 5 μg ml^{-1} gentamicin, and incubated for 24 h. Following this, RAW 264.7 cells were washed twice

with PBS, and lysed using 500 µl PBS containing 0.5% Triton X-100. Samples were withdrawn to determine bacterial numbers and the remaining bacteria were pelleted by centrifugation. Harvested bacterial cells were assayed for β-galactosidase production as described previously, with the following alterations. Arbitrary expression units were calculated as a measure of substrate cleavage (4-MUG) by β-galactosidase into 4MU, which was evaluated by measuring the fluorescence of each sample at 355 / 460 nm, 0.1 sec, divided by the CFU ml^{-1}. Samples collected from the initial inocula were analyzed for β-galactosidase activity, and used as a measure of baseline expression to identify changes in transcription during infection, as described previously (O'Croinin and Dorman, 2007; Walthers *et al.*, 2007). The data presented was generated from 6 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

DNA Sequencing of the *sigS* Promoter Region. The *sigS* promoter region was amplified via PCR with primer pairs OL281/OL OL-1570. The PCR product was then analyzed via gel electrophoresis and the resulting band extracted via the QIAGEN QIAquick PCR Purification kit according to the manufacturer's instructions. Briefly, the appropriate band was excised from the gel and allowed to dissolve in QG buffer (guanidine isothiocyanate) at $50^{o}C$. The DNA was then bound to a QIAquick spin column and washed with a 500 µl aliquot of QG buffer (QIAGEN). This is followed by addition of PE buffer and drying via centrifugation for 2 min in order to remove any residual buffer. The membrane was placed over a clean eppendorf and the DNA eluted in

50 µl diH_20. The purified DNA was then sent to Eurofins MWG Operon for sequencing with the primers used in the original PCR reaction.

Mutation via N-methyl-N'-nitro-N-nitrosoguanidine (NTG) Exposure. An overnight culture was synchronized and allowed to grow to mid-exponential phase at which point NTG was added to a final concentration of 50 µg ml^{-1} and allowed to incubate at 37^{o}C for 60 min (Iordanescu and Surdeanu, 1976). The cells were then collected via centrifugation at 3,500 rpm, washed, resuspended with tryptic soy broth (TSB) and allowed to recover at 37^{o}C for 2 h (Adelberg and Pittard, 1965). Following the recovery period, one ml aliquots were collected via centrifugation at 3,500 rpm for 10 min and resuspended in TSB glycerol (20% v/v) and stored in the $^{-}$80^{o}C for future analysis. The CFU ml^{-1} for the culture was determined both prior to NTG exposure and after in order to determine if the survival rate is $\geq$ 50%. For analysis, the frozen samples were allowed to thaw and were then serially diluted in sterile PBS (8 g l^{-1} NaCl, 1.4 g l^{-1} Na_2HPO_4, 0.2 g l^{-1} KCl and 0.2 g l^{-1} KH_2PO_4) and the 10^0 through the 10^{-5} dilutions plated and CFU ml^{-1} determined.

Transposon Mutagenesis and DNA Sequencing of Insertion Sites. Mutagenesis was carried out in strain HKM18 (Table 1) as previously described (Shaw *et al.*, 2006). Strain HKM18 was created via PCR amplification of the *sigS-lacZ* region from pLES205 utilizing primer pair OL281/OL909 (Table 1) which was subsequently TA cloned into pSC-A utilizing StrataClone PCR Cloning Kit as per the manufacturer's instructions. In order to create a tetracycline marked fusion strain, primer pair OL522/OL523 was used to

amplify the *tet* cassette from pDG1515 (Guerout-Fleury *et al.*, 1995) then digest with Xba1 and ligated into similarly cut pSC-A containing *sigS-lacZ* to create pHKM5 (Table 1). *S. aureus* RN4220 was transformed with this construct, with clones confirmed by PCR analysis, using a combination of gene and vector specific primers (OL-281/OL-909). A representative clone was selected to transduce the 8325-4 wild-type background. Clones were again confirmed by PCR analysis, creating strain HKM17. This strain was then used as a receptor for the temperature sensitive Tn*551* transposon containing plasmid pRN3208 (Kornblum *et al.*, 1986) and grown at 30^{o}C to aid in plasmid replication. In order to construct the Tn*551* mutant library, this strain was first grown overnight at 30^{o}C on a TSA plate containing 0.25 mM $CdCl_2$, 5 μg ml^{-1} Ery, 5 μg ml^{-1} Tet (CET). A flask of 100 ml TSB_{CET} was inoculated from the plate and grown overnight at 30^{o}C. A 5 ml volume from the overnight was washed once with TSB and resuspended in 100 ml TSB containing Ery (5 μg ml^{-1}) and grown at 43^{o}C. After 4 hours of growth, another 5 ml of culture was transferred to 100 ml TSB containing Ery (5 μg ml^{-1}) and allowed to grow at 43^{o}C until the OD_{600} reached 1.5 (Kornblum *et al.*, 1986). One ml aliquots were then collected via centrifugation at 3,500 rpm for 10 min and resuspended in TSB glycerol (20% v/v) and stored in the -80^{o}C for future analysis. The CFU ml^{-1} and insertion rate of the library was determined via serial dilution plating on TSA containing Ery (5 μg ml^{-1}) and TSA containing $CdCl_2$ (0.25 mM). The glycerol stocks were then plated on TSA containing Ery (5 μg ml^{-1}) at approximately 200 colonies per plate and analyzed for the appearance of blue colonies. The blue colonies were then selected and glycerol stocked as before. To confirm relevance of the insert, the transposon was transduced into 8325-4, SH1000 and Houston and the blue resulting transductants

glycerol stocked and whole genomic DNA collected utilizing the QIAGEN DNeasy Tissue Kit according to the manufacturer's instructions. Briefly, 5 ml of an overnight culture was collected via centrifugation at 4,500 rpm for 10 min and resuspended in 180 µl Enzymatic lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM NaEDTA, 1.2% Triton X-100, 50 µg ml^{-1} RNase A, 5 µl Lysostaphin (5 mg ml^{-1}) and incubated at 37^{o}C for 30 min. This was followed by an addition of 25 µl proteinase K and 200 µl Buffer AL which was then vortexed and incubated at 60^{o}C for 30 min. Post incubation, 200 µl of 100% EtOH was added to the lysate and vortexed. The lysate was then applied to a DNeasy column, washed and eluted in 400 µl of di$H_2$0 (QIAGEN). The concentration of DNA was measured utilizing the NanoDrop Spectrophotometer and 10 µl of 500-1000 ng ml^{-1} sample was sent to Eurofins MWG operon for sequencing. Sequencing was performed utilizing single-strand DNA sequencing using primer OL1130 specific to Tn*551*. Sequencing runs through the end of Tn*551* and into flanking genomic DNA which allows for approximately 500 bp of sequenced DNA outside of the transposon. Insertion sites were then identified utilizing NCBI BLAST analysis.

Disk Diffusion Analysis for Phenotypic Characterization. Disk diffusion analysis was carried out as previously described for transcriptional analysis, with the following modifications. Top agar overlay was seeded with cultures standardized to the same OD_{600} and without the addition of X-GAL. Additionally, evaluation of survival ability was determined as a measure of the diameter of the zone of inhibition of mutant versus wild-type parent strain.

Competitive Growth Analysis. Coculture studies were performed by inoculating complex liquid media with a 1:1 ratio of both mutant and wild-type strains. These were allowed to grow for defined periods of time, before colony enumeration via serial dilutions onto both TSA and TSA containing tetracycline. Because the mutant strain is marked with a tetracycline resistance cassette, only it will grow in the presence of tetracycline, whereas both parent and mutant will grow on TSA. As such, the CFU per ml of both parent and mutant strains can be obtained and a competitive index (CI) calculated as a measure of the relative proportion of the two strains.

Sensitivity Assays. Exponentially growing cultures were washed and resuspended in PBS before the addition of DNA damaging agents (5 mM H_2O_2, 25 mM MMS, 5 mM ethidium bromide (EtBr), 100 mM acridine orange, 25 mM ethyl methanesulfonate and 450 mM 4-nitroquinoline N-oxide). These were then placed at 37^{o}C with shaking, and aliquots removed at the time intervals specified. Samples were serially diluted, and CFU ml^{-1} determined. Control samples were also removed prior to exposure, and processed in an identical manner. Percent survival was calculated by comparing initial CFU ml^{-1} to final CFU ml^{-1} from three independent assays and the data was analyzed using a Student *t* test, where $p < 0.05$ was considered statistically significant. Data is presented as fold change of each percent survival relative to the percent survival of the wild-type strain.

Ultraviolet Radiation Survival Assay. This assay was performed as previously described (Chen *et al.*, 2007). Briefly, cultures were synchronized to an OD_{600} of 0.05 and allowed to grow for 4 h. Cultures were then serially diluted, and the 10^{-2} through 10^{-}

^{6} dilutions plated on TSA. Dilutions 10^{-2} and 10^{-3} were then subjected to UV irradiation at 4,000 μJ/cm^{2} using a CL-1000 Ultraviolet Crosslinker (UVP). Dilutions 10^{-4} through 10^{-6} served as unexposed controls. All plates were incubated in the dark at 37^{o}C overnight. Survival rates were calculated from three independent experiments, and the data was analyzed using a Student *t* test, where $p < 0.05$ was considered statistically significant.

Affymetrix *S. aureus* GeneChip Array. The RN4220 wild-type and *sigS* mutant strains were grown in TSB under standard conditions to 3h, which corresponds to a window of maximal *sigS* expression. Bacterial cell pellets were collected, stored and total bacterial RNA was isolated, processed and labeled as described previously with the following modifications. Bacterial pellets were resuspended in 100 μl of TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and cells lysed via bead beating utilizing FastPrep lysing matrix tubes for 2 cycles of 40 sec with 2 min cooling intervals on ice (MP Biomedicals LLC). Total bacterial RNA was isolated using the RNeasy Mini Columns according to the manufacturer's directions (Qiagen, Inc., Valencia, Calif.) Post isolation, RNA was freed of contaminating DNA via DNase treatment with RNase-free DNase 1 (Ambion®). RNA quantity and quality was checked utilizing Agilent 2100 Bioanalyzer (Agilent Technologies). The GeneChip *S.aureus* Genome Array containing 3,300 ORFs and 4,800 intergenic regions was used. Data is from three independent experiments and was analyzed for a fold cut-off of ≥ 2 utilizing a Student *t* test where $p<0.05$ was considered statistically significant. ORF IDs in the list of differentially expressed genes were then mapped to *S. aureus* COL IDs where possible.

Intracellular Proteomic Analysis. Intracellular proteins were harvested from synchronized cultures of 8325-4 wild-type and *sigS* mutant grown to 5 h in 2.5 mM MMS via centrifugation for 10 minutes at 4,150 rpm. The pellets were washed with PBS and resuspended in 1 ml of UDS buffer (6 M urea, 5 mM DTT, 1 % SDS, 50 mM Tris-HCl pH 8) (Maresso and Schneewind, 2006). After addition of 0.1 mm disruption beads, the cells were lysed at 4^{o}C with a BioSpec Mini-BeadBeater for a total of 5 min allowing for cooling periods after every min. The intracellular proteins were then separated from the cell wall debris by two rounds of centrifugation for 10 minutes each at 13,300 rpm and supernatants collected. Resulting protein concentrations were determined using a Pierce 660 nm protein assay kit and subsequently standardized to 1 mg ml^{-1}. The resulting samples were then reduced using dithiothreitol and alkylated via exposure to iodoacetamide, followed by a trypsin digest overnight at 37^{o}C. The digested proteins underwent de-salting before being fractionated via HPLC. Approximately 20 fractions were collected at one min intervals from the time of initial peptide elution. Fractions were then dried in a speed vacuum and resuspended in 20 µL of 0.1% formic acid (Rivera *et al.*, 2012). The resulting peptides were then identified via an LTQ XL mass spectrometer. The experiment was performed in triplicate and the resulting proteins combined and evaluated for statistical significance.

Purine Enzyme Activity Assays. Bacterial extracts were harvested from 100 ml cultures grown in TSB to 3 h, pelleted via centrifugation, washed in PBS and lysed in 1 ml of PBS containing lysostaphin (100 µg ml^{-1}) and incubated at 37^{o}C for 1 h. Resulting

lysates were cleared via centrifugation and protein concentration determined via the Pierce 660 nm protein assay (Thermo Scientific) as per the manufacturer's protocol. Equal amounts of protein were used in individual experiments. Activity assays were carried out as previously described (Hoffee *et al.*, 1978) with the following modifications. All measurements were carried out utilizing the ND-1000 UV-Vis spectrophotometer (NanoDrop) with a light path of 0.0001 cm. Specific activity was calculated from three independent experiments where the data was analyzed using a Student *t* test, where $p < 0.05$ was considered statistically significant.

Determination of Intracellular Nucleotide Pools. Intracellular nucleotide pools were extracted as described previously (Rajagopal *et al.*, 2005). Briefly, USA300 LAC wild-type and *sigS* mutant strains were grown in 100 ml of TSB for 3 h. The cells were washed in an equal volume of PBS and the cell pellet was resuspended in 10 ml of ice-cold 0.2 N HPLC grade formic acid (Fisher). Nucleotides were extracted at 4°C for 2 h and centrifuged for 10 min at 6000 *g*. Supernatants were filtered using a formic acid compatible 0.22 μm pore-size filter (Millipore, USA) and dried in a speed-vac for 1 h. The dried sample was then resuspended in 400 μl of buffer A (5 mM NH4H2PO4 pH 2.8). Intracellular nucleotide concentrations were determined by HPLC using a Hypersil SAX column (4.6 × 250mm, Thermo Hypersil-Keystone, Bellefonte, PA) equipped with a guard column. Twenty μl of sample were injected into the column, which was pre-equilibrated with buffer A. Nucleotides were eluted using a discontinuous gradient of 5mM NH4H2PO4 pH 2.8 (buffer A) and 750 mM NH4H2PO4 pH 3.7 (buffer B) as per manufacturer's instructions. The gradient employed was an increase in buffer B, from

0% to30% over 25 min and 30% to 100% buffer B, over the next 10 min at a constant flow rate of1.0 ml min−1. The absorbance was recorded at 254 nm and the peaks generated and those peaks corresponding to purines adenine and guanine were identified by comparison to standard HPLC nucleotide markers. These mono-, di- and triphosphate markers were purchased individually and combined at a 1:1 ratio. The markers were analyzed in a manner identical to that of the samples and standard curves were obtained for individual experiments from the resulting peaks and the known standard concentrations. The nucleotide concentrations of the individual samples were determined from the peak areas and standard curves, as described previously (Sigoillot *et al.*, 2003).

Whole Human Blood Survival Assay. Synchronized cultures of the USA300 LAC wild-type, *sigS* and *ecfX* mutants and the respective complement strains were grown to exponential phase and subsequently washed three times with PBS and placed in 1 ml of whole human blood. The initial inocula for each strain was determined retroactively by serially dilution and plating on TSA. Blood cultures were allowed to incubate at 37°C with shaking for three hours, before the CFU ml^{-1} of each strain was determined, again by serial dilution and plating. The data presented is the percentage survival of each strain compared to the beginning inoculum. Data is representative of three independent experiments where the data was analyzed using a Student *t* test, where $p < 0.05$ was considered statistically significant.

Macrophage Cell Culture and *S. aureus* Intracellular Survival Assay. Infections were carried out as described above for transcription studies, with the following

alterations. RAW 264.7 cells were infected with *S. aureus* strains resuspended in cell culture medium at 2.5 x 10^6 CFU per well to give an MOI of 1. Samples were withdrawn 24 hours post phagocytosis and CFU ml^{-1} determined via serial dilution and plating on TSA. The data presented was generated from 3 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Murine Models of Abscess Formation. Analysis using a murine model of wound formation was performed by infecting hairless SKH1-E female mice via subcutaneous injection. Ten mice were injected on the thigh with an inoculum of 1.00 x 10^8 CFU per mouse with the wild-type strain while another 10 received the same inoculum of the triple σ mutant strain. Each inoculum contained 0.01 ml of sterile Cytodex beads to act as foreign material microcarriers to aid in establishing infection (Bunce *et al.*, 1992; Ford *et al.*, 1989). The lesions were evaluated based on size for a period of 6 days at which time the mice were humanely euthanized and abscesses recovered. The lesions were then analyzed for the presence of *S. aureus* by homogenizing the abscesses in PBS and serially diluting the suspension onto TSA plates which were incubated overnight at 37^{o}C, and the resulting colonies counted to calculate CFU per abscess. Individual CFU per abscess values were averaged and percent recovery calculated as a measure of CFU per abscess over inoculum CFU. Data was analyzed using a Student *t* test, where $p < 0.05$ was considered statistically significant.

CHAPTER 3:

ANALYSIS OF THE ENVIRONMENTAL INDUCIBILITY OF *sigS* EXPRESSION

Note To Reader. Portions of these results have been previously published (Miller *et al.*, 2012; Shaw *et al.*, 2008) and are utilized with permission of the publisher (Appendix 1).

Background. Many components govern the adaptive nature of bacteria, including complex regulatory networks, which allow it to respond to constantly changing environments via rapid shifts in gene expression. There are a number of different elements that mediate this fine-tuning, including DNA-binding proteins, two-component systems, regulatory RNAs and alternative σ factors (1944; Cheung *et al.*, 1992; Deora *et al.*, 1997b; Fournier *et al.*, 2001; Giraudo *et al.*, 2003; Janzon *et al.*, 1986; McNamara *et al.*, 2000; Novick *et al.*, 1995; Novick and Jiang, 2003a). This latter class act by binding to core-RNA polymerase and redirecting promoter recognition to coordinate gene expression, bringing about expedient and wide-reaching alterations within the cell. These σ factors include the essential housekeeping factors (σ^A or σ^{70}), which are responsible for the majority of transcription as well as alternative σ factors, which are important for niche-specific transcriptional regulation in response to environmental change (Gruber and Gross, 2003; Helmann, 2002; Lonetto *et al.*, 1992; Lonetto *et al.*, 1994). During times of

stress, the housekeeping sigma factor is substituted for alternative σ factors, which control distinct regulons to circumvent detrimental condition. These regulators are highly specific to the respective stress that they respond to and are often only transcribed when needed. For example, σ^H of *Mycobacterium tuberculosis* displays minimal transcription during exponential phase growth compared to other σ factors in this organism. Even after exposure to stresses, such as SDS, low aeration and low temperatures, the amount of *sigH* transcript remains low; however, following exposure to heat shock, its levels increase significantly (Manganelli *et al.*, 1999). The ECF (extracytoplasmic function) σ factors are a group of alternative sigma factors, which typically respond to perturbations in the cell envelope. These are by far the most numerate of all sigma factors (Helmann, 2002); for example, *Streptomyces coelicolor* contains approximately 65 σ factors, around 50 of which are of the ECF subtype . Examples include RpoE of *E. coli*, which responds to stress triggered by misfolded proteins in the periplasm or outer membrane as a result of heat shock or osmotic stress (Dartigalongue *et al.*, 2001). The ECF sigma factors, σ^M , σ^W and σ^X of *B. subtilis* all respond to extracytoplasmic stresses elicited by certain cell wall targeting antimicrobials (Cao *et al.*, 2002a; Cao *et al.*, 2002b; Helmann, 2006). Additionally a number of ECF sigma factors, including σ^{FecI} of *E. coli* respond to iron-limiting conditions through transmembrane signaling (Enz *et al.*, 2000). Furthermore, there are examples within the ECF group of alternative sigma factors that respond to cytoplasmic stresses. These include RpoE from *R. sphaeroides* and σ^R from *S. coelicolor*, which both respond to thiol specific oxidative stresses induced by diamide (Campbell *et al.*, 2007; Dufour *et al.*, 2008; Paget *et al.*, 2001a); and Ecf of *Neisseria gonorrhoeae*, which responds to oxidative stress (Gunesekere *et al.*, 2006). Furthermore,

there are some ECF sigma factors which are constitutively expressed. Specifically, σ^R of *Streptomyces coelicolor* is constitutively expressed from a downstream promoter through the action of the housekeeping sigma factor, σ^A, and then subsequently upregulates itself from an upstream promoter during times of induction (Kim *et al.*, 2009). As such, it is clear that ECF-sigma factors have extensive variations in their inducibility by external conditions. Furthermore, they are employed to combat such stress in a variety of different ways. As such, the focus of this chapter will be to explore the impact of external conditions on expression profile of the lone ECF σ factor of *S. aureus*, σ^S. Specifically, we seek to identify the environmental cues that lead to its upregulation. We previously demonstrated that σ^S acts to upregulate itself from its own promoter region; therefore, in order to elucidate this promoter and any others, we set out to map the *sigS* promoter(s).

Results

Evaluation of the *sigS* Transcriptional Start Site Reveals Three Upstream Promoters and a Fourth Unique Internal Promoter. We previously demonstrated that σ^S autoregulates itself from its own promoter region; therefore, in order to elucidate the specific promoter sequences responsible for this, we set out to map the *sigS* transcriptional start site. To this end, we performed 5' rapid amplification of cDNA ends (RACE) analysis. This technique converts total cellular mRNA into full length cDNA, which through subsequent steps, can be used as a template for amplification and sequencing of the 5' end of mRNAs using gene specific primers. This was carried out in

the laboratory strain, SH1000, the same strain previously used for our preliminary characterization of σ^S. (Shaw *et al.*, 2008) Cells were grown under standard conditions and collected at 3 h, before total mRNA was extracted utilizing the Qiagen RNeasy Kit as per the manufacturer's protocol. The *sigS* mRNA transcript was converted to cDNA utilizing a 5' end-phosphorylated primer, which will convert the entire transcript through to the unknown 3' end. The cDNA was then ligated to form concatemers and used as a template for PCR where primers specific to known regions of the template will be used to PCR across the unknown 3' region. The resulting products were introduced into pSC-A via the StrataClone PCR Cloning Kit (Stratagene) then were sent to Eurofins MWG Operon for sequencing using primer T7 specific to pSC-A. The resulting sequencing reaction will generate DNA sequences from pSC-A across the insertion site of the PCR product containing the unknown 3' region of the *sigS* mRNA transcript. A total of 3 independently generated pSC-A constructs were analyzed by DNA sequencing, all of which identified a transcriptional start site 85 nucleotides downstream of the putative start codon, as displayed in Figure 7. Later work conducted in our laboratory utilizing primer extension analysis with a primer positioned 174 nucleotides downstream of the *sigS* translation start site confirmed the existence of this internal promoter (P4). Further analysis with a second primer located 12 nucleotides downstream of the *sigS* initiation codon led to the identification of 3 unique transcription start sites (Figure 7), the longest of which bears an adenine +1 residue, located 150 bp upstream of the translation start site. This is 7 nt away from a putative σ^A promoter, denoted as P1, with a sequence of aTtACA, followed by a 17bp spacer, and then TATtta. Promoter P2 is located 126 nt upstream of the translational start site, beginning with a thymine residue, and appears to

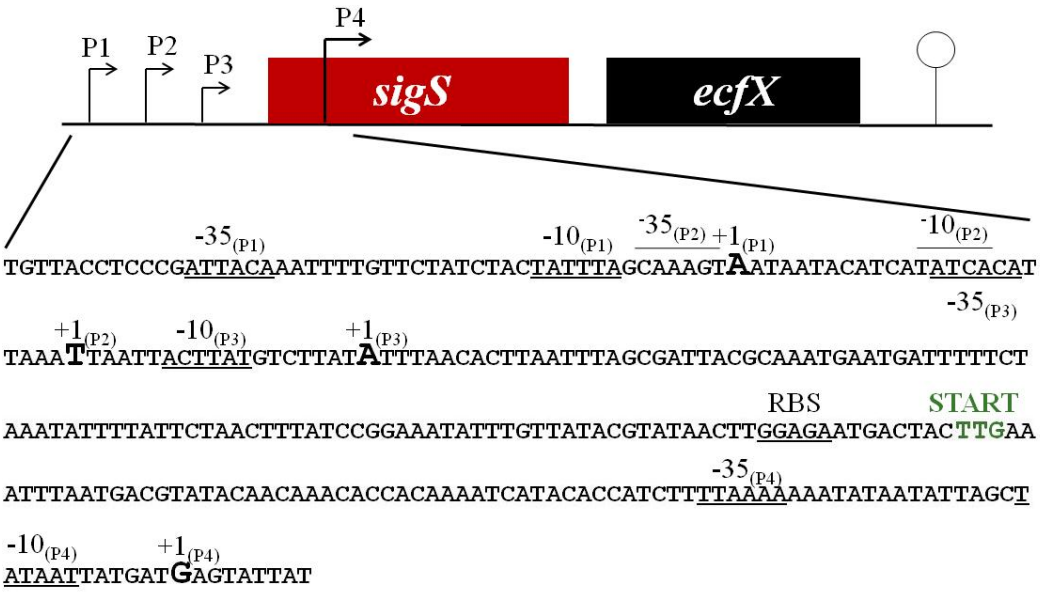


Figure 7. Promoter Mapping of *sigS* Reveals the Existence of an Internal Promoter and Three Upstream Promoters. 5' RACE analysis was performed on the *sigS* operon resulting in the identification of an internal promoter (P4) depicted in the cartoon above. Analysis of the sequence upstream of the identified +1 results in a putative weak σ^A promoter as marked. Later analysis in our laboratory utilizing primer extension revealed the existence of three other, upstream promoters (P1-P3). Both P1 and P3 contain putative σ^A consensus sequences, while P2 contains a putative σ^S consensus sequence. Adapted from Miller *et al*., 2012.

have no notable σ^A or σ^B promoter sequences. It does, however, bear a putative σ^S promoter of CAAAGT 12 bp upstream of TATCA. The third transcription start site, positioned 107 nt upstream of the coding region, contains an adenine +1 residue and is 7 nucleotides away from a putative σ^A promoter, denoted as P3, with a sequence of aTcACA, followed by an 11 bp spacer, and then acTtAT.

Transcriptional Profiling of *sigS* Reveals Conditions Under Which it is Expressed. Transcriptional analysis of *sigS* was performed utilizing reporter gene fusion constructs in plasmid pAZ106, by placing the *lacZ* gene, which encodes the enzyme β-galactosidase, under the control of the *sigS* promoters. Figure 8 details construction of this strain, which is produced via chromosomal integration of the vector by homologous recombination, resulting in duplication of the four *sigS* promoters. One copy controls the *lacZ* gene, whilst the other controls a functional *sigS* gene, downstream of the insertion site. As this construct contains all four *sigS* promoters (P1, P2, P3 and P4), it is termed *sigS-lacZ*$_{(P1\text{-}P4)}$ (Figure 9). A second fusion construct was created in order to assess just the upstream promoters (P1, P2 and P3) denoted as *sigS-lacZ*$_{(P1\text{-}P3)}$ (Figure 10). Production of β-galactosidase is then evaluated in these strains through the use of X-gal and 4-MUG, which, when cleaved by β-galactosidase, result in the production of either a blue color or a quantifiable fluorescent absorbance, respectively. Thus, each of these can be used to measure expression from the *sigS* promoters.

Expression of *sigS* is Increased in the RN4220 Background Under Standard Conditions. Transcriptional analysis using the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion was first performed

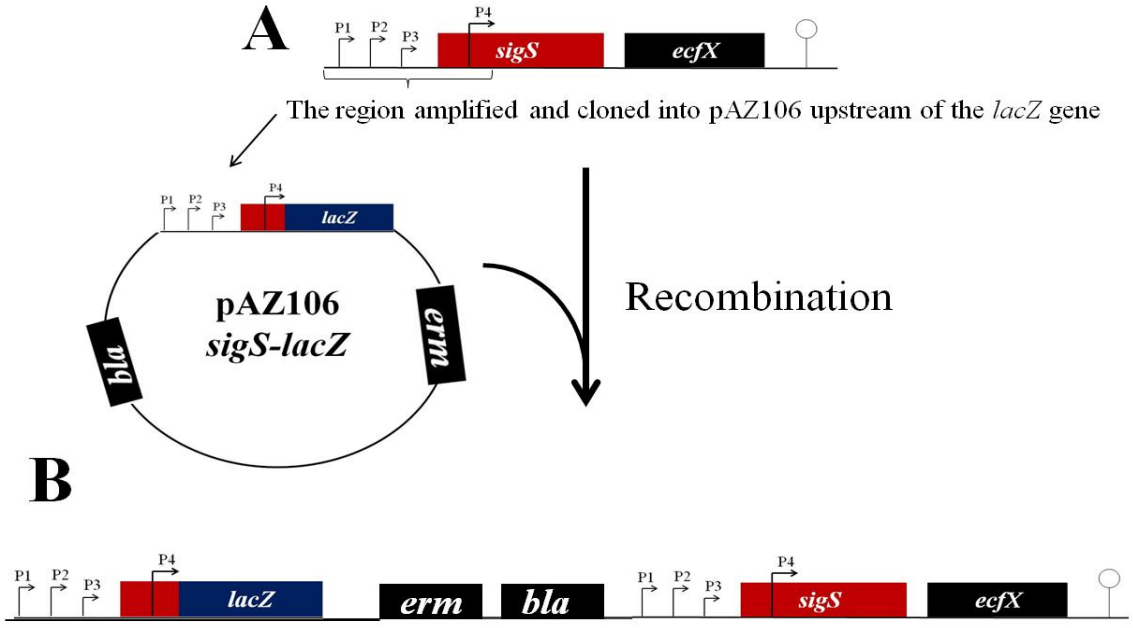


Figure 8. Construct of a *sigS-lacZ* Fusion. **(A)** Depiction of the *sigS-ecfX* locus in the *S. aureus* chromosome. A portion of DNA 5' of P1 to 3' of P4 was amplified and cloned into pAZ106, upstream of a promoterless *lacZ* gene. The pAZ106 plasmid contains both *bla* and *erm* for resistance against ampicillin and erythromycin, respectively. Integration of pAZ106 *sigS-lacZ* results in **(B)** creation of a *sigS-lacZ* fusion, which contains a duplication of the *sigS* promoters, placing *lacZ* under their control, followed by the rest of the pAZ106 plasmid. Note that the *sigS-ecfX* locus is functional and uninterrupted.

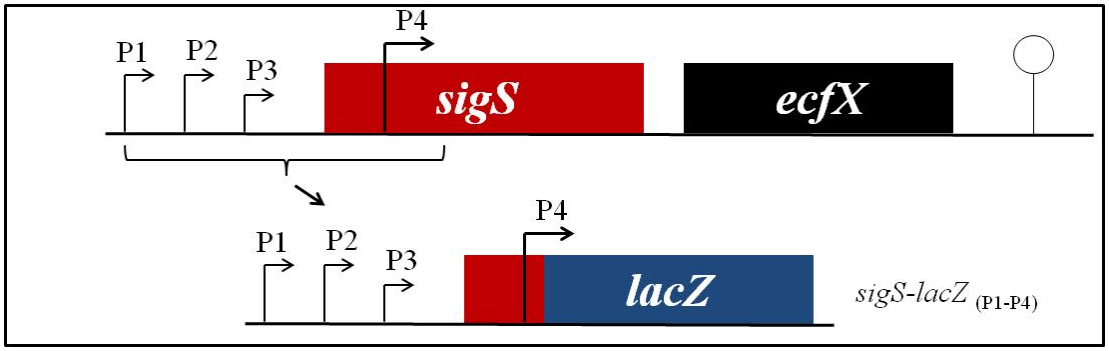


Figure 9. Promoter Map of the *sigS-lacZ*$_{(P1-P4)}$ Fusion Construct. The *sigS* operon is shown containing three upstream promoters (P1, P2 and P3) and one internal promoter (P4), identified by 5' RACE and primer extension analysis. The fusion strain contains all four *sigS* promoters, and is termed *sigS-lacZ* $_{(P1-P4)}$.

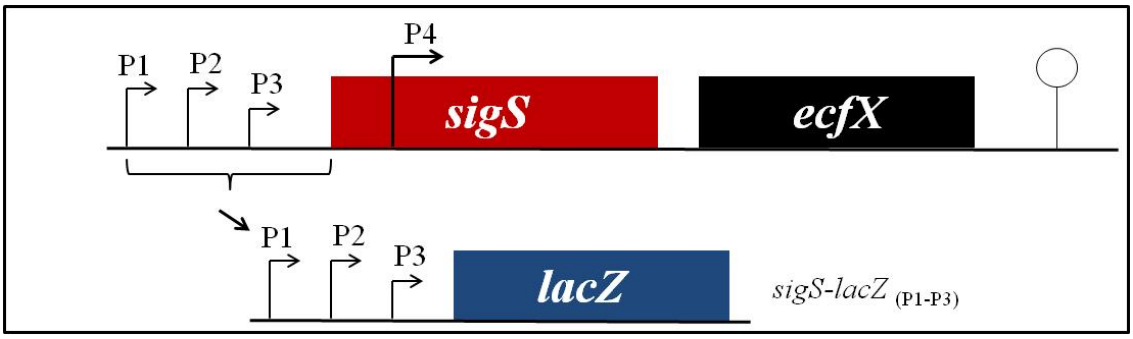


Figure 10. Promoter Map of the *sigS-lacZ*$_{(P1-P3)}$ Fusion Construct. The *sigS* operon is shown containing three upstream promoters (P1, P2 and P3) and one internal promoter (P4), identified by 5' RACE and primer extension analysis. The fusion strain contains only the three upstream *sigS* promoters, and is denoted as *sigS-lacZ* $_{(P1-P3)}$.

by examining the strains for production of a blue coloration when plated on TSA containing X-gal, indicating expression from the *sigS* promoter. As displayed in Figure 11A, a variety of laboratory strains, including RN4220, 8325-4, SH1000 and Newman, as well as the USA300 clinical isolates, Houston, LAC and FPR were analyzed with only RN4220 demonstrating expression. We then confirmed our findings in complex liquid media where again expression of *sigS* exhibited only baseline expression over a 24 h period in every strain, apart from RN4220 (Figure 11B). In this latter strain, we observed an approximate 5-fold increase in *sigS* expression when compared to other *S. aureus* isolates, suggesting that there are features unique to this strain that allow for upregulation of *sigS* under standard conditions. To ensure our findings were not an artifact of the fusion construct, we performed quantitative real-time PCR on these wild-type strains including RN4220, 8325-4, SH1000 and Newman, as well as the USA300 clinical isolates, Houston, LAC and FPR during a window of maximal *sigS* expression (3 h). The primer pair utilized, termed $sigS_{(P1\text{-}P4)}$, is located 357 bp downstream of the *sigS* translation start site, and will detect transcript from any of the *sigS* promoters that extends to the end of the *sigS* coding region. Using this approach we again observed robust expression of *sigS* in RN4220, with minimal transcription detected in the other isolates (Figure 12). Curiously, whilst low expression was observed for the other backgrounds, transcriptional activity in Newman was almost entirely negligible.

The Differential Expression Observed for *sigS* in *S. aureus* Wild-Type Strains is True Across the *sigS* Locus. We repeated the analysis using the *sigS-lacZ*$_{(P1\text{-}P3)}$ RN4220 fusion by again growing it on TSA containing X-gal. We found that unlike the *sigS*-

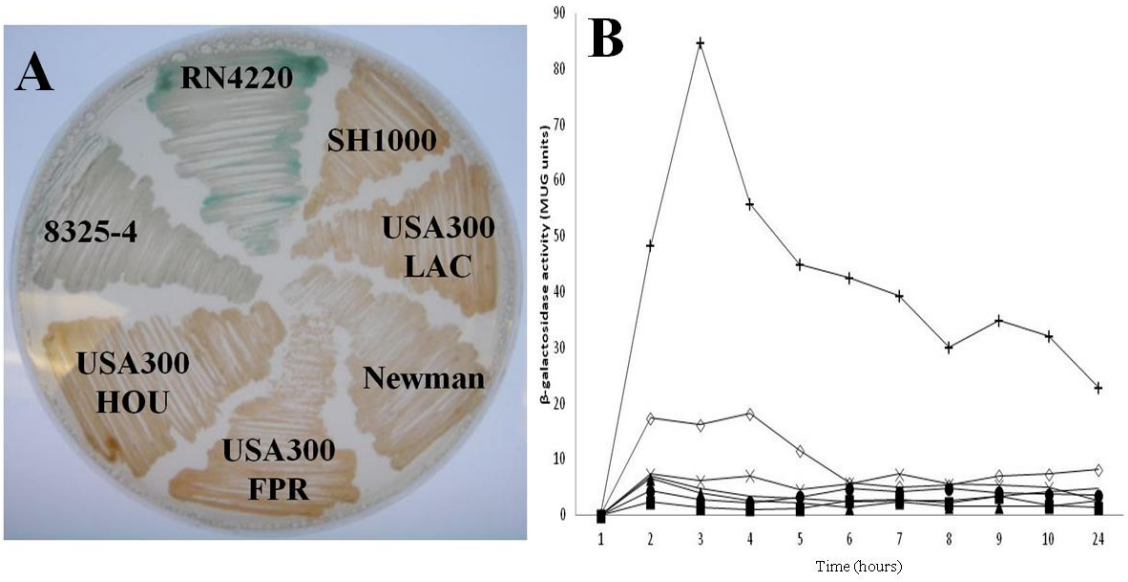


Figure 11. Transcription Profiling of *sigS* in a Variety of *S. aureus* Wild-Type Strains. (**A**) *sigS-lacZ*$_{(P1-P4)}$ fusion strains were allowed to grow on TSA containing X-gal at 37^{o}C overnight. Blue coloration indicates *sigS* upregulation. (**B**) *sigS-lacZ*$_{(P1-P4)}$ fusion strains in RN4220 (+), 8325-4 (◊), USA 300 HOU (X), USA 300 FPR (●), SH1000 (▲), USA300 LAC (■) and Newman (□) were grown in TSB at 37^{o}C and sampled every hour for 10 h and again at 24 h. β-galactosidase activity was measured to determine levels of *sigS* expression. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent experiments that showed less than 10 % variability. Adapted from Shaw *et al*., 2008 and Miller *et al*., 2012.

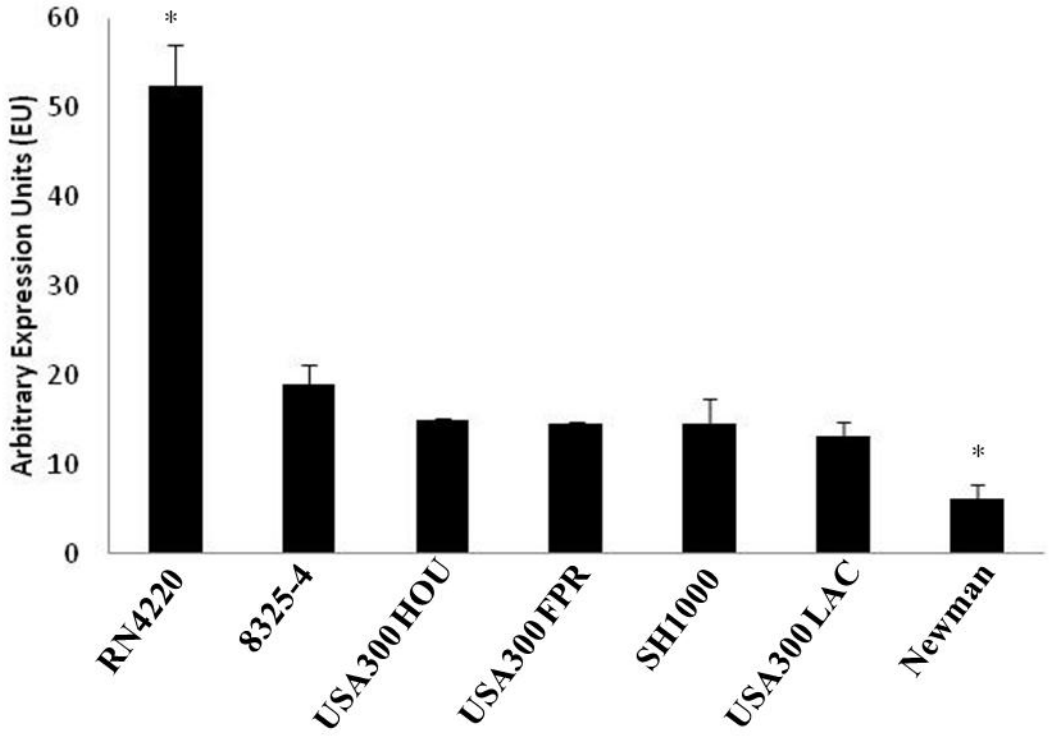


Figure 12. Transcription Profiling of *sigS* in a Variety of *S. aureus* Wild-Type Strains. Quantitative real-time PCR was performed on *S. aureus* wild-type strains grown for 3 h in TSB at 37^{o}C, with primers specific to $sigS_{(P1\text{-}P4)}$. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test. Adapted from Miller *et al*., 2012.

lacZ$_{(P1\text{-}P4)}$ fusion, growth of RN4220 *sigS-lacZ*$_{(P1\text{-}P3)}$ led to no blue coloration, a finding that was mirrored in the β-galactosidase expression assays of the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion (Figure 13). These findings suggest that the majority of expression observed in the RN4220 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain is a direct result of expression from the internal P4 promoter. We then continued our analysis to include evaluation of transcript throughout the *sigS* locus utilizing three separate primer pairs as displayed in Figure 14. The first of which, termed *sigS*$_{(P1\text{-}P3)}$, is located 47 bp upstream of P4; as such analysis with these primers will include only transcript from the upstream promoters, excluding transcript from the internal P4 promoter. The second primer pair, termed *sigS*$_{(P1\text{-}P4)}$, is the same as utilized before and is located 357 bp downstream of the *sigS* translation start site, and will detect transcript from any of the *sigS* promoters that extends to the end of the *sigS* coding region. The *sigS* locus contains an additional gene that is likely to be transcriptionally linked, located 112 bp downstream of *sigS*, SACOL1828, which we have termed *ecfX*. The final primer pair, *ecfX*, is located in this downstream gene, at 254 bp 3' of its translation start site, and will target transcripts that run through to the end of the *ecfX* coding region. Using this approach, we observed that expression is elevated for the *sigS*$_{(P1\text{-}P3)}$, *sigS*$_{(P1\text{-}P4)}$ and *ecfX* primer pairs in RN4220, with minimal transcription detected from each of the three primer pairs in the other isolates again with negligible expression in the Newman background (Figure 15). The ability to detect transcript utilizing our upstream *sigS*$_{(P1\text{-}P3)}$ qRT-PCR primer pair conflicts with our *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion findings, suggesting that transcript generated from the upstream promoters may terminate prior to the fusion site of the *lacZ* construct. Additionally, the blue coloration observed for the *sigS-lacZ*$_{(P1\text{-}P4)}$ may be a result of a combined effect of all four

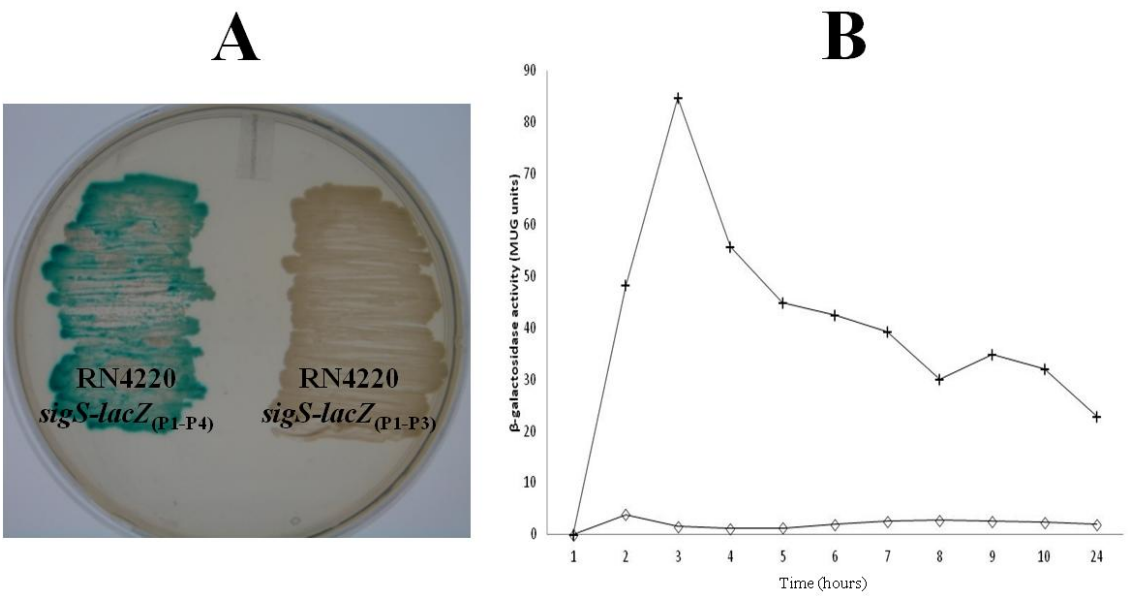


Figure 13. Transcription Profiling of *sigS*$_{(P1-P3)}$ in RN4220. (A) The RN4220 *sigS-lacZ*$_{(P1-P3)}$ and *sigS-lacZ*$_{(P1-P4)}$ fusion strains were grown on TSA containing X-gal at 37^{o}C overnight. The appearance of blue coloration indicates expression of *sigS*. **(B)** The *sigS-lacZ*$_{(P1-P3)}$ (◊) and *sigS-lacZ*$_{(P1-P4)}$ (+) fusion strains in the RN4220 background were grown in TSB at 37^{o}C and sampled every hour for 10 h and again at 24 h. β-galactosidase activity was measured to determine levels of *sigS* expression. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability.

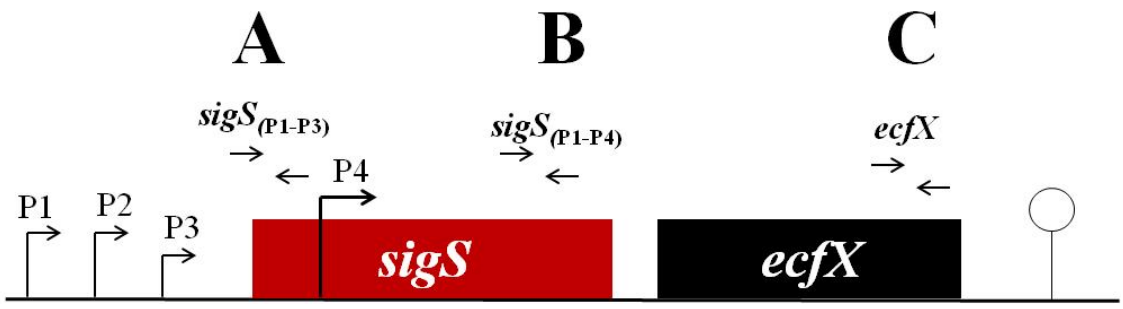


Figure 14. The qRT-PCR Primer Locations in the *sigS* Operon. Displayed is the *sigS* operon and the location of each of primer pair used in qRT-PCR analysis. Three separate primer pairs were generated: (**A**) termed $sigS_{(P1\text{-}P3)}$ located 47bp upstream of P4, (**B**) termed $sigS_{(P1\text{-}P4)}$ located 357bp downstream of the *sigS* translation start site, and (**C**) *ecfX*, located 254bp downstream of the *ecfX* translation start site.

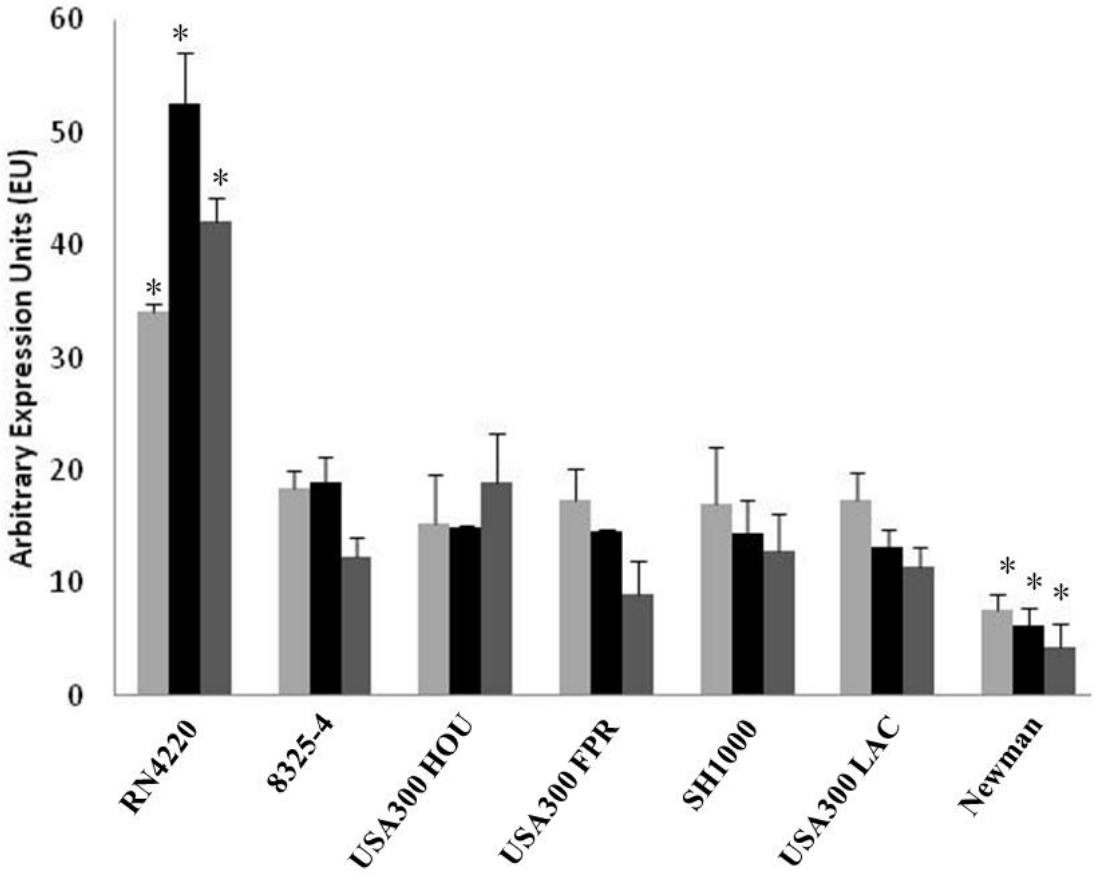


Figure 15. Transcription Profiling of the *sigS* Locus in a Variety of *S. aureus* Wild-Type Strains. Quantitative real-time PCR was performed on *S. aureus* wild-type strains grown for 3 h in TSB at 37^{o}C, with primers specific to $sigS_{(P1\text{-}P3)}$ (light grey), $sigS_{(P1\text{-}P4)}$ (black) or *ecfX* (dark grey). The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test.

promoters acting on the *lacZ* fusion gene, while the strength of the upstream promoters alone may not lead to observable β-galactosidase activity.

Expression of *sigS* is Inducible in Response to External Stimuli. Given the low level of *sigS* expression observed amongst the majority of *S. aureus* strains under standard conditions, we next set out to explore whether transcription could be induced from this locus via external stress, as with other ECF σ factors (Helmann, 2002; Raivio and Silhavy, 2001; Staron *et al.*, 2009). This was performed using a modified disk diffusion assay where agar plates were overlayed with top agar containing X-gal, and seeded with the relevant fusion strain. Subsequent to this, sterile filter disks are added to the plates, and impregnated with appropriate stress chemicals. Following overnight incubation, plates were screened for the appearance of a blue ring around the zone of inhibition, indicating expression from the *sigS* promoters. The *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strains in 8325-4, Newman, SH1000 and USA300 LAC were grown in the presence of a plethora of stress conditions (Table 2). Whilst we were unable to detect upregulation of *sigS* in most strains, we did observe significant inducibility in 8325-4 (Figure 16). Specifically, we noted *sigS* expression in the presence of a variety of chemicals (Table 3), including a number of agents that induce cell wall stress, as well as compounds know to elicit DNA damage. We also noted *sigS* upregulation in amino acid limiting media, metal ion limiting media, and during growth on pig serum. Again to rule out artifacts of the screen, we sought to verify these findings during continuous growth in liquid media. This was performed with the 8325-4 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain grown in TSB containing sub-lethal concentrations of select chemicals (MMS, H_2O_2 and NaOH) from Table 3, and

Table 2. Compounds Used to Screen for Expression of the *sigS-lacZ* Reporter Fusion.

Stress	Agent	Concentration
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
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
Antibiotics	Penicillin G	20 µg
	Nalidixic Acid	50 µg
	Ciprofloxacin	50 µg
	Oxacillin	50 µg

Table 2. Continued.

Stress	Agent	Concentration
	Cefotaxime	50 µg
	Chloramphenicol	50 µg
	Phosphomycin	20 µg
	Vancomycin	20 µg
	Spectinomycin	50 µg
	Ampicillin	1 mg
	Tetracycline	50 µg
	Erythromycin	50 µg
	Lincomycin	250 µg
	Kanamycin	500 µg
	Neomycin	500 µg
	Puromycin	250 µg
	Mupirocin	20 µg
Disulfide	Diamide	500 mM
Miscellaneous	Berberine Chloride	128 µg
	Peracetic Acid	4.2 M

Table 3. Compounds Found to Induce Expression of a *sigS-lacZ*$_{(P1-P4)}$ Reporter Fusion in Strain 8325-4.

Agent / Condition	Stress / Mode of Action	Overall Effect
Sodium Hydroxide	Alkali Stress	DNA damage
Hydrogen Peroxide	Oxidative Stress	DNA damage
Methyl methanesulfonate	Alkylates DNA	DNA damage
Ethyl methanesulfonate	Alkylates DNA	DNA damage
Ciprofloxacin	Inhibits DNA gyrase	DNA damage
Nalidixic Acid	Inhibits DNA gyrase	DNA damage
Chloramphenicol	Inhibits protein synthesis	Miscellaneous
Pig Serum	Humoral immune system components	Miscellaneous
Amino acid limiting	Minimal media	Miscellaneous
Metal limiting	Minimal media	Miscellaneous
Cefotaxime	Inhibits transpeptidation	Cell wall weakening/disruption
Ampicillin	Inhibits transpeptidation	Cell wall weakening/disruption
Oxacillin	Inhibits transpeptidation	Cell wall weakening/disruption
Sodium Dodecyl Sulfate	Disrupts cell walls	Cell wall weakening/disruption
Phosphomycin	Inhibits UDP-N-aceytglucosamine-3-enolpyruvyltransferase	Cell wall weakening/disruption

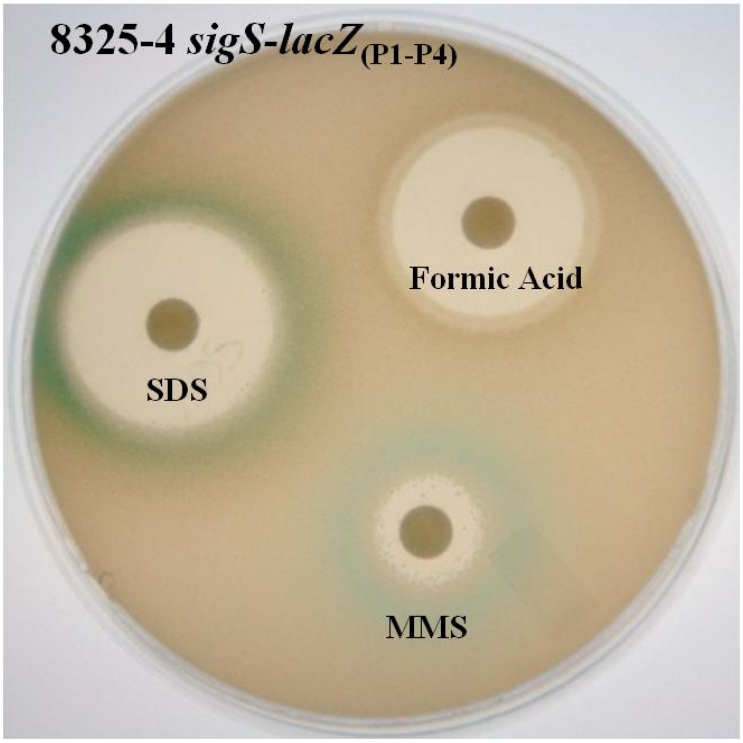


Figure 16. Transcription of *sigS* is Inducible in Response to External Stress. A modified disk diffusion assay was carried out to determine stress conditions that lead to *sigS* expression. TSA plates were overlayed with top agar seeded with the 8325-4 *sigS-lacZ*$_{(P1-P4)}$ fusion and 0.2 mg X-gal. Sterile filter disks were placed on the solidified agar and 10 µl of appropriate chemical added. The appearance of a blue ring around the zone of inhibition, as displayed here, indicates *sigS* activity.

revealed increased expression of *sigS* in each instance (Figure 17A-C). In the presence of MMS, expression peaked at 5 h, with a 48.7-fold increase compared to standard conditions. Maximal expression with both NaOH and H_2O_2 occurred at 10 h, with fold increases of 10 and 4.4 respectively, compared to unsupplemented media. Additional qRT-PCR analysis was performed (Figure 17D) to verify this data, and again confirmed that the greatest fold increase in *sigS* expression was induced by exposure to MMS, resulting in a 102.6- fold increase in transcription. Transcription of *sigS* upon exposure to NaOH and H_2O_2 was 63.3- and 57.2-fold higher, respectively.

To explore if this upregulation was conserved for other *S. aureus* strains, but perhaps below the limit of detection for our plate based assay, we performed experiments with MMS, NaOH and H_2O_2 using SH1000 and USA300 LAC *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strains. Interestingly, despite a lack of blue coloration in the plate based assay, we again detected upregulation of *sigS* during growth in liquid media with sub-lethal concentrations of MMS and H_2O_2 (Figure 18A-B). Expression with MMS in both SH1000 and USA300 LAC was highest at 2 h, with fold increases of 3.6 and 8.1, respectively, compared to standard growth conditions. In the presence of H_2O_2, *sigS* expression in SH1000 increased by 2.6-fold (2 h), and by 2.3-fold in USA300 LAC (7 h). Conversely, we observed no increase in expression for NaOH when grown in these conditions, suggesting that greater, and perhaps more lethal concentrations of this agent may be required to induce expression. We again confirmed this data by qRT-PCR in SH1000 and USA300 LAC grown in the presence of MMS and H_2O_2 (Figure 18C). SH1000 displays a 16.9-fold increased in *sigS* expression when cultured with MMS, and a 13.5-fold increase

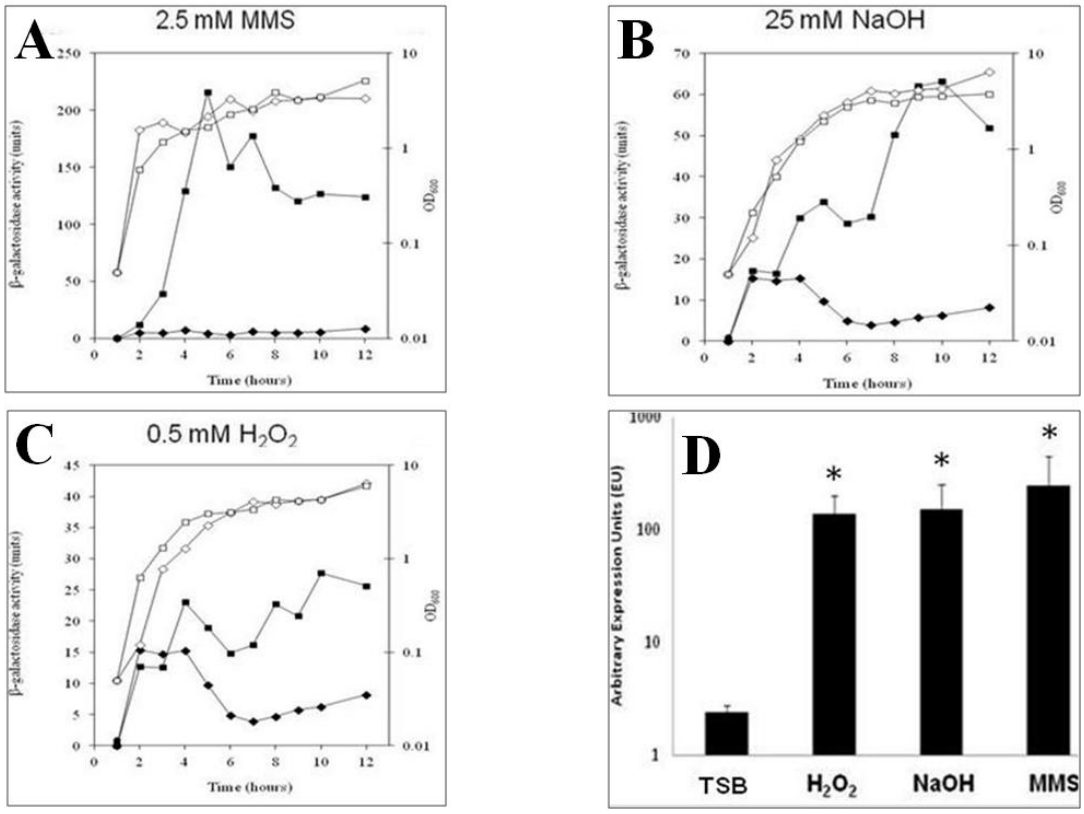


Figure 17. Transcription of *sigS* is Inducible in Response to External Stress. The 8325-4 *sigS-lacZ*$_{(P1-P4)}$ strain was grown (**A-C**) in either TSB (♦) or TSB supplemented with sub-lethal concentrations of the indicated stress chemicals (■). Cultures were sampled every hour for 10 h, and again at 24 h, to determine β-galactosidase activity. Additionally, growth was monitored via OD_{600} at the times indicated for both standard (◊) and supplemented (□) growth conditions. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability. (**D**) Quantitative real-time PCR analysis was performed with strain 8325-4 grown for 5 h under conditions identical to **A-C**, using primers specific to *sigS*$_{(P1-P4)}$. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test (Miller *et al*., 2012).

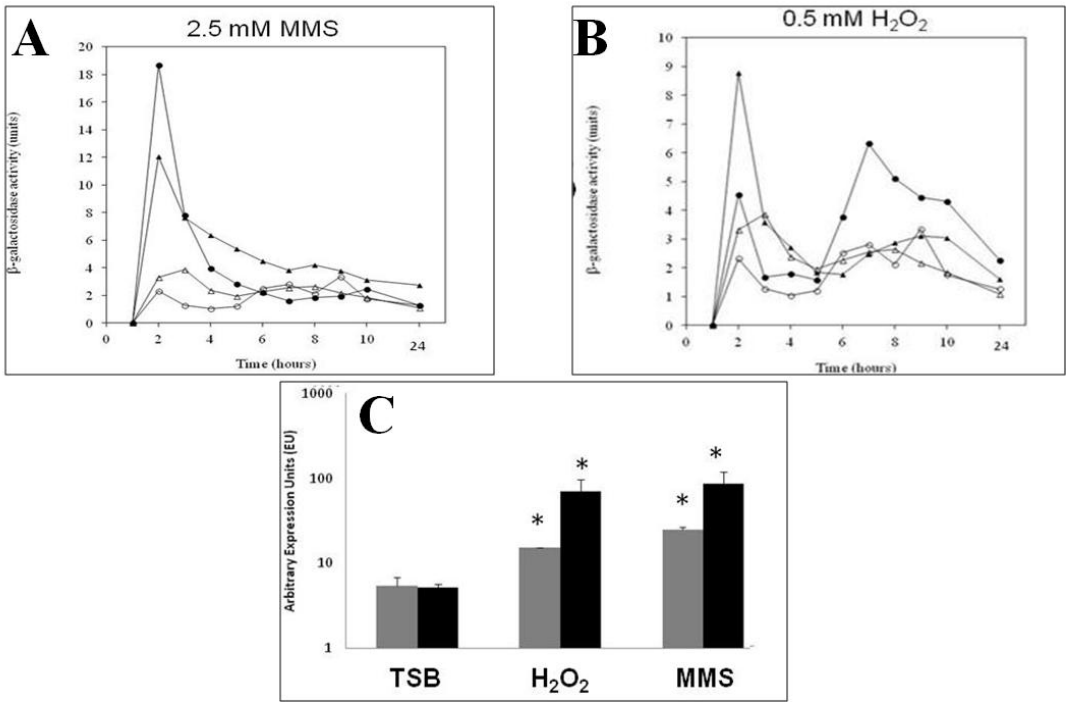


Figure 18. The Inducibility of *sigS* Expression is Conserved Across *S. aureus* Strains. The SH1000 (Δ) and USA300 LAC (○) *sigS-lacZ* fusion strains were grown (**A-B**) in either TSB (open symbols) or TSB supplemented with sub-lethal concentrations of the indicated stress chemicals (closed symbols). Cultures were sampled every hour for 10 h, and again at 24 h, to determine β-galactosidase activity. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability. (**C**) Quantitative real-time PCR analysis was performed with strain USA300 LAC (grey) and SH1000 (black) grown for 2 h under conditions identical to **A-B**, using primers specific to $gS_{(P1-P4)}$. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test, and represent significant variation from standard conditions (TSB) (Miller *et al*., 2012).

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when cultured with H_2O_2. Expression of *sigS* in USA300 LAC grown with MMS or H_2O_2 displayed 4.6- and 2.8-fold increases in transcription, respectively. Collectively, these findings demonstrate a significant inducibility of *sigS* in response to external stimuli that is conserved across *S. aureus* strains.

Transcription is Increased Across the *sigS* Locus in 8325-4 Upon Exposure to External Stress. Given our findings regarding expression from the upstream promoters (P1-P3) in RN4220, we next set out to examine the *sigS-lacZ*$_{(P1-P3)}$ in those stress conditions previously shown to induce expression of the *sigS-lacZ*$_{(P1-P4)}$ fusion. This analysis was again carried out in the 8325-4 as we observed the highest level of environmental inducibility from this strain. We found that none of the conditions leading to expression for the *sigS-lacZ*$_{(P1-P4)}$ fusion resulted in observable expression with the *sigS-lacZ*$_{(P1-P3)}$ fusion using the disk diffusion assay (Figure 19). In order to assess whether transcription was increased, but again below the limit of detection for our plate based assay, we performed qRT-PCR under these conditions with the *sigS*$_{(P1-P3)}$ and *ecfX* primer pairs. Interestingly, while the *sigS-lacZ*$_{(P1-P3)}$ fusion strain produced no visible expression via disk diffusion analysis, we did observed significant increase in *sigS*$_{(P1-P3)}$ transcript in the presence of each of the chemicals examined, although not to the levels observed for *sigS*$_{(P1-P4)}$ and *ecfX*. Specifically, we observed 6.1-, 4.5- and 5.8-fold increases for *sigS*$_{(P1-P3)}$ in the presence of MMS, NaOH and H_2O_2, respectively compared to growth in TSB (Figure 20A). Levels of *ecfX* transcript were also increased in the presence of MMS, NaOH and H_2O_2 with fold changes of 81.1, 42.5 and 38.6 respectively, as displayed in Figure 20A. Our findings again suggest that the

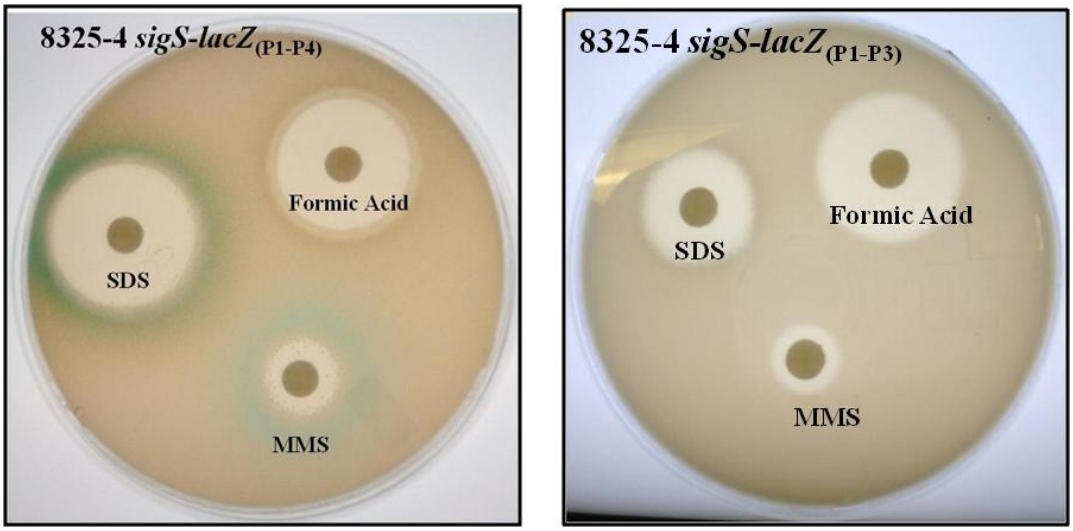

Figure 19. The Expression of *sigS* is Notable Only in the 8325-4 *sigS-lacZ*$_{(P1-P4)}$ Fusion Strain in Response to External Stress. A modified disk diffusion assay was carried out to determine stress conditions that lead to *sigS* expression. TSA plates were overlayed with top agar seeded with either the 8325-4 *sigS-lacZ*$_{(P1-P4)}$ or the 8325-4 *sigS-lacZ*$_{(P1-P3)}$ fusion and 0.2 mg X-gal. Sterile filter disks were placed on the solidified agar and 10 µl of appropriate chemical added. The appearance of a blue ring around the zone of inhibition, as displayed here, indicates *sigS* activity.

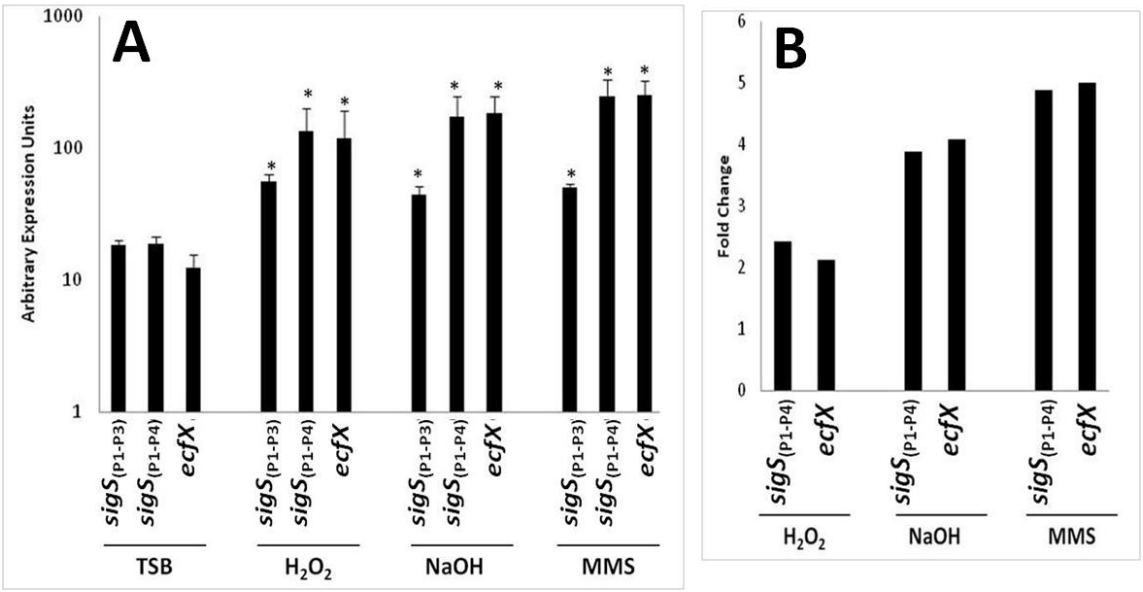

Figure 20. Transcription is Increased Across the *sigS* Locus in 8325-4 Upon Exposure to External Stress. (A) Quantitative real-time PCR analysis was performed with strain 8325-4 grown for 5 h in TSB, or TSB supplemented with various stress chemicals (0.5 mM H_2O_2, 25 mM NaOH or 2.5 mM MMS). Three separate primer pairs were again used as described in figure 14. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test, and represent significant variation from standard conditions (TSB). **(B)** The fold changes are displayed for transcript measured from the $sigS_{(P1\text{-}P4)}$ and *ecfX* primer pairs relative to the transcript detected using the $sigS_{(P1\text{-}P3)}$ primer pair. Specifically this shows the fold increase directly relating to transcript generated from the internal P4 promoter. The results presented herein were representative of three independent replicates that showed less than 10 % variability.

conflicting results generated between our reporter gene fusion and qRT-PCR studies may be a result of truncated *sigS* transcript. Additionally, this would suggest that the strength of the internal promoter combined with that of the upstream promoters leads to our observed β-galactosidase activity. Comparison of the fold change for transcript measured by the $sigS_{(P1\text{-}P3)}$ primer pair compared to that of the $sigS_{(P1\text{-}P4)}$ and *ecfX* is displayed in Figure 20B. Specifically this measures the fold increase directly relating to transcript generated from the internal P4 promoter. During growth in H_2O_2, we observed a 2.4- and 2.1-fold increase for the $sigS_{(P1\text{-}P4)}$ and *ecfX* primer pairs compared to the $sigS_{(P1\text{-}P3)}$ primer pair respectively. This difference was further enhanced during growth in NaOH, where we observed fold increases of 3.9 and 4.1 for $sigS_{(P1\text{-}P4)}$ and *ecfX* respectively. Finally, the greatest fold increase was observed during growth in the presence of MMS with 4.9- and 5-fold increases for $sigS_{(P1\text{-}P4)}$ and *ecfX* respectively.

Transcription is Increased Across the *sigS* Locus in Both the SH1000 and USA300 LAC Backgrounds During Growth in the Presence of DNA Damage Agents. To explore if the upregulation found across the *sigS* locus in the 8325-4 background was conserved for other *S. aureus* strains, we performed experiments with MMS, NaOH and H_2O_2 using SH1000 and USA300 LAC with both the $sigS_{(P1\text{-}P3)}$ and *ecfX* primer pairs. We determined that expression from $sigS_{(P1\text{-}P3)}$ and *ecfX* was also increased upon exposure to MMS and H_2O_2 to levels comparable to that of $sigS_{(P1\text{-}P4)}$. Specifically, in the SH1000 background, fold changes of 20.1 and 16 were observed for $sigS_{(P1\text{-}P3)}$ and *ecfX* in the presence of MMS (2 h), respectively (Figure 21). Analysis using the $sigS_{(P1\text{-}P3)}$ and *ecfX* primer pairs in the presence of H_20_2 at 2 h led to 13.1- and

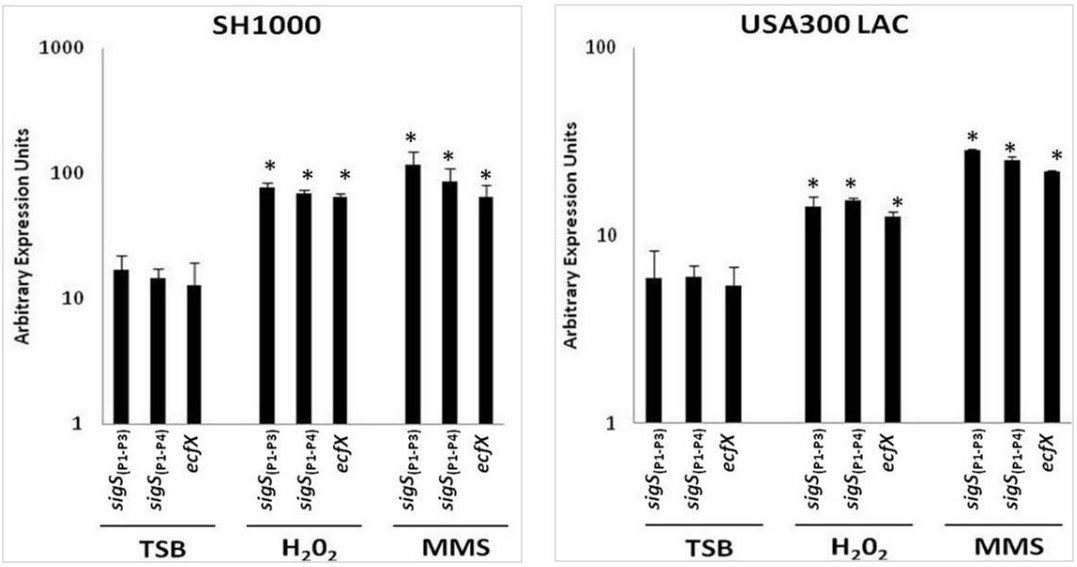

Figure 21. Transcription is Increased Across the *sigS* Locus in Both SH1000 and USA300 LAC Upon Exposure to External Stress. Quantitative real-time PCR analysis was performed with strains SH1000 and USA300 LAC grown for 3 h in TSB, TSB supplemented with 0.5 mM H_2O_2 and TSB supplemented with 2.5 mM MMS. Three separate primer pairs were used (A) located 47bp upstream of P4 (B) located 357bp downstream of the *sigS* translation start site and (C) located 254bp downstream of the *ecfX* translation start site. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test, and represent significant variation from standard conditions (TSB).

15.9- fold increases, respectively in the SH1000 background. As displayed in Figure 21, analysis in the USA300 LAC background at 2 h in the presence of MMS led to fold increases of 4.8 and 3.6 for *sigS*$_{(P1-P3)}$ and *ecfX* respectively. Finally, 2.4- and 2.1-fold increases were observed for *sigS*$_{(P1-P3)}$ and *ecfX* respectively in the presence of H_2O_2 at 2 h. Collectively, these findings demonstrate significant inducibility of the transcripts across the *sigS* operon, which is conserved across *S. aureus* strains. Interestingly, these findings suggest that the internal promoter, P4, is disregulated in 8325-4 and RN4220, but not in the SH1000 and USA300 LAC backgrounds, suggesting that the observable β-galactosidase activity in the aforementioned strains may be a direct result of increased transcript from the internal promoter.

Expression of *sigS* is Strongly Upregulated During Growth in Pig Serum. Despite limited expression under standard laboratory growth conditions, we have previously demonstrated a role for σ^S in the virulence of *S. aureus* (Shaw *et al.*, 2008). Working on a hypothesis that *sigS* transcription would be increased during infection, we performed expression profiling of both *sigS-lacZ* fusion constructs upon *ex vivo* challenge by components of the innate immune system. Indeed, our plate based analysis already suggests that *sigS* expression is increased by exposure to pig serum, at least from P1-P4 (Table 3). In order to confirm these findings, and quantify this increase across different strains, we performed transcriptional analysis using both the *sigS-lacZ*$_{(P1-P4)}$ and *sigS-lacZ*$_{(P1-P3)}$ fusions strains grown in TSB, and then subcultured into pig serum. We determined that after just 1 h of growth in serum, *sigS* expression increased by 3.4-fold in strain 8325-4 *sigS-lacZ*$_{(P1-P4)}$, compared to TSB. Expression continued to rise over time,

with fold increases of 21.2 and 20.5 observed at hours 5 and 24, respectively (Figure 22A). Additionally we observed a similar effect in strains SH1000 and USA300 LAC. Specifically, over the course of growth, we noted fold increases of 13.8, 36.2 and 44.0 in SH1000 *sigS-lacZ*$_{(P1\text{-}P4)}$ for hours 1, 5 and 24, respectively (Figure 22A). Finally, in USA300 LAC *sigS-lacZ*$_{(P1\text{-}P4)}$, *sigS* expression increased 6.8-, 15.6- and 26.3-fold at hours 1, 5 and 24, respectively (Figure 22A). Analysis performed in the 8325-4 *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strain led to increases of 6.4-, 7.3- and 22-fold observed at hours 1, 5 and 24 respectively (Figure 22B). In the SH1000 *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion, expression increased 2.4-, 1.6- and 12.5-fold over 1, 5 and 24 hours respectively (Figure 22B). Finally, expression in USA300 LAC increased 1.6- and 1.3 fold during hours 1 and 5, with a significant increase of 8.6-fold by hour 24 (Figure 22B). Comparison of the *sigS-lacZ*$_{(P1\text{-}P4)}$ and *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strains shows the highest increases occurring in the 8325-4 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion, with the greatest increase of 4-fold observed for hour 24 (Figure 22C). These findings further support our expression analysis which suggests that the internal promoter, P4, is disregulated in the 8325-4 background.

Expression of *sigS* is Strongly Upregulated During Phagocytosis. We continued this line of investigation by performing expression profiling of *sigS-lacZ*$_{(P1\text{-}P4)}$ and *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strains upon phagocytosis by RAW 264.7 macrophage-like cells. Accordingly, macrophages were infected with strains 8325-4, SH1000, and USA300 LAC bearing either the *sigS-lacZ*$_{(P1\text{-}P4)}$ or the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion. β-galactosidase expression was measured both prior to infection, as well as 24 h post phagocytosis (Figure 23). Expression from the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion was significantly increased after

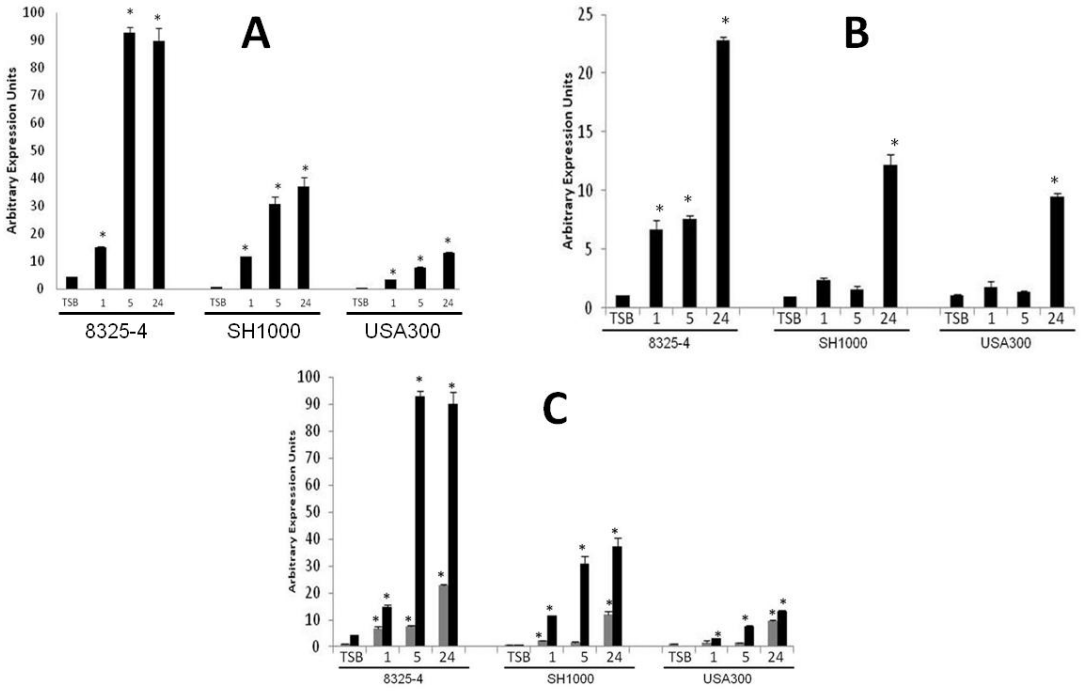

Figure 22. Profiling of *sigS* Expression During Growth in Pig Serum. (**A**) The *sigS-lacZ*$_{(P1-P4)}$, (**B**) the *sigS-lacZ*$_{(P1-P3)}$ fusion, and (**C**) both the *sigS-lacZ*$_{(P1-P4)}$ (black) and the *sigS-lacZ*$_{(P1-P3)}$ (grey) fusion strains were assayed for β-galactosidase activity prior to (TSB), and during (1h, 5h and 24h), growth in pig serum. The data presented is from at least 3 independent experiments. Error bars are presented as +/- SEM, *= $p<0.001$ using a Student *t* test. Adapted from Miller *et al*., 2012.

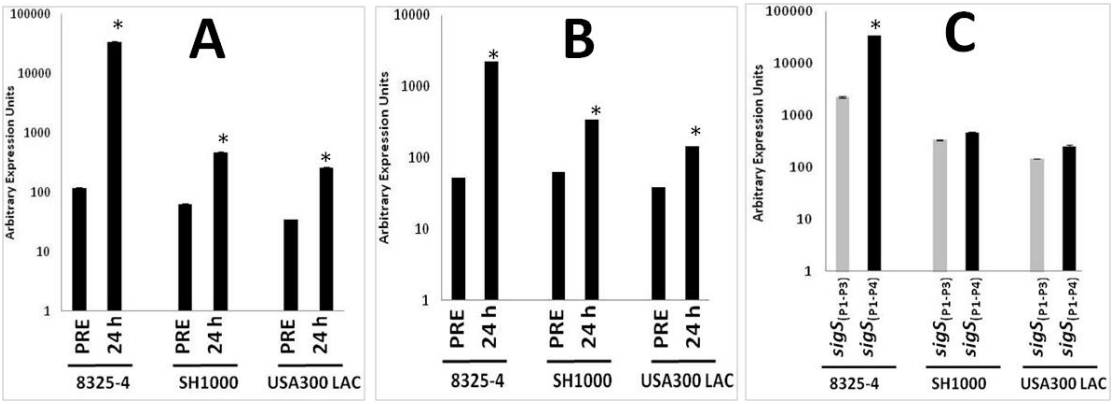

Figure 23. Profiling of *sigS* Expression Post Phagocytosis. (**A**) The *sigS-lacZ*$_{(P1-P4)}$, (**B**) the *sigS-lacZ*$_{(P1-P3)}$ fusion, and (**C**) both the *sigS-lacZ*$_{(P1-P4)}$ (black) and the *sigS-lacZ*$_{(P1-P3)}$ (grey) fusion strains were assayed for β-galactosidase activity prior to phagocytosis (grey bars) and 24 h post phagocytosis (black bars) by RAW 264.7 murine macrophage-like cells. Cells were infected at an MOI of 1:100 and incubations carried out at 37^{o}C in a humidified atmosphere of 5% CO_2. The data presented is from at least 3 independent experiments. Error bars are presented as +/- SEM, *= $p<0.001$ using a Student *t* test. Adapted from Miller *et al*., 2012.

phagocytosis in all strains tested (Figure 23A), with the highest levels again observed in 8325-4. In this strain we found a 286.6-fold increase in *sigS*$_{(P1\text{-}P4)}$ expression compared to pre-phagocytosis levels. In SH1000 and USA300 expression increased by 7.5- and 7.4-fold, compared to background levels. Analysis with the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strains again led to significantly increased expression in all of the strains tested (Figure 23B). As with the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain, the highest level of expression was observed in the 8325-4 background with a fold increase of 43.2. The expression increase in SH1000 and USA300 was 5.4-, 3.8-fold respectively. Upon examination of the *sigS-lacZ*$_{(P1\text{-}P3)}$ against the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion, we observed expression profiles similar to that for our previous findings. Specifically during phagocytosis, we noted an approximately 15-fold increase for the 8325-4 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion compared to the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strain (Figure 23C). In contrast, only slight increases in expression were observed for the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion compared to the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strain in SH1000 and USA300 LAC. This data suggests that both the upstream and internal promoters are active during challenge by components of the innate immune system; however, the internal promoter again appears to be disregulated in the 8325-4 background. Collectively, this further supports our hypothesis that suggests that σ^S is required during the interaction of *S. aureus* with its host.

Expression of *sigS* is Not Influenced by Either *agr* or *sigB*, Two Major Global Regulators of *S. aureus*, Under Standard Conditions. Due to the variable expression observed amongst strains of *S. aureus*, we sought to determine if this variation was due to mutation of the global regulators *agr* and *sigB*. Specifically, under standard conditions

we noted that RN4220 had markedly higher levels of *sigS* expression compared to other wild-type strains. Of the many mutations within its genome, RN4220 carries both a *cis* SNP in *agr* as well as a *trans* SNP in *sigB*, mutations which render them nonfunctional (Nair *et al.*, 2011). As such, we sought to determine the effect of these individual disruptions on the expression of *sigS*. We introduced the *sigS-lacZ*$_{(P1-P4)}$ fusion into a SH1000 *sigB::tet* mutant strain, and analyzed it via β-galactosidase assays. It was found that there was no observable difference in *sigS* expression in this strain when compared to the wild-type (Figure 24A). The *sigS-lacZ*$_{(P1-P4)}$ fusion was then introduced into an SH1000 *agr::tet* mutant strain, and the analysis repeated. Again we found that expression of *sigS* in this strain led to no observable change compared to the parent (Figure 24B). This shows that neither *agr* nor *sigB* individually regulate expression of *sigS*. With this, we then sought to examine *sigS* expression in 8325-4 *agr::tet*, a strain containing both the same *trans* SNP in *sigB* that exists in RN4220 as well as a mutation introduced into *agr*, thereby creating a double mutant. Expression in this strain again showed no observable increases (data not shown). These findings indicate further that the altered expression profile of RN4220 is likely attributed to other SNPs within its genome.

The Observed *sigS* Transcriptional Inducibility is Not Dependent on a Functional σ^B. As demonstrated earlier, the laboratory strain 8325-4 displays inducible *sigS* expression, indicating that there may be characteristics unique to this strain that allow for amplified expression of *sigS*. The closely related strain SH1000 is almost identical to 8325-4, but contains a functional *rsbU* gene (Horsburgh *et al.*, 2002). RsbU is a positive

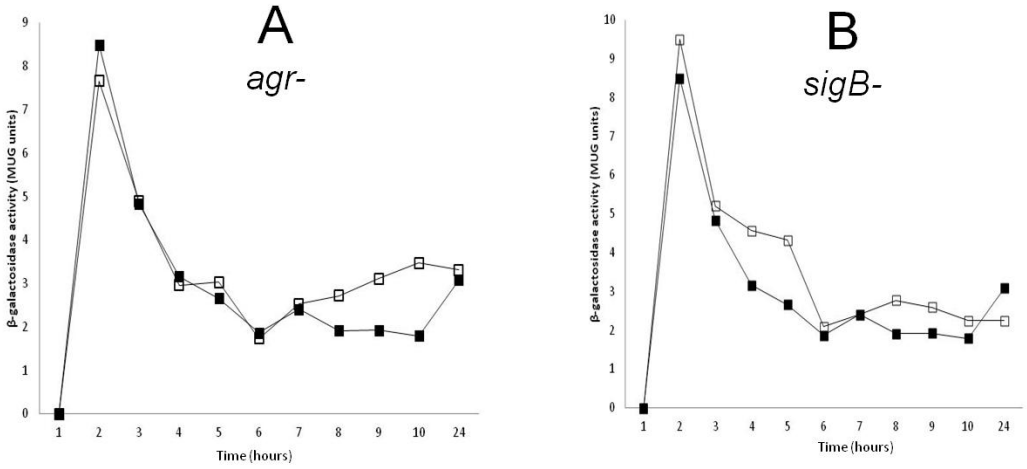

Figure 24. Expression of *sigS* is Not Influenced by the Global Regulators *agr* or *sigB* in *S. aureus*. Mutations in *agr* **(A)** and *sigB* **(B)** were introduced into the SH1000 *sigS-lacZ*$_{(P1-P4)}$ fusion strain. Both the wild-type fusion (■) and the fusion bearing the respective mutation (□) were grown in TSB at 37^{o}C, and sampled every hour for 10 h, and again at 24 h. β-galactosidase activity was measured to determine levels of *sigS* expression. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability.

regulator of the general stress response σ factor, σ^B, which is non-functional in 8325-4 as a result of a naturally occurring 11-bp deletion (Giachino *et al.*, 2001). Strain SH1000 was created by introducing a functional *rsbU* gene into 8325-4, thereby restoring σ^B expression (Horsburgh *et al.*, 2002). Therefore, if the increase in σ^B expression is responsible for decreased *sigS* expression in SH1000, then mutation of *sigB* in SH1000 should restore *sigS* expression to that of 8325-4 levels. As mentioned previously, mutation of *sigB* in the SH1000 background under standard conditions led to no observable increase in expression; however, we sought to examine the expression of *sigS* in this background under inducing conditions. We found, however, that even under inducing conditions, such as growth in MMS, the SH1000 *sigB* mutant strain was not comparable to strain 8325-4 in terms of *sigS* expression, as shown in Figure 25. This further suggests that σ^B is not solely responsible for the differential regulation of *sigS* between these 2 strains. Given recent findings showing a role for RsbU outside of its influence of σ^B activity (Truong-Bolduc and Hooper, 2010), this led us to believe that these differences may be directly related to *rsbU*, the gene disrupted in 8325-4, rather than directly to *sigB*. Therefore, we analyzed the expression of *sigS* in the RN6390 background. 8325-4 and RN6390 are identical, in that they originate from the NCTC8325 strain, have been UV phage cured, and both possess a mutation in *rsbU* (Traber and Novick, 2006). We found the inducible expression profile of *sigS* in RN6390 does not mirror that of 8325-4, indicating that there are likely additional factors beyond *rsbU* that are responsible for the variable expression amongst these strains (Figure 26). As such, it is likely that SNPs may exist in the RN6390 background compared to 8325-4 that have yet to be determined, which may impact *sigS* expression. Indeed, the RN6390

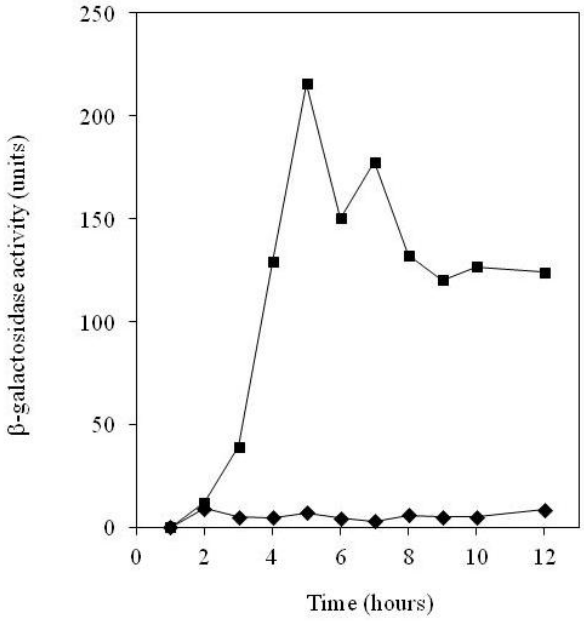

Figure 25. The *sigS* Transcriptional Inducibility is Not Dependent on a Functional σ^B. The *sigS-lacZ*$_{(P1-P4)}$ fusion in 8325-4 (■) and SH1000 *sigB*::*tet* (♦) backgrounds were grown in TSB containing 2.5 mM MMS at 37^{o}C, and sampled every hour for 10 h and again at 24 h. β-galactosidase activity was measured to determine levels of *sigS* expression. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability.

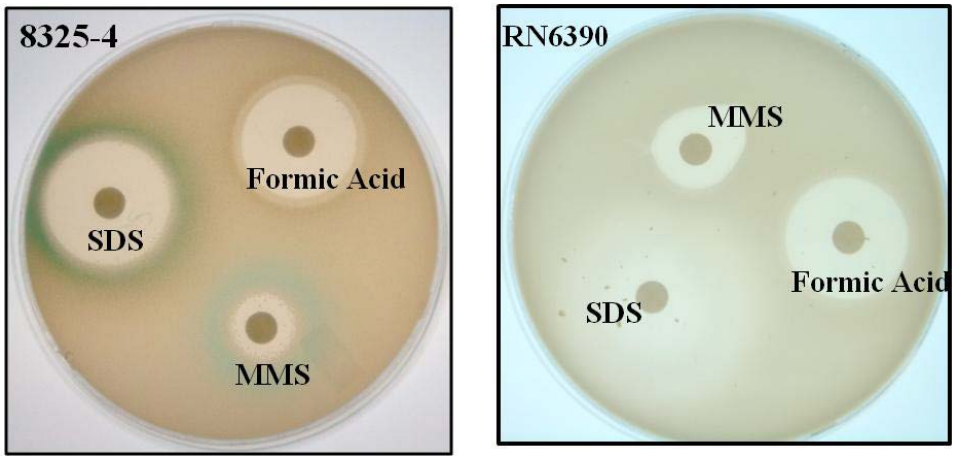

Figure 26. Expression of *sigS* is Not Dependent on *rsbU*. A modified disk diffusion assay was carried out to determine if *sigS* expression is comparable between 8325-4 and RN6390. TSA plates were overlayed with top agar seeded with 0.2 mg X-gal and the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strains in the 8325-4 and RN6390 backgrounds (both *rsbU*$^-$ strains). Sterile filter disks were placed on the solidified agar and 10 µl of appropriate chemical added. The appearance of a blue ring around the zone of inhibition indicates *sigS* activity, which is observed only in the 8325-4 background.

strain was subjected to insertion of a TN*551* containing plasmid and subsequently cured of this plasmid leading to the creation of the current strain; therefore, it is likely that this process allowed for the acquisition of additional SNPs unique to RN6390 (Traber and Novick, 2006).

DNA Sequencing and Alignment of the *sigS* Operon Reveals Only Minor Differences Between Strains of *S. aureus.* In order to evaluate which *cis* modifications, if any, may be attributed to the variable expression observed between *S. aureus* strains, we performed sequence analysis of the *sigS* locus. Specifically, this was performed from 336 nt 5' of the *sigS* start codon to the *sigS* stop codon for strains RN4220, RN6390, 8325-4, SH1000, Newman, USA300 FPR and USA100 (Figure 27). Surprisingly, homology in this region is highly consistent between strains, with only minor deviations occurring in the 8325 lineage strains (RN4220, RN6390, 8325-4 and SH1000) and strain USA100. As displayed in Table 4, the 8325 lineage strains each carry the same mutations in the 11th codon, replacing CAC (Histidine) with CAG (Glutamine), as well as the 16th codon, replacing CAT (Histidine) with TAT (Tyrosine). Additionally, USA100 carries different mutations, in the 63rd codon replacing TTT (Phenylalanine) with TAT (Tyrosine), and in the 111th codon, where CAT (Histidine) replaces CAA (Glutamine). As such, it appears that the *sigS* locus, and specifically the promoter region, is well conserved amongst *S. aureus* strains. Additionally, as there are no unique mutations in those strains exhibiting increased *sigS* expression (RN4220 and 8325-4), the differential expression observed cannot be attributed to SNPs (single-nucleotide polymorphisms) within the *sigS* operon.

Table 4. Genetic Polymorphisms Specific to the *sigS* Locus Between Laboratory and Clinical Isolates of *S. aureus*, Relative to Reference Genome COL.

Strain	Nucleotide Change	Amino Acid Change
NCTC8325 Lineage Strains (RN4220, RN6390, 8325-4, SH1000)	C -> G	$H_{11}Q$
	C -> T	$H_{16}Y$
Newman	NONE	NONE
USA300 LAC	NONE	NONE
USA100	T -> A	$F_{63}Y$
	T -> A	$H_{111}Q$

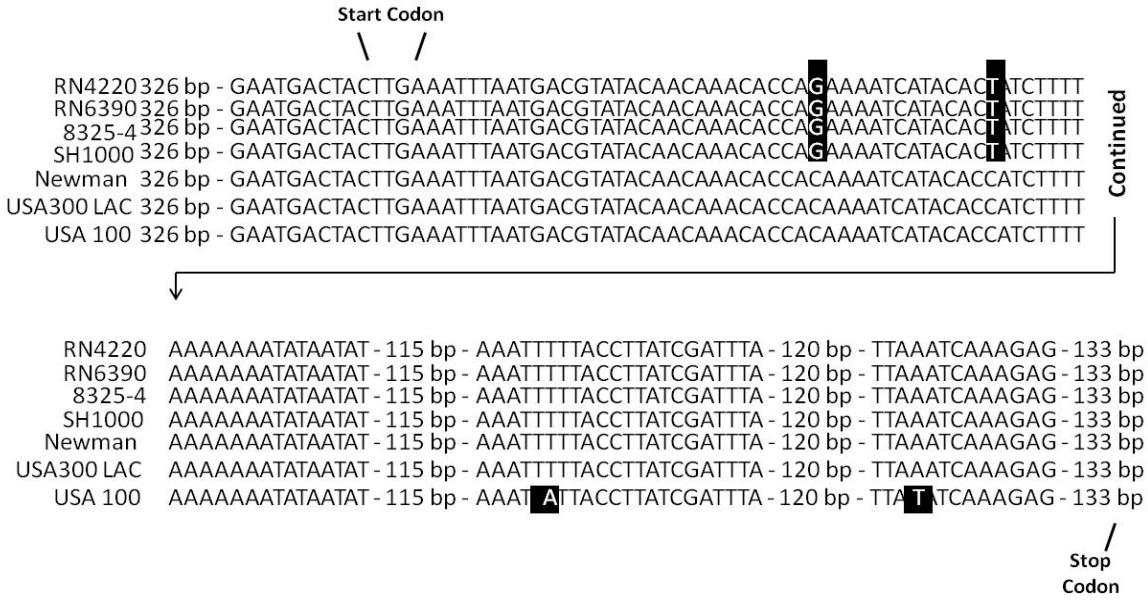

Figure 27. Sequencing Alignment of the *sigS* Locus Reveals no SNPs Accountable for the Variable Expression Profiles Amongst *S. aureus* Strains. DNA sequencing of the *sigS* locus including 336 bp upstream of the putative start codon through the end of the *sigS* gene was performed. Displayed below is the multiple sequence alignment of all strains analyzed. Numbered base pair (bp) labels denote stretches of DNA with no differences in sequence. Black boxes highlight the nucleotides where changes occur and the putative start and stop codons are denoted as such.

Exploration of Additional *sigS* Transcriptional Start Sites as a Result of Environmental Inducibility. As our previous promoter mapping analysis was performed under conditions where *sigS* expression is minimal, we sought to reevaluate the transcription start site under conditions shown to induce *sigS* expression. Evaluation under these conditions may potentially reveal the existence of alternative promoter(s) only active under induction conditions. As done previously, we performed 5' rapid amplification of cDNA ends (RACE) analysis, using the laboratory strain 8325-4, in the presence of 2.5 mM of the DNA damage agent methyl methanesulfonate (MMS) at hour 5, a condition previously determined to elicit maximal expression of *sigS*. Total mRNA was again extracted and *sigS* mRNA transcript converted to cDNA utilizing the 5' end-phosphorylated primer. Subsequently, PCR amplification and TA cloning was carried out as before. A total of 14 independently generated pSC-A constructs were analyzed by DNA sequencing, each of which identified the identical transcriptional start site as before, located 85 nucleotides downstream of the putative start codon (Table 5). We then repeated these experiments in other *S. aureus* strains, in order to ensure that our findings were not specific to 8325-4. In SH1000 in the presence of MMS at 2 h, we again saw 2 of the three sequences identifying the exact +1 as before; however, the third sequence presented a start site 82 nucleotides downstream of the putative start codon.

Discussion. Promoter mapping of the *sigS* locus reveals four discrete transcriptional start sites. Promoters, P1 and P3, both appear to be under the control of the housekeeping σ factor, σ^A, however both are severely corrupted from consensus sequences and/or spacing. Due to the relative weakness of these promoters it is likely that other regulatory

Table 5. Promoter Mapping of the *sigS* Locus. 5' rapid amplification of cDNA ends (RACE) was employed to determine the transcriptional start site(s) of *sigS*. Analysis was carried out in a number of backgrounds and environmental conditions as displayed below.

Strain	Conditions[a]	# Submitted	Sequences Returned[bc]	
SH1000	Standard Conditions 3 h	3	3	(**G**AGTATTATCAACT)
8325-4	2.5 mM MMS 5 h	14	14	(**G**AGTATTATCAACT)
SH1000	2.5 mM MMS 2 h	3	2	(**G**AGTATTATCAACT)
			1	(**GC**TGAGTATTATCAACT)

[a] Conditions utilized are during windows of maximal *sigS* expression as determined via *lacZ* fusion analysis.

[b] Underlined nucleotides represent the experimentally determined +1 of the *sigS* transcript.

[c] Red nucleotides indicate those that differ from the published sequence.

elements must act to activate transcription from these sequences. This likely explains the low levels of *sigS* expression observed in the majority of *S. aureus* strains, and argues for a genetic regulatory network that controls expression of this regulator. In previous work, our group demonstrated that *sigS* can direct transcription from its own promoter in a type of autoregulation akin to many other ECF sigma factors (Helmann, 2002; Missiakas *et al.*, 1997; Shaw *et al.*, 2008). During our mapping of the *sigS* promoters through primer extension analysis, we have identified a potential σ^S driven promoter, P2 (CAAAGT -12 bp- TATCA), with an AAG motif 27 nucleotides upstream of the +1. Typically ECF sigma factor consensus sequences display a conserved AAC motif in the -35 region (Helmann, 2002; Missiakas *et al.*, 1997); however, exceptions exist. Specifically, the ECF sigma factor of *Neisseria gonorrhoeae* does not recognize an AAC motif (Gunesekere *et al.*, 2006), while σ^R of Streptomyces coelicolor recognizes an AAT motif (Paget *et al.*, 2001a). More importantly, σ^X, an ECF sigma factor in several Pseudomonas spp, specifically recognizes an AAG motif, as seen here for σ^S (Brinkman *et al.*, 1999). Furthermore, AAC motifs appear to contribute to the interaction between σ factor and promoter (Lane and Darst, 2006). As such, the imperfect AAG sequence may act as an additional level of regulation for the *sigS* promoter.

ECF σ factors typically have significant divergence, and decreased homology, within their region 2.4 (Lonetto *et al.*, 1992; Missiakas *et al.*, 1997), which specifically recognizes -10 promoter elements. Accordingly, such sites are often difficult to ascertain, however the identified putative -10 element is strikingly similar to the TCTGA recognized sequence of RpoE in *E. coli* (Dartigalongue *et al.*, 2001). Promoter 4 is

unusual in that it lies within the *sigS* coding region; however, this situation is not exclusive to *sigS*, as the ECF sigma factor σ^R of *Streptomyces coelicolor* contains a promoter internal to its coding region as well. This promoter is constitutively expressed through the action of the housekeeping sigma factor, σ^A. This is interesting, as analysis of P4 also reveals a relatively strong consensus sequence for σ^A. During times of induction, σ^R upregulates itself from a promoter located further upstream leading to an elongated σ^R translated from an upstream start codon (Kim *et al.*, 2009). This system may be utilized for *sigS* as well; however, no notable ribosome bindings and start codon pairing could be identified within the *sigS* coding region 3' of P4. Another example of an ECF sigma factor containing a promoter within the coding region is RpoE of *Xylella fastidiosa.* This promoter is recognized by RpoE in order to increase transcription of the cotranscribed downstream gene, encoding its anti-sigma factor, which enables RpoE to negatively regulate its own activity (da Silva *et al.*, 2007). As suggested above, however, the *sigS* P4 promoter contains a notable σ^A motif, more closely resembling consensus than P1 and P2; thereby suggesting that this internal promoter does not exist as a means for negative regulation by σ^S. However, transcript initiated from this promoter potentially includes the downstream gene, *ecfX*, leading to an extended 5' untranslated region, which may serve as an additional level of regulation.

We demonstrate herein that expression of *sigS* is minimal during growth under standard laboratory conditions in strain SH1000 (Shaw *et al.*, 2008). This phenomenon is conserved across a variety of *S. aureus* strains, including laboratory (8325-4, SH1000 and Newman) and clinical isolates (USA300 LAC, FPR and HOU). In each case, we again

observed low levels of expression of *sigS* during growth under standard conditions in rich media. These results may not be surprising, as the majority of ECF σ factors are employed to protect the cell during times of stress, and are often only transcribed when required (Helmann, 2002; Raivio and Silhavy, 2001; Staron *et al.*, 2009). Interestingly, we did observe robust *sigS* expression in the highly mutated laboratory strain, RN4220. Amongst the many mutations present in this strain are those that render the activity of other global regulators nonfunctional, including *agr* and *sigB* (de Azavedo *et al.*, 1985; Traber and Novick, 2006). This increased expression of *sigS* does not appear to be mediated directly through either Agr or σ^B, as we have demonstrated that mutations in these genes alone, at least in the SH1000 background, do not affect *sigS* expression. We mention previously that the laboratory strain 8325-4 contains a naturally occurring 11-bp deletion in RsbU, a positive regulator of the general stress response σ factor, σ^B. Therefore, one would suggest that the differences observed between strain 8325-4 and SH1000 and USA300 LAC are likely attributed directly to the lack of a functional σ^B in strain 8325-4. However, we show herein that mutation of it in the SH1000 background does not restore this strain to 8325-4 levels even in the presence of the inducer MMS. As such, it is possible that as yet unidentified SNPs exist in 8325-4 and RN4220 that lead to its higher activity in these backgrounds. Therefore, based on our findings regarding the differential expression of *sigS* observed across *S. aureus* strains we analyzed the *sigS* promoter region of each strain in order to determine which modifications, if any, may be attributed to the variable expression profile between strains. In each case, we found this region to be highly conserved across all strains, indicating that *sigS* activity amongst

these strains cannot be attributed to SNPs within the promoter or regulatory regions of *sigS*.

Mutation of the *sigS* promoter region cannot account for its increased expression in the RN4220 background. As such, the regulatory circuits in place in this strain are likely to be highly disordered, potentially explaining *sigS* disregulation, and therefore upregulation. Recent sequencing of the RN4220 genome has revealed a total of 121 SNPs (single-nucleotide polymorphisms) and 4 deletions relative to the parent NCTC 8325. Of interest, a number of these are involved in DNA metabolism, replication, recombination and repair (Berscheid *et al.*, 2012; Nair *et al.*, 2011). Most notably, RN4220 carries a SNP in UvrC, a component of the UvrABC exonuclease which in *Escherichia coli* repairs DNA damage induced by a number of mechanisms, including UV light (Sancar and Rupp, 1983). Additionally, SNPs in RN4220 are located in a putative helicase, SAOUHSC_02790 as well as a truncated resolvase, SAOUHSC_02392. Collectively, these observations suggest that RN4220 is perhaps far more prone to DNA damage than other wild-type strains, as a result of mutated and non-functional repair pathways. Indeed we have observed that the varying levels of *sigS* activity between different wild-type strains directly correlates with their sensitivity to DNA damaging agents, such as MMS (Figure 28). As such, this might explain why *sigS* is upregulated in RN4220 at levels greater than other *S. aureus* wild-type strains.

This correlates with our environmental inducibility analysis, which revealed those chemicals that induce DNA damage, such as methyl methanesulfonate (MMS), are the

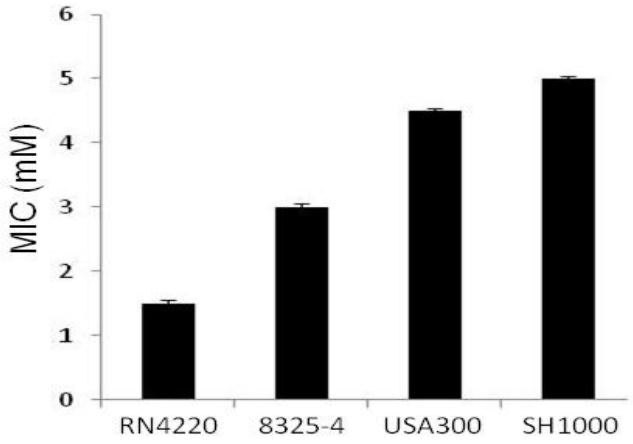

Figure 28. *S. aureus* Wild-Type Strains Exhibit Variable Sensitivity to the DNA Damage Agent, MMS. The RN4220, 8325-4, USA300 and SH1000 strains, were grown in TSB containing increasing concentrations of the DNA damage agent, MMS, in a 96-well plate format. The cultures were allowed to grow overnight at 37^{o}C and subsequently analyzed for the minimum inhibitory concentration (MIC). The data is representative of at least three independent experiments that showed less than 10% variability.

most effective in upregulating *sigS*. Specifically, MMS, an alkylating agent, and EMS, a methylating agent, both result in base mispairing and replication blocks (Beranek, 1990). The damages are often repaired via base excision repair and DNA alkyltransferases (Lindahl and Wood, 1999). Exposure of *S. aureus* to the antibiotics ciprofloxacin and nalidixic acid also lead to strong induction of *sigS*, further supporting the notion that it responds to DNA damage, as these compounds target DNA gyrase, leading to inhibition of DNA replication and subsequent chromosomal damage (LeBel, 1988). Of note, evidence exists that suggests these antibiotics may also lead to DNA damage similar to that observed for oxidative stresses (Gurbay *et al.*, 2006). Interestingly, a number of agents were identified that are not typically thought of as inducing DNA damage, but can also induce this kind of stress. For example, H_20_2 resulted in *sigS* upregulation, and can react with intracellular iron to form hydroxyl radicals, which cause damage to DNA (Chang *et al.*, 2006a; Imlay *et al.*, 1988; Imlay and Linn, 1988; Repine *et al.*, 1981). Further to this, a number of ECF σ factors have been shown to be involved in the response to oxidative stress through cytoplasmic sensing, such as RpoE of *Rhodobacter sphaeroides*, and σ^R from *Streptomyces coelicolor* (Campbell *et al.*, 2007; Dufour *et al.*, 2008; Paget *et al.*, 2001a) As such, it is plausible that σ^S behaves in an analogous manner, by responding to oxidative challenge. Additionally, SOS and DNA damage repair genes have previously been shown in *Escherichia coli* to be upregulated during alkali stress caused by excess NaOH, which also upregulates *sigS* expression (Goodson, 1989; Schuldiner *et al.*, 1986). Finally, the protein synthesis inhibiting antibiotic, chloramphenicol upregulated *sigS*, and has been shown to lead to the degradation of dsDNA and the inhibition of DNA synthesis (Murray *et al.*, 1983).

A consideration with these studies is that a large number of *sigS* inducing agents lead to damage in DNA. Therefore, it is possible that they are not directly upregulating *sigS* expression, but are perhaps causing mutations within the *S. aureus* genome, leading to SNPs. In such a scenario this could result in disregulation of regulatory circuits, leading to *sigS* upregulation in a manner akin to that proposed for RN4220. To examine this, we analyzed *sigS-lacZ* fusion strains exposed to DNA damaging agents for a 24 h period. Upon removal of the stressor, strains were grown on agar plates containing X-gal. We found that no blue coloration was detectable on such plates, indicating DNA damage induced upregulation of *sigS* does not appear to be mediated via heritable SNPs, but results directly from exposure to these agents. As such, the increase in expression of *sigS* is due solely to exposure to agents such as MMS, and suggests that σ^S may be utilized by the cell to adapt during times of DNA damage.

In addition to DNA repair pathway factors, RN4220 also carries mutations in two genes involved in peptidoglycan synthesis, UDP-N-acetylglucosamine 1-carboxyvinyltransferase (*murA*) and a putative UDP-N-acetylmuramyl tripeptide synthetase (Gunetileke and Anwar, 1968; Wickus and Strominger, 1973). MurA is targeted by the antibiotic phosphomycin, and as shown here, *sigS* is induced upon exposure to this drug (Eschenburg *et al.*, 2005). As such, there may be increased instability of the cell envelope in this strain, leading to enhanced *sigS* expression. This is further supported by our studies focused on *sigS* inducibility in response to external stress. Specifically, we observe substantial increases in expression of this element post

exposure to a number of cell-envelope targeting chemicals, such as cefotaxime, ampicillin, oxacillin, phosphomycin and SDS; again suggesting a role for σ^S in protection against this type of stress. These findings may not be surprising as a unifying feature of the ECF sigma factors is involvement in cell-envelope functions, such as secretion, transport and extracytoplasmic stress (Helmann, 2002). Specifically, σ^W and σ^M of *Bacillus subtilis* both respond to cell wall biosynthesis inhibiting antibiotics, and σ^M is important for survival during exposure to phosphomycin (Cao *et al.*, 2002a; Cao *et al.*, 2002b; Thackray and Moir, 2003). Furthermore, the ECF sigma factor, σ^E of *Mycobacterium tuberculosis* responds to challenge by SDS (Manganelli *et al.*, 1999). We also found *sigS* to be upregulated during growth on metal limiting media, and previous reports demonstrate that divalent cations can stabilize the cell wall in Gram positive organisms (Kihm *et al.*, 1994; Marquis, 1968; Murray *et al.*, 1998). As such, the absence of metals may lead to destabilization of the *S. aureus* cell wall, necessitating *sigS* upregulation. Furthermore, a correlation between ECF sigma factors and a requirement for metal ions has been established in *S. coelicolor*, as strains containing a mutation in *sigE* require the presence of divalent cations in the media for normal growth and sporulation (Paget *et al.*, 1999). This expression analysis also corroborates our previous work, which demonstrates that σ^S mutants are more susceptible to Triton X-100 induced lysis (Shaw *et al.*, 2008). Taken together these findings implicate σ^S in the protection of *S. aureus* against cell envelope instability.

Previous reports have demonstrated increased expression of *sigS* during exposure to peracetic acid and berberine chloride (Chang *et al.*, 2006b; Wang *et al.*, 2008). However,

our analysis of the fusion strains in the presence of either chemical led to no observable induction of expression (data not shown), possibly owing to the different strains used between the studies, as we have demonstrated here that expression of *sigS* can vary between *S. aureus* backgrounds. With that said, peracetic acid does lead to oxidative damage (Nde *et al.*, 2011), thus there is a correlation with our findings here that *sigS* may be involved in the response to this type of stress. Additionally, microarray analysis has been carried out by others in some of the conditions that we show here to induce expression of *sigS*; however, those works do not indicate changes in its transcription. Specifically, global transcriptomic analysis of *S. aureus* in the presence of hydrogen peroxide, ciprofloxacin and the cell wall targeting antibiotic, oxacillin has been performed (Chang *et al.*, 2006a; Cirz *et al.*, 2007); however, as we have demonstrated here, *sigS* expression is increase in the presence of these chemicals and it appears to be tightly regulated leading to maximum expression within a certain window, even under inducing conditions. Therefore, as microarray analysis may not have been performed under a period of peak *sigS* expression, its effects on the cell in the presence of these stressors may have been as yet overlooked.

We demonstrate here that *sigS* activity is increased when *S. aureus* is challenged by complement during growth in pig serum. Further to this, we also present evidence for *sigS* upregulation during *ex vivo* infection, as we demonstrate a strong upregulation (regardless of background) upon phagocytosis by murine macrophage-like cells. This supports our previous work, which reveals a major requirement for σ^S during virulence (Shaw *et al.*, 2008). As part of the microbicidal mechanism employed by macrophages,

reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) are excreted at very high levels, leading to DNA damage in invading organisms during infection (Kennedy *et al.*, 1997; Lancaster, 1997; MacMicking *et al.*, 1997; Salgo *et al.*, 1995; Spencer *et al.*, 1996). Moreover, it has been observed that pathogenic organisms such as *Burkholderia* spp., *B. abortus* and *Vibrio cholerae* defective in DNA damage repair pathways are attenuated in virulence, underscoring that pathogenic bacteria are bombarded by DNA damaging conditions during infection (Cuccui *et al.*, 2007; Davies *et al.*, 2011; Roux *et al.*, 2006; Yeager *et al.*, 2001). Together this suggests that, upon entry into the host, bacterial pathogens are faced with an array of DNA damaging conditions, which requires extensive use of repair pathways, and likely goes some way to explaining the avirulence of *sigS* mutants. Furthermore, we have also presented here findings which indicate that *sigS* activity is influenced by cell-envelope stress; a stable cell wall has been implicated in the virulence of many pathogenic organisms, including *S. aureus* (Bae *et al.*, 2004; Miyazaki *et al.*, 2012). In *L. monocytogenes*, penicillin-binding proteins are important for *in vivo* survival of using a murine model of intraperitoneal infection (Guinane *et al.*, 2006). During infection, *S. aureus* is faced with a multitude of stresses; therefore it is likely that the response of *sigS* to both DNA damage as well as cell-envelope destabilization go some way to explaining the virulence defect observed in σ^S mutants.

Through the use of our *lacZ* reporter gene fusions, we have demonstrated that during growth in inducing conditions, the majority of transcription appears to be a direct result of upregulation of the internal promoter, P4. However, upon examination of the *sigS*

locus via qRT-PCR analysis, we find conflicting results in that transcript originating from the upstream promoters (*sigS*$_{(P1\text{-}P3)}$) is only slightly decreased in the RN4220 and 8325-4 backgrounds compared to transcript measured towards the end of the *sigS* coding region (*sigS*$_{(P1\text{-}P4)}$). Furthermore, in the SH1000 and USA300 LAC backgrounds, mRNA levels are comparable across the *sigS* locus, which suggests that transcript originating from the upstream promoters terminates prior to the *sigS* coding region. Specifically, transcript originating from the upstream promoters and continuing through the end of *sigS* will be measured by both the *sigS*$_{(P1\text{-}P3)}$ and *sigS*$_{(P1\text{-}P4)}$ primer pairs; whereas, transcript from P4 will only be measured by *sigS*$_{(P1\text{-}P4).}$ Following this logic, levels of *sigS*$_{(P1\text{-}P4)}$ would be equal to that of *sigS*$_{(P1\text{-}P3)}$ if P4 were not generating transcript. However, if P4 is active as suggested by our *lacZ* fusion analysis, then *sigS*$_{(P1\text{-}P4)}$ should be greater than that of *sigS*$_{(P1\text{-}P3)}$, with the increase being a direct result of transcript from P4. Furthermore, if the upstream promoters are active, as demonstrated here by our qRT-PCR analysis, then the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strain should display increased expression levels with higher levels observed for the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain, owing to added activity from P4. However, our findings were different from expected, in that we were only able to observe increased expression from the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain with no notable increases observed for the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion. A possible explanation for these discrepancies is that the majority of transcription may terminate prior to the *lacZ* gene in the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strain. As displayed in Figure 29, the *sigS* mRNA contains a hairpin loop spanning a 62 nucleotide region beginning 69 nucleotides upstream of the start codon with minimum free energy of -9.09 kcal/mol. In this scenario, the *sigS* mRNA would be detected via qRT-PCR analysis as the beginning of the reverse primer pair is contained

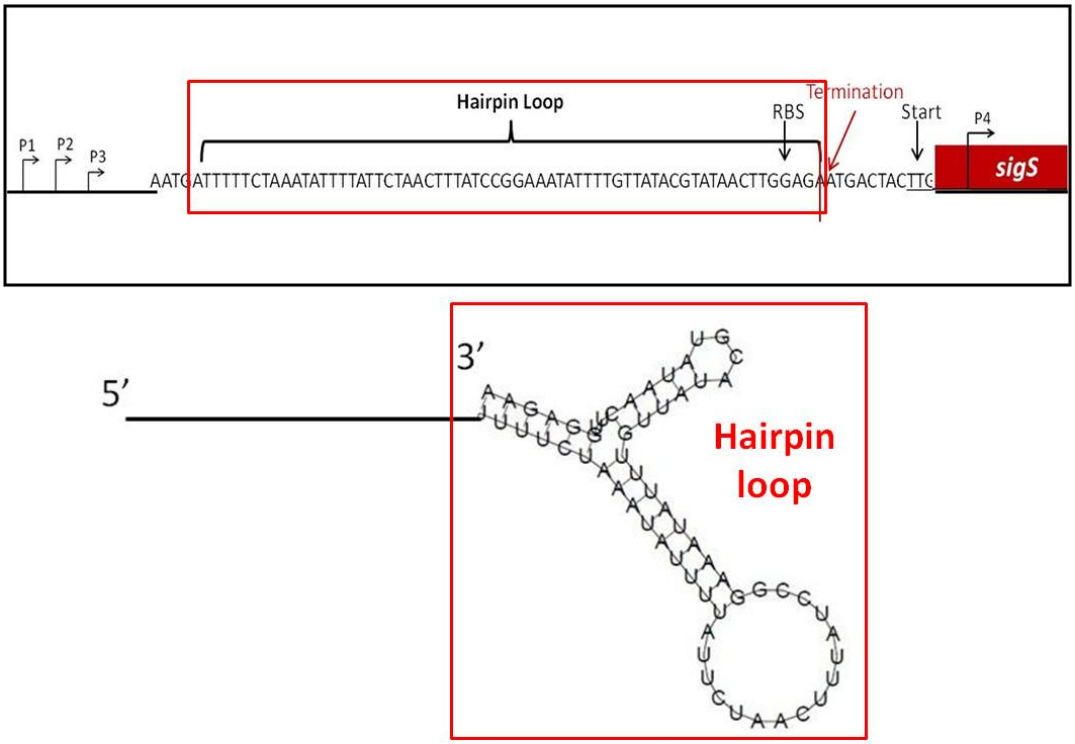

Figure 29. Possible Premature Termination May Result in a Truncated *sigS* mRNA. The predicted structure of the truncated *sigS* mRNA transcript terminated due to the formation of a hairpin loop, with a minimum free energy of -9.09 kcal/mol.

within this region (Figure 30). However, termination would occur prior to the *lacZ* gene, as shown in Figure 31, which would explain why qRT-PCR primer pairs $sigS_{(P1\text{-}P3)}$ and $sigS_{(P1\text{-}P4)}$ display similar levels of transcript, yet the *sigS-lacZ*$_{(P1\text{-}P3)}$ fusion strain is much weaker than that of *sigS-lacZ*$_{(P1\text{-}P4)}$. Such a model of attenuation occurs throughout nature as a means for regulation of gene expression. The classic example of this is the *trp* operon in *E. coli*, where an attenuator lies 3' of the promoters and 5' of the coding region, resulting in premature termination of the transcript in the presence of tryptophan (Morse *et al.*, 1969; Yanofsky, 1981). As such, transcription of *sigS* may be regulated in a similar manner. Further, it is possible that this increased activity from the upstream promoters during exposure to inducing conditions may help drive some transcript through the terminator in order to upregulate *sigS* when needed by the cell.

The higher level of expression observed in both 8325-4 (upon induction) and RN4220 (under standard conditions) appear to be a result of increased transcript across the entire *sigS* locus (from both the upstream P1, P2, P3 and internal promoters, P4), as we observed higher level of transcript in these backgrounds from each of the primer pairs examined, compared to SH1000 and USA300 LAC. The higher level of β-galactosidase activity in the RN4220 and 8325-4 backgrounds can also be attributed to disregulation of the internal promoter, P4. Specifically, in RN4220, there was a difference of 2 fold for the downstream primer pair compared to that of the upstream primer pair. In 8325-4, we observe fold increases between 2 and 5, depending on the conditions tested, for transcript measured by the primer pair located at the end of the *sigS* coding region compared to that of the upstream primer pair. These increases are not found for SH1000 and USA300

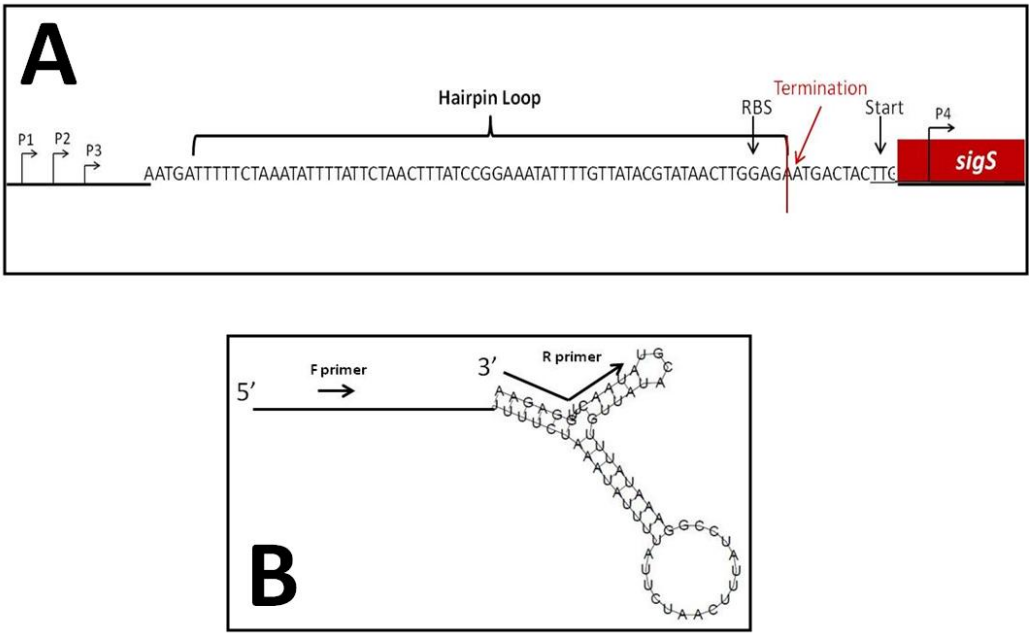

Figure 30. The Terminated *sigS* mRNA Transcript is Recognized by the qRT-PCR *sigS*$_{(P1\text{-}P3)}$ Primer Pair. (A) The *sigS* operon is shown with the upstream promoters, and putative hairpin loop, ribosome binding site, transcription termination site, start site and finally the internal promoter. During transcription, the predicted architecture of the truncated *sigS* mRNA transcript contains a hairpin loop, with a minimum free energy of -9.09 kcal/mol. This structure may lead to premature termination of the *sigS* transcript. **(B)** As displayed above, the *sigS*$_{(P1\text{-}P3)}$ primers are able to amplify this truncated *sigS* transcript due to the location of the primers.

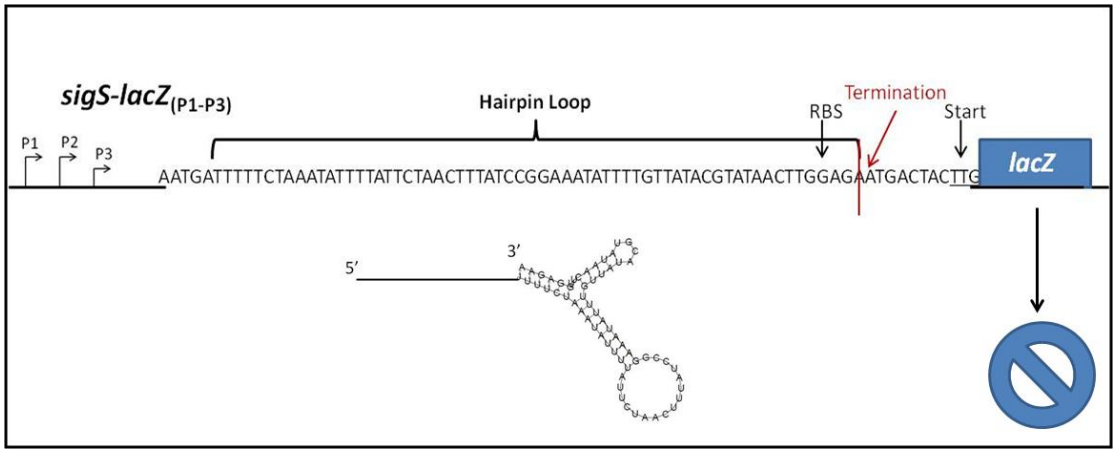

Figure 31. Premature Termination Inhibits Transcription of β-galactosidase in the *sigS-lacZ*$_{(P1-P3)}$ Fusion Strain. The *sigS-lacZ*$_{(P1-P3)}$ fusion is shown above with the upstream promoters and putative hairpin loop, ribosome binding site, transcription termination site and start site labeled. During transcription, the predicted architecture of the truncated *sigS* mRNA transcript contains a hairpin loop, with a minimum free energy of -9.09 kcal/mol. As displayed above, this hairpin formation leads to termination of the transcript prior to the *lacZ* gene. Therefore, no *lacZ* bearing transcript is produced, which may explain the lack of β-galactosidase activity observed for the *sigS-lacZ*$_{(P1-P3)}$ fusion strain.

LAC, as the transcript measured by the two primer pairs is comparable. These findings suggest that higher levels of transcript are generated from the P4 internal promoter than the upstream promoters in the RN4220 and 8325-4 backgrounds. This goes some way to explain why these strains both produce visible blue coloration on TSA containing X-gal under inducing conditions; whereas, the other backgrounds examined do not. This disregulation is likely due to mutation of regulatory networks that control the internal promoter in the RN4220 and 8325-4 backgrounds.

CHAPTER 4:

ANALYSIS OF THE GENETIC REGULATION OF *sigS*

Note To Reader. Portions of these results have been previously published (Shaw *et al.*, 2008) and are utilized with permission of the publisher (Appendix 1).

Background. Alternative σ factors are subject to coordinated and temporal regulation by a number of different mechanisms; in order to ensure their effects are expediently brought about, only when needed. Some σ factors are controlled at the transcriptional level via regulation at key steps during initiation. For example, transcription of the ECF sigma factor, σ^T of *Caulobacter crescentus,* is controlled by the two-component system PhyRK (Lourenco *et al.*, 2011). Further to this, in order to be transcribed, promoters of genes encoding ECF sigma factors must first be recognized by a sigma factor bound to RNAP. In many cases within the ECF subfamily, the ECF sigma factor autoregulates itself, as we have previously demonstrated for σ^S. However, there are examples of ECF sigma factor promoters being recognized by housekeeping sigma factors, other alternative sigma factors (including other ECF sigma factors), or a combination of the above. For example, the downstream promoter for σ^R of *Streptomyces coelicolor* is transcribed by the housekeeping sigma factor, σ^A (Kim *et al.*, 2009), while σ^M of *Corynebacterium glutamicum* is controlled by another ECF sigma factor, σ^H (Nakunst *et*

al., 2007). Further to this, σ factor synthesis is also controlled via mRNA stability and translation efficiency. For example, RpoS of *E. coli* is controlled by three different small non-coding RNAs, OxyS, DsrA and RprA. Both DsrA and RprA interfere with a hairpin structure at the 5' end of the *rpoS* transcript to expose the ribosome binding site for translation (Majdalani *et al.*, 1998; Updegrove *et al.*, 2008). In contrast, OxyS reduces levels of RpoE by a mechanism not fully understood. It has been shown that OxyS may form a complex with *rpoS* and Hfq, a Sm-like protein that has a high affinity for many sRNAs and is required for efficient translation of *rpoS* (Muffler *et al.*, 1996; Zhang *et al.*, 1998; Zhang *et al.*, 2002). Additional regulation of σ factors occurs at the post-translational level through protein-protein interaction, and complex proteolytic cascades. *S. aureus* employs this type of regulation with its general stress response σ factor, σ^B. Specifically, the *sigB* locus consists of *rsbU, rsbV, rsbW* and *sigB* (Kullik and Giachino, 1997; Wu *et al.*, 1996). RsbW acts as an anti-σ factor, which binds free σ^B, thereby blocking its activity (Miyazaki *et al.*, 1999); while both RsbU, a serine phosphatase, and RsbV, an anti- anti-σ factor, act as positive regulators of σ^B (Palma and Cheung, 2001). Regulation occurs as a result of stress, such as low pH, activating RsbU, which in turn dephosphorylates RsbV, leading to interaction with RsbW (Senn *et al.*, 2005). This results in the release and activation of free σ^B. Examples of this type of post-translational regulation are also common within the ECF family of proteins as well. For example, the ECF σ factor RpoE of *E. coli* is transcribed and translated into a functional protein in the cell; yet is sequestered by the cotranscribed anti-σ factor, RseA when its activity is not required. This type of regulation allows for a rapid response to detrimental environmental conditions as functional σ factor protein is already present in the cell and

can become active through a series of rapid proteolytic steps. Specifically, RpoE responds to misfolded proteins in the periplasm as a result of heat shock (Mecsas *et al.*, 1993; Missiakas *et al.*, 1996a; Missiakas *et al.*, 1996b; Missiakas *et al.*, 1997; Rouviere *et al.*, 1995). These proteins are sensed by the serine protease, DegS, which initiates a regulated intramembrane proteolysis (RIP) cascade. DegS first processes the anti-σ factor, RseA, in its periplasmic region in a mechanism known as site 1 cleavage. Subsequently, the remaining RseA becomes a substrate for site 2 cleavage in the inner membrane spanning region by the metalloprotease, RseP. The remaining RseP/RpoE complex is released from the membrane where it is recognized by the adapter protein, SspB, which directs it to ClpXP for final degradation (Ades *et al.*, 1999; Alba *et al.*, 2002; Flynn *et al.*, 2004; Grigorova *et al.*, 2004; Kanehara *et al.*, 2002). At this point, freed RpoE is now able to associate with core-RNAP, directing it to transcribe genes essential for growth during high temperatures, including numerous chaperones and proteases involved in the repair of misfolded cytoplasmic proteins (Erickson and Gross, 1989; Hiratsu *et al.*, 1995; Wang and Kaguni, 1989). The process of ECF σ factor regulation can vary drastically at each stage, from different signaling proteins/proteases, anti-σ factors and adapter proteins involved in regulation. As such, the focus of this chapter is on exploring the regulation of the lone ECF σ factor of *S. aureus*, σ^S. Specifically, we seek to identify the regulatory network involved in its control.

Results

Expression of *sigS* is Positively Regulated by the Downstream Gene, EcfX. Typically, ECF σ factors are cotranscribed with a downstream gene whose product acts as an inhibiting anti-σ factor. Located 113 bp 3' of *sigS* is the gene *ecfX*, whose translational product may fulfill such a function due to its close proximity. To investigate this, we began by examining whether *ecfX* was cotranscribed with *sigS*. To this end, we utilized reverse transcription of total cellular mRNA using primers that would amplify the 113 bp intergenic region between *ecfX* and *sigS*. The primers employed are displayed in Figure 32A, with the forward located 247 bp into *sigS* and the reverse positioned 107 bp into *ecfX*. Analysis showed the presence of a 462 bp band demonstrating that transcript exists between *sigS* and *ecfX*, which is absent in the control lane containing no reverse transcriptase (Figure 32B). Our data shows potential transcriptional linkage of *ecfX* with *sigS*; thus if it functions as an anti-σ factor, then its inactivation should result in free σ^S protein, and upregulation of the *sigS* gene via autoregulation. To this end, we introduced an *ecfX*::*tet* mutation into our *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strains in SH1000, 8325-4 and USA300 LAC backgrounds. Each of the fusions were then grown on TSA containing X-gal, and in each case, no notable increase in *sigS* expression was observed (data not shown). We then performed our analysis in complex liquid media in SH1000, in order to see if there were any alterations in expression not observed on solid media. Analysis showed that the presence of a mutation in *ecfX* led to no change in expression of *sigS* in the SH1000 background under standard conditions (Figure 33A). Next we sought to determine if an *ecfX* mutation had any effect under conditions in which *sigS* is expressed

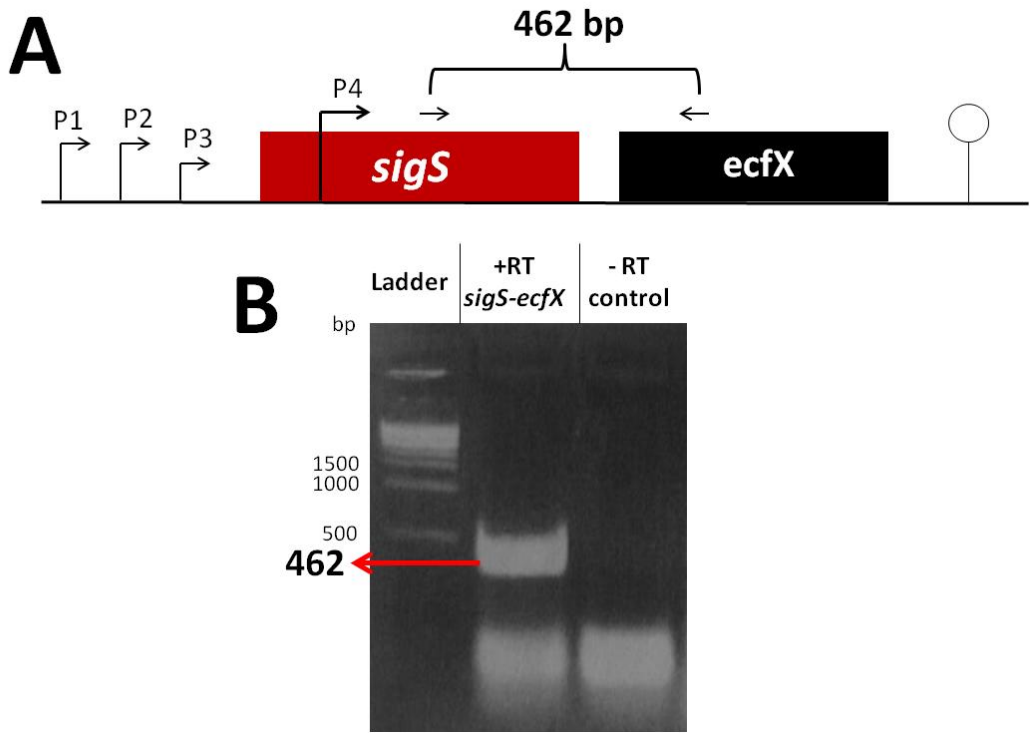

Figure 32. *sigS* and *ecfX* are Cotranscribed. Reverse transcriptase (RT) PCR was performed on total cellular mRNA collected from SH1000 grown under standard conditions to hour 3 utilizing primer pairs encompassing the *sigS-ecfX* intergenic region. (**A**) the *sigS* locus displaying the primer pairs used for reverse transcriptase (RT) analysis; (**B**) a 2% agarose gel showing the resulting RT-PCR product. A 462 bp band corresponding to amplification of the transcript between the displayed primer pairs is highlighted.

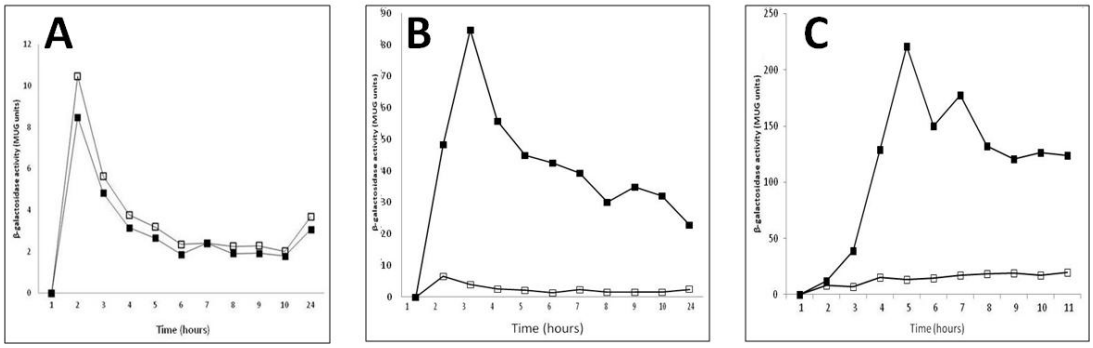

Figure 33. EcfX Acts as a Positive Regulator of *sigS*. The *sigS-lacZ*$_{(P1-P4)}$ fusion wild-type (■) and *ecfX* mutant (□) strains were grown in: **(A)** SH1000 and **(B)** RN4220 in TSB at 37^{o}C; or (**C**) in 8325-4 grown in TSB containing 2.5 mM MMS at 37^{o}C. Each was sampled every hour for 10 h and again at 24 h. β-galactosidase activity was measured to determine levels of expression. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability. Adapted from Shaw *et al*., 2008.

and/or upregulated. Therefore, we repeated these experiments using conditions identified in chapter 3 that lead to increased *sigS* expression. We introduced the *ecfX*::*tet* mutation into our RN4220 *sigS-lacZ*$_{(P1-P4)}$ fusion strain, and, when plated on TSA containing X-gal, a condition previously shown to result in *sigS* expression, we observed no blue coloration of the *ecfX*::*tet* fusion strain (data not shown). Our analysis was then carried out in complex liquid media where we found an approximately 21-fold decrease in *sigS* expression in the *ecfX* mutant background (Figure 33B). We next repeated our transcriptional disk diffusion analyses in 8325-4 using chemicals that upregulate *sigS* expression. In each case, the transcriptional upregulation was abrogated upon mutation of *ecfX* (data not shown). To confirm our findings, we again performed liquid β-galactosidase activity assays in liquid media in the presence of sub-MIC levels of MMS. Mutation of *ecfX* led to an approximately 16.9-fold decrease in *sigS* expression in this analysis (Figure 33C). Due to close proximity of the integration sites for the reporter-fusion and *ecfX* mutation, we next regenerated the *ecfX* mutant strains via an alternative method. Where the RN4220 *sigS-lacZ*$_{(P1-P4)}$ fusion strain had previously been used as the recipient strain for the *ecfX* construct, we created the new strain in the opposite manner, using the fusion as the donor strain. Resulting clones were confirmed and 2 representatives were selected for analysis. We found that regeneration of the strain again led to no detectible upregulation of *sigS* (data not shown). Collectively, this data argues that *ecfX* functions to enhance σ^S expression, rather than repress it.

Induction of *sigS* Expression Can be Achieved Through Random Genome Mutation. Given the inducible nature of *sigS* expression, and the differential expression between

wild-type strains, we hypothesized that genetic components within the *S. aureus* cell serve to modulate expression from this locus. In an effort to test this hypothesis, we assessed whether induction of *sigS* expression could be achieved through the introduction of random mutations throughout the genome, by exposure to the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG). NTG works by introducing base substitutions into DNA during replication, and can also result, albeit rarely, in small deletions. NTG is commonly utilized as a mutagen as it creates a high number of mutations, while maintaining a 50% or greater survival rate of treated organisms (Burns *et al.*, 1988). To this end, an SH1000 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain was grown to mid-exponential phase, at which point NTG was added to a final concentration of 50 μg ml^{-1}, and allowed to incubate at 37^{o}C for 60 min. This was then followed by washing to remove NTG, and growth in TSB at 37^{o}C for a period of 2 h to allow recovery. Following this period, 1 ml aliquots were collected via centrifugation at 3,500 rpm for 10 min and resuspended in TSB containing 20% glycerol, before being stored at -80^{o}C. The CFU ml^{-1} for the culture was determined both prior to NTG exposure and immediately following in order to ensure a survival rate of $\geq$ 50%. For analysis, the frozen samples were allowed to thaw and subsequently plated on TSB containing X-gal, where the appearance of blue colonies indicate increased expression of *sigS* as a result of mutations within the genome. Post exposure, upwards of 10,000 colonies were screened for increased expression of *sigS* based on a blue coloration when plated on X-gal containing TSB. Interestingly, we found no colonies had acquired mutations that led to augmented *sigS* expression (data not shown). As such, a 1 ml aliquot of this first round of treatment was allowed to grow overnight, and was then subjected to a second round of NTG exposure. Examination of

approximately 5,000 colonies from the resulting library following this secondary treatment led to the isolation of 98 colonies expressing high levels of *sigS* (Figure 34A). Two representative clones, as displayed in Figure 34B, were chosen to quantify expression by measuring β-galactosidase levels in complex liquid media. The strains, termed NTG A and NTG B, both resulted in high levels of *sigS* transcription, equating to approximately 75.4-fold higher levels of *sigS* expression at 5h (Figure 34C), when compared to the un-mutated strain. In order to determine if the observed expression increases were due to *cis* SNPs, we transduced the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion from 10 blue NTG isolates into a clean SH1000 background. Transduction of the fusion using bacteriophage phi 11 results in the transfer of approximately 40 kb of additional sequences flanking the fusion. If the increased expression is a result of mutation within the *sigS* locus, or surrounding DNA, then any increase in *sigS* expression will be carried over to the clean background post transduction. We found that transduction of these strains led to a loss of *sigS* expression in each example (data not shown), indicating that any *sigS* modulating SNPs are *trans* acting in nature. As such, in addition to significant environmental inducibility, *sigS* expression is seemingly also controlled internally at the genetic level. This observation likely explains the differential nature of transcription for this element across *S. aureus* strains, perhaps resulting from *trans* SNP variations, or alterations in global regulatory circuits, between isolates.

Exploring the Molecular Mechanisms of *sigS* Regulation. Given our findings regarding the induction of *sigS*, and altered levels of its expression amongst wild-type strains, we hypothesized that regulatory cascades exist to control the expression of this σ

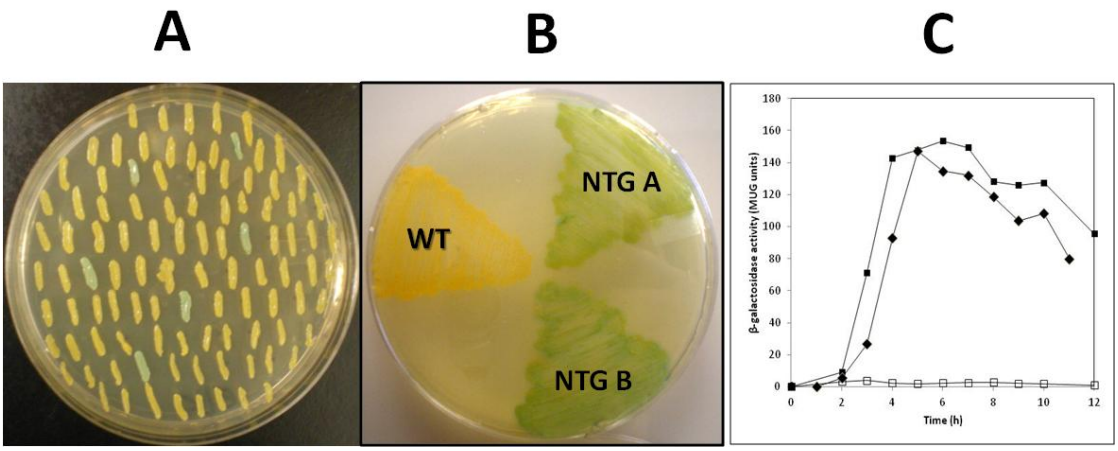

Figure 34. Profiling of *sigS* Expression Following Random Mutagenesis. The SH1000 *sigS-lacZ*$_{(P1-P4)}$ fusion strain was subjected to treatment with the mutagen NTG, and the resulting library of mutants analyzed for increased expression of *sigS* expression via growth on TSA containing X-gal (**A**). Two representative colonies (**B**) were selected for further analysis during growth in TSB at 37^{o}C (**C**). *sigS-lacZ*$_{(P1-P4)}$ fusions in SH1000 (□), NTG A (■) and NTG B (♦) were grown alongside the parent, with samples removed every hour for 10 h, and again at 24 h. β-galactosidase activity was measured to determine levels of *sigS* expression. Assays were performed on duplicate samples and the values averaged. The results presented herein were representative of three independent replicates that showed less than 10 % variability.

factor. Therefore, we performed a transposon screen using Tn*551* and a *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion. We performed this screen using strain 8325-4 as it has already proven to be the most sensitive to *sigS* modulation, and provides results that are conserved in other wild-type strains. The disruption by Tn*551* of a gene involved in the regulation of *sigS* may lead to its upregulation; therefore, analysis of the transposon insertion sites for those strains that display increased *sigS* expression will elucidate potential members of the *sigS* regulatory cascade. During a screen of >10,000 colonies, we identified 123 isolates with increased *sigS* expression. We performed a secondary screen on 96 of these mutants, transducing them into a clean 8325-4 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion background in order to ensure that the observed increases in expression were due directly to disruption by Tn*551* insertion and not a result of indirect effects through accumulation of SNPs during library construction. We again observed blue coloration of each of the strains during growth in the presence of X-gal. The strains were then subjected to sequence analysis to identify sites of insertion that impact *sigS* expression. Of the 96 mutants analyzed, 51 were unique insertions in different genes (Table 6), whilst the rest were duplicate insertions in already identified loci. In order to verify that our findings were not specific to strain 8325-4, we transduced 18 of these Tn*551* mutations into SH1000 and USA300 backgrounds containing the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion. In each case, a blue coloration on X-gal, indicating *sigS* upregulation, was observed (data not shown).

Disruption of DNA Replication, Recombination and Repair Pathways Leads to Increased Expression of *sigS*. We have previously shown that exposure of *S. aureus* to DNA damage inducing chemicals leads to increases in expression of *sigS*. Interestingly,

Table 6. Transposon Insertions Resulting in Increased Expression of *sigS*.

Accession Number	Tn*551* Insertion Site	Gene	Hits[a]	Unique[b]
DNA metabolism: DNA replication, recombination, and repair				
SACOL0566	nucleoside permease	*nupC*	1	1
SACOL1315	DNA mismatch repair protein	*hexA*	1	1
SACOL1382	Exonuclease	*sbcC*	3	2
SACOL1413	Hypothetical Protein (putative		1	1
SACOL1479	putative, 5'-3' exonuclease		2	1
INTERGENIC				
SACOL1412	hydrolase-related protein/ hypothetical		2	1
Regulators				
SACOL0513	transcriptional regulatory protein	*glcT*	1	1
SACOL1136	Hypothetical Protein (nucleic acid		1	1
SACOL1393	transcriptional antiterminator LicT,	*licT*	3	1
SACOL1436	Msa (modulator of SarA)	*msa*	1	1
SACOL2086	transcriptional regulator, TenA family	*tenA*	1	1
Toxin Production and Resistance				
SACOL1441	tellurite resistance protein, putative		3	1
Cell Envelope Associated				
SACOL1411	Aminoacyltransferase	*femB*	1	1
SACOL1472	cell wall associated fibronectin-	*ebh*	6	2
SACOL1490	Penicillin binding protein 2	*pbp2*	1	1
Transporters				
SACOL0682	putative antiporter		1	1
SACOL0684	Na+/H+ antiporter, MnhE component,		2	1
SACOL0993	oligopeptide ABC transporter, ATP-	*oppD*	1	1
SACOL1319	glycerol uptake facilitator protein		5	1
SACOL1392	sodium:alanine symporter family protein		3	1
SACOL1414	peptide ABC transporter, ATP-binding		4	1
SACOL1416	peptide ABC transporter, permease		1	1
SACOL1443	branched-chain amino acid transport	*brnQ3*	3	1
INTERGENIC				
SACOL1441	Tellurite resistance protein, putative /		2	1
SACOL1893	ABC transporter, ATP-binding		1	1
Amino Acid Biosynthesis				
SACOL0168	glutamate N-acetyltransferase/amino-	*argJ*	2	
SACOL1349	Hypothetical Protein (Aspartate		1	1
SACOL1429	Aspartate-semialdehyde dehydrogenase		1	1
SACOL1434	Alanine racemase family		1	1
SACOL1448	2-oxoglutarate dehydrogenase, E2		1	1
SACOL1449	2-oxoglutarate dehydrogenase, E1		3	1
SACOL1478	alanine dehydrogenase		1	1
SACOL2045	Ketol-acid reductoisomerase		1	1

Table 6. Continued.

Accession Number	Tn*551* Insertion Site	Gene	Hits[a]	Unique[b]
	INTERGENIC			
SACOL1447	conserved hypothetical protein/ 2-		1	1
SACOL1449	2-oxoglutarate dehydrogenase, E1	*/arlS*	2	1
	Metabolism			
SACOL2188	lactose phosphotransferase system		5	1
	Protein Synthesis and Modification			
SACOL1369	50S ribosomal protein L33		4	1
SACOL1397	peptide methionine sulfoxide reductase	*msrA*	4	1
SACOL1455	Carboxyl-terminal protease		1	1
SACOL1480	Hypothetical Protein (ribosome		1	1
	INTERGENIC			
SACOL1369	50S ribosomal protien L33/ 30S		1	1
SACOL1396	5' side: fmtC protein; 3' side: peptide	*fmtC*	1	1
SACOL1555	peptidase, M20/M25/M40 family/		1	1
	Biosynthesis of Cofactors			
	INTERGENIC			
SACOL1820	riboflavin biosynthesis protein RibD/1	*ribD*	1	1
	Unknown Function			
SACOL1452	PAP2 family protein	*pap2*	1	1
SACOL1602	metallo-beta-lactamase family protein		1	1
	INTERGENIC			
SACOL1451	DNA-binding response regulator ArlR/	*arlR/*	1	1
	Hypothetical Proteins			
SACOL0940	Hypothetical Protein (No conserved		1	1
SACOL1469	Hypothetical Protein (No conserved		1	1
SACOL1556	Hypothetical Protein (No conserved		1	1
SACOL2642	Hypothetical Protein (No conserved		1	1
	INTERGENIC			
SACOL1445	CbbQ/NirQ/NorQ/GpvN family		1	1
SACOL1620	Conserved hypothetical protein/ CBS		1	1

[a] The hits refers to the total number of Tn*551* insertion sites identified for a particular gene.

[b] The unique number of insertions sites refers to those insertions that are a result of distinctive insertion events.

our screen identified that the disruption of genes involved in the response to DNA damage also resulted in enhanced levels of *sigS* expression. Specifically, insertions were found in two exonucleases, SACOL1479, a putative 5'-3' exonuclease, and SACOL1382, which encodes the SbcC component of the SbcDC exonuclease. Both exonucleases were identified multiple times, two of which were unique insertions into *sbcC*. In *S. aureus*, SbcDC is believed to be involved in the SOS response and aids in survival during UV induced damage (Chen *et al.*, 2007). A mutation was identified in SACOL1413, a putative helicase demonstrating homology to a Snf2 family protein believed to be involved in DNA damage repair (Durr *et al.*, 2005). This mutation was identified by multiple insertions, of which 2 are unique including an intergenic insertion located 5' of SACOL1413. The DNA mismatch repair protein, SACOL1315 or *hexA*, was also identified in this screen. HexA, also termed MutS, is a major component of the methyl-mismatch repair system (O'Neill, 2010). Finally, disruption of SACOL0566, *nupC*, which encodes a nucleoside permease, increased expression of *sigS*. Collectively these findings suggest that *sigS* is employed in response to impairment in the ability of *S. aureus* to effectively repair DNA damage.

A Number of Tn*551* Insertions in *S. aureus* Regulators and Genes Whose Products are Involved in Protein Modification/Biosynthesis Leads to Augmented *sigS* Transcription. Our analysis of *sigS* expression thus far suggests that it is under the control of transcriptional regulation. During our examination of the transposon mutations that lead to increased expression of *sigS*, we found a number of regulators. Specifically, mutation of SACOL0513, encoding GltC, a transcriptional regulator involved in

glutamate synthesis, led to increased expression of *sigS*. Disruption of SACOL1393, which encodes a putative LicT transcriptional antiterminator also raised the level of *sigS* transcript in the cell and was identified multiple times. In addition, disruption of *msa*, a modulator of *sarA* expression (Sambanthamoorthy *et al.*, 2006), led to increased *sigS* transcription, suggesting that it may be a global regulator of gene expression in *S. aureus*. SACOL2086 belonging to the TenA family of transcriptional regulators was also identified in this screen. We have previously shown that expression of *sigS* is increased during exposure to oxidative stress induced by hydrogen peroxide. Interestingly, we found multiple disruptions in the oxidative stress response gene, SACOL1397, which encodes a peptide methionine sulfoxide reductase (MsrA) involved in oxidative stress repair, which led to increased expression of *sigS*. We also identified an insertion in the carboxyl-terminal protease, *ctpA* or SACOL1455 that led to increased expression of *sigS*.

Transcription of *sigS* Increases as a Result of Disruptions in Transporters and Genes Involved in Cell Wall Biosynthesis. Our expression analysis of *sigS* demonstrated inducibility post exposure to cell wall weakening antimicrobial agents. In this screen we find that *sigS* expression is also dependent on a number of cell wall associated proteins. Multiple insertions of Tn*551* into SACOL1319, which encodes a glycerol uptake facilitator protein, increased the level of *sigS* transcript. Insertions were also identified in SACOL1392, a predicted sodium:alanine symporter, which belongs to a family that functions to import L-alanine and Na^+ into the cell (Kamata *et al.*, 1992; MacLeod and MacLeod, 1992). We also identified a disruption in SACOL0684, a sodium/hydrogen antiporter involved in pH and sodium homeostasis (Padan and

Schuldiner, 1993). Transposon insertion into a number of peptide, amino acid and amine ABC transporters also lead to increased *sigS* expression, including SACOL1414 (4 insertions), SACOL1416 and SACOL0993; as well as the branched-chain amino acid transport system carrier protein, *brnQ3*. Further to this, multiple disruptions of *ebh*, a cell wall associated fibronectin-binding protein led to increased expression of *sigS*, of which two were unique insertions. Finally, inactivation of both SACOL1411 (*femB),* as well as *pbp2,* a penicillin binding protein, both of which are involved in cell wall biosynthesis, also affected *sigS* expression.

Elevation of *sigS* Expression is Observed Upon Disruption of Amino Acid Biosynthesis Pathway Genes. Growth of the 8325-4 *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion strain on amino acid limiting media led to an increase in its expression (Chapter 3). In support of this, during our analysis of the transposon screen, we identified a number of insertions into amino acid biosynthesis pathways that also increase the expression of *sigS*. These insertions include SACOL1429, involved in the biosynthesis of the amino acid, aspartate as well as SACOL0168 (*argJ*), encoding a glutamate N-acetyltransferase. Two insertions into genes involved in alanine biosynthesis were identified, including alanine dehydrogenase (SACOL1478), and alanine racemase (SACOL1434). Collectively these findings further suggest the involvement of σ^S in *S. aureus* amino acid biosynthesis.

Validation of Select Tn*551* Transposon Mutants and Their Impact on *sigS* Expression. To ensure that our findings did not result from an artifact of our fusion system, we performed qRT-PCR analysis to quantify *sigS* transcription in three wild-type

strains (lacking the *sigS-lacZ*$_{(P1\text{-}P4)}$ fusion), into which we had transduced representative mutations of *sbcC,* an exonuclease involved in DNA damage repair post UV exposure, *msrA* a peptide methionine sulfoxide reductase involved in repairing proteins following oxidative damage and *msa* a modulator of the global regulator, SarA. Analysis in 8325-4 during exponential growth (3 h, a window of maximal *sigS* expression in RN4220), displayed increases of 3.2-, 3.5-and 4.6-fold for *sigS*$_{(P1\text{-}P3)}$, *sigS*$_{(P1\text{-}P4)}$ and *ecfX* primer pairs, respectively, in the *sbcC* Tn*551* mutant background (Figure 35). Mutation of *msrA* in 8325-4 led to a substantial fold increase of 35 for *sigS*$_{(P1\text{-}P3)}$, yet only fold increases of 2.6 and 3.8 for the downstream primer pairs, *sigS*$_{(P1\text{-}P4)}$ and *ecfX*, respectively. In the presence of a mutation in *msa* in the 8325-4 background, a 5.9-fold increase of *sigS*$_{(P1\text{-}P3)}$ was observed, with fold increases of 3.3 and 4.6 for *sigS*$_{(P1\text{-}P4)}$ and *ecfX* respectively.

The Impact of Tn*551* Transposon Mutants on *sigS* Expression is Conserved Across *S. aureus* Backgrounds. We performed further qRT-PCR confirmation of the transposon screen using the alternate wild-types SH1000 and USA300 LAC to determine if these findings were conserved, as suggested by transduction analysis. In each case, we again observed increased *sigS* expression in the presence of the mutations examined (Figures 36A and 36B respectively). Specifically, upon disruption of *sbcC,* fold increases of 5.6-, 8.6- and 3.9 where noted for *sigS*$_{(P1\text{-}P3)}$, *sigS*$_{(P1\text{-}P4)}$ and *ecfX*, respectively, in the SH1000 background. When using USA300 LAC, we determined fold increases of 9.0-, 12- and 5.9 for *sigS*$_{(P1\text{-}P3)}$, *sigS*$_{(P1\text{-}P4)}$ and *ecfX*, respectively. Using an *msa* transposon mutant, increases for *sigS*$_{(P1\text{-}P3)}$, *sigS*$_{(P1\text{-}P4)}$ and *ecfX* were 4.5-, 6.4- and 4.4–fold, respectively, in SH1000; while the same conditions in the USA300 LAC led to

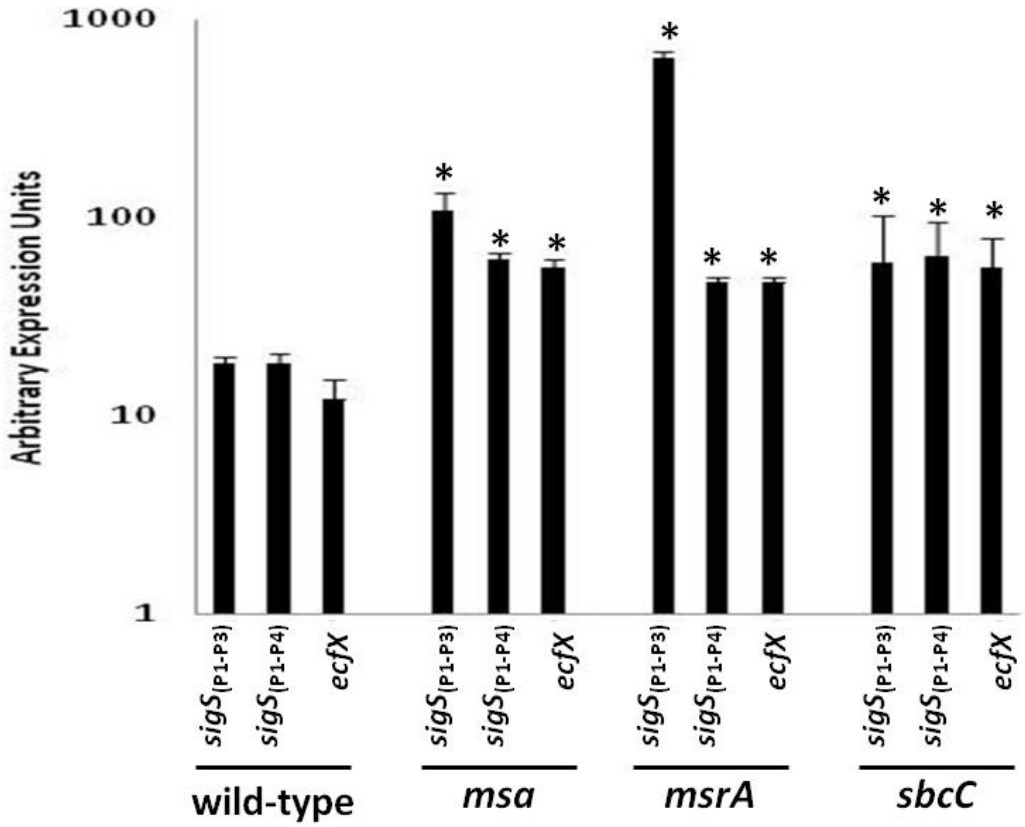

Figure 35. Transcription is Increased Across the *sigS* Locus in 8325-4 *msa, msrA* and *sbcC* Tn*551* Mutants. Quantitative real-time PCR analysis was performed with wild-type 8325-4, or derivatives bearing *msa*::Tn*551*, *msrA*::Tn*551* or *sbcC*::Tn*551* mutations, grown for 3 h in TSB. Three separate primer pairs were used: (***sigS*$_{(P1-P3)}$**) located 47bp upstream of P4, (***sigS*$_{(P1-P4)}$**) located 357bp downstream of the *sigS* translation start site, and (**ecfX**) located 254bp downstream of the *ecfX* translation start site. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test, and represent significant variation from the wild-type.

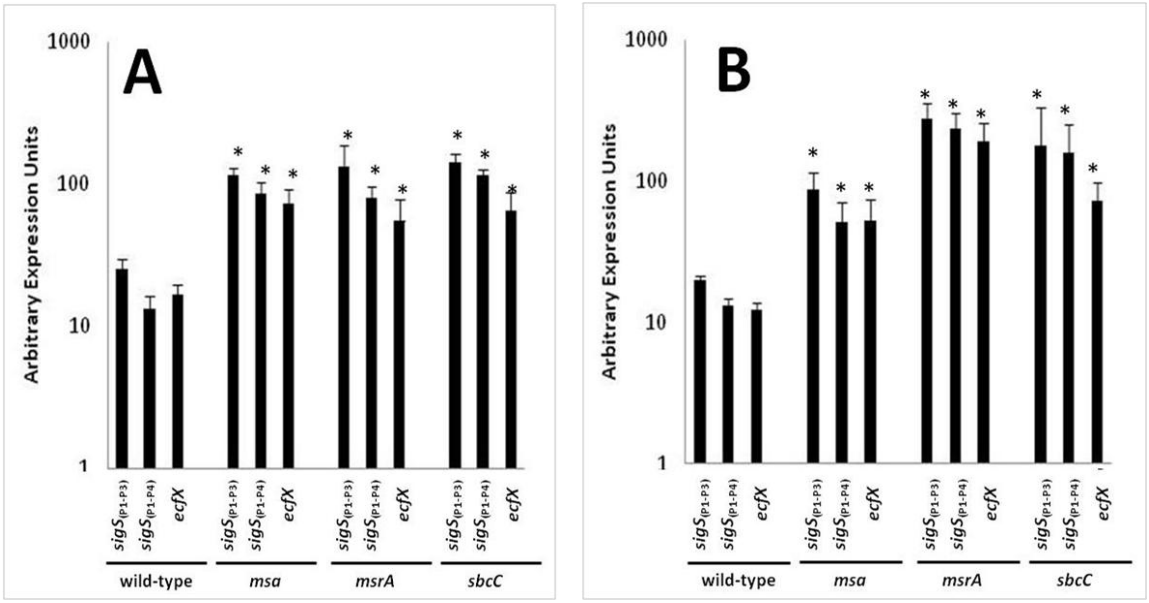

Figure 36. The Impact of *msa, msrA* and *sbc* Disruption on *sigS* Expression is Conserved Across *S. aureus* Wild-Type Strains. Quantitative real-time PCR analysis was performed with **(A)** SH1000 and **(B)** USA300 wild-types, or derivatives of which bearing *msa*, *msrA* and *sbcC* Tn*551* mutations, grown for 3 h in TSB. Three separate primer pairs were used: (***sigS*$_{(P1-P3)}$**) located 47bp upstream of P4, (***sigS*$_{(P1-P4)}$**) located 357bp downstream of the *sigS* translation start site, and (**ecfX**) located 254bp downstream of the *ecfX* translation start site. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test, and represent significant variation from the wild-type.

increases of 4.4-, 3.9- and 4.3-fold. Finally, we examined a mutation in *msrA* and observed fold increases of 5.2, 5.9 and 3.3 for the $sigS_{(P1\text{-}P3)}$, $sigS_{(P1\text{-}P4)}$ and *ecfX* transcripts, respectively, in the SH1000 background. In the USA300 LAC background we observe 14-, 17.6- and 15.5-fold increases in $sigS_{(P1\text{-}P3)}$, $sigS_{(P1\text{-}P4)}$ and *ecfX* respectively. As such, these findings suggest that, in addition to significant environmental inducibility, *sigS* expression is also controlled internally at the genetic level. This observation likely explains the differential nature of transcription for this element across *S. aureus* strains, perhaps resulting from SNP variations, or alterations in global regulatory circuits/signaling pathways, between isolates.

Discussion. As discussed previously, ECF sigma factors are typically sequestered by a membrane bound anti-sigma factor during times of non-induction, in order to keep the sigma factor inactive when not needed by the cell. These anti-sigma factors are typically cotranscribed downstream of the sigma factor. In this work we investigated the gene located immediately downstream of *sigS* as a possible anti-σ factor. This downstream gene, *ecfX*, is atypical of the classic model of anti-sigma factors in that it does not contain any membrane spanning regions. However, examples exist for soluble anti-sigma factors that regulate ECF sigma factors that respond to cytoplasmic stresses, including RpoE from *Rhodobacter sphaeroides*, which is inhibited by binding to a cytoplasmically located, zinc-dependant anti-σ factor, ChrR (Campbell *et al.*, 2007; Dufour *et al.*, 2008). Due to our findings regarding the response of *sigS* to the cytoplasmic stress created by DNA damage, *ecfX* may be an ideal candidate anti-sigma factor. However, we observed that mutation of this gene led to no notable change in *sigS* expression in strain SH1000

under standard laboratory conditions. We then sought to evaluate the effect of this mutation during conditions of *sigS* expression. Our data herein actually demonstrates a requirement for a functional EcfX for expression of *sigS,* suggesting that this gene acts as a positive activator. These findings appear counterintuitive as the general mechanism previously established for ECF σ factors include negative regulation by a downstream co-transcribed gene (Helmann, 2002). Bioinformatic analysis of EcfX reveals a ComK-like domain located in its C-terminus. In *B. subtilis*, ComK acts as a DNA-binding protein involved in the upregulation of itself and other competence genes (van Sinderen *et al.*, 1995). Therefore, EcfX may act as a DNA-binding protein in a manner akin to that of ComK, which would suggest novel regulation of this transcriptional regulator. Additionally, we have sequenced SACOL1828 in each of the backgrounds discussed herein to elucidate whether any SNPs exist that could account for the differential expression exhibited between strains. We have determined that no notable disparity is detectable in the sequence of this gene in any manner (data not shown).

We have previously shown that the highly mutated laboratory strain RN4220 displayed markedly higher levels of *sigS* expression under standard conditions compared to other wild-type strains. We attributed this increase to mutations in regulatory networks that control the expression of *sigS.* As such, we attempted to reproduce these findings by introducing random mutations into the SH1000 background, which displays low levels of *sigS* expression under standard conditions. Such treatment did indeed prove successful, as, post exposure to the alkylating agent NTG, we were able to create a number of SH1000 mutants which display elevated levels of *sigS* expression. We have verified that

these mutations are *trans*, which confirms our hypothesis that regulatory networks are in place in the *S. aureus* cell acting to govern *sigS* expression. In order to elucidate which mutations contribute to this disregulation, we are currently performing whole genome sequencing of strains NTG A and NTG B.

As an extension of this approach, we next performed a transposon mutagenesis screen, which provided evidence not only that *sigS* appears to be regulated genetically within the *S. aureus* cell, but also supporting information for its response to cell-envelope and DNA damage stresses. Specifically, we observed upregulation of *sigS* in multiple *sbcC* insertions, which encodes part of the SbcDC exonuclease. Interestingly, in *E. coli*, this exonuclease functions to repair DNA and maintain genome stability through cleavage of DNA hairpins caused by intrastrand base pairing of palindromic DNA (Chalker *et al.*, 1988; Connelly *et al.*, 1998; Connelly *et al.*, 1999; Leach and Stahl, 1983). Its function in *S. aureus* is not completely understood; however, it has been documented to be induced by the SOS response, and has a role in protection against DNA damage created by UV irradiation (Chen *et al.*, 2007). Previously, *sbcC* as well as a number of DNA damage repair genes were also shown to be induced upon exposure to heat shock in *S. aureus* (Fleury *et al.*, 2009). In our earlier work, we demonstrated a role for σ^S during growth in increased temperatures (Shaw *et al.*, 2008). As such, our current findings suggest that the sensitivity of the σ^S mutant to increased temperatures may be attributed to the DNA damage that occurs during the exposure of *S. aureus* to high heat. Additionally, expression of *sigS* was increased upon disruption of SACOL1479, which encodes another 5'-3' exonuclease and was identified by multiple insertions. While little

is known about this protein, it has been demonstrated that *E.coli* deficient in 5'-3' exonucleases are more susceptible to DNA damage induced by UV exposure (Chase and Masker, 1977). Therefore, it is plausible that this 5'-3' exonuclease and SbcC may be necessary to repair DNA in *S. aureus* that has been damaged by UV irradiation. A mutation was also identified in HexA (also termed MutS), a DNA mismatch repair protein. Disruption of this gene leads to a mutator phenotype in *S. aureus* (O'Neill, 2010), meaning that strains acquire random SNPs at a higher frequency. In *E. coli*, MutS has a role in preventing homologous recombination; however, in *S. aureus* it appears to only have a minor role in this process (Matic *et al.*, 1994; Prunier and Leclercq, 2005; Rayssiguier *et al.*, 1989). Collectively, these findings further suggest involvement of σ^S in DNA damage repair of *S. aureus*.

The nucleoside permease NupC was also identified in our transposon screen as being involved in the expression of *sigS*. NupC from *E. coli* functions to import nucleosides (excluding guanosine or deoxyguanosine), and is dependent on proton motive force for function. These imported nucleosides serve as precursors for DNA, RNA and histidine, as well as other various co-factors (Komatsu, 1973; Munch-Petersen *et al.*, 1979; Saier, 2000). Additionally, *nupC* is repressed by CytR, a regulatory protein important for expression of genes involved in nucleoside utilization (Munch-Petersen and Mygind, 1976). These findings would suggest that *sigS* is utilized by *S. aureus* in response to a limitation in the acquisition of exogenous nucleotides. Another gene in our screen with multiple insertions was *msrA*, which encodes a peptide methionine sulfoxide reductase. This protein is widely conserved across all forms of life and functions to reduce

methionine sulfoxide in oxidatively damaged proteins to methionine, for repair purposes (Boschi-Muller *et al.*, 2005; Lowther *et al.*, 2002; Weissbach *et al.*, 2002; Weissbach *et al.*, 2005). In Chapter 3 we demonstrate the increased expression of *sigS* in the presence of the oxidative stress inducing chemical H_2O_2. This perhaps suggests a role for σ^S in the response to oxidative stress, which, as detailed earlier, may also subsequently lead to DNA damage (Singh *et al.*, 2001). Of note, another ECF sigma factor, encoded by *Neisseria gonorrhoeae*, has previously been shown to have a role in regulation of *msrA* (Gunesekere *et al.*, 2006). Disruption of SACOL1602, encoding a metallo-beta-lactamase protein, led to increased expression of *sigS*. This is not the first example of a correlation between this type of gene and ECF sigma factors; σ^P of many Bacillus species is also involved in the control of β lactamase genes (Ross *et al.*, 2009). Furthermore, this protein contains a domain that demonstrates homology to a predicted exonuclease, and it is characteristic of proteins in this group to possess functional nuclease activity. Indeed, a number have been identified as possessing a role in RNA and DNA metabolism (Dominski, 2007). Additionally, downstream of SACOL1602 are genes that encode competence proteins; therefore this transposon insertion may also affect expression of these genes. It has been demonstrated in *Bacillus subtilis* that DNA damage-inducible genes, as well as other SOS response genes, are transcriptionally active during the development of competence (Love *et al.*, 1985; Lovett *et al.*, 1989). Thus, there may also be a link between *S. aureus* competence genes and DNA damage that is mediated by σ^S.

Further analysis of *sigS* expression through the transposon screen reveals mutations in a number of genes involved in cell-envelope metabolism and integrity. These findings

further support our expression data, which suggests involvement of σ^S in the extracytoplasmic stress response, similar to that of other ECF sigma factors. Specifically, we identified disruptions in cell wall associated genes, as well as other genes involved in cell-envelope metabolism, all leading to increased expression of *sigS*. These include *ebh*, a cell wall associated protein of *S. aureus*, which binds human fibronectin and is involved in cellular adhesion during infection (Clarke *et al.*, 2002). Also identified was a mutation in *pbp2*, which is one of 5 such proteins in *S. aureus*, involved in catalyzing the transpeptidase reaction during cell wall synthesis (Georgopapadakou and Liu, 1980a, b; Waxman and Strominger, 1983; Wyke, 1984). In *S. aureus*, Pbp2 has been shown to contribute to β-lactam resistance, and we have demonstrated here that *sigS* expression is increased in the presence of the β-lactam antibiotics cefotaxime, ampicillin and oxacillin (Chambers and Miick, 1992; Leski and Tomasz, 2005). Further to this, a glycerol uptake facilitator protein, SACOL1319 was identified in this screen, and previous work has demonstrated the importance of glycerol for the synthesis of membrane components, therefore disruption of this gene may lead to a weakening of the cell wall (Ray *et al.*, 1972; Ray and White, 1972). The aminoacyltransferase, FemB, encoded by SACOL1411, was also identified as having an effect on expression of *sigS*, and is involved in cell wall assembly as it is aids in the formation of the pentaglycine cross-bridge (Henze *et al.*, 1993). Further to this, inactivation of *femB* leads to increased susceptibility of *S. aureus* to β-lactam antibiotics, which again also leads to expression of *sigS*. As stated previously, our prior work suggests a role for σ^S during the survival of *S. aureus* to cell-envelope stress, specifically from Triton X-100 induced lysis. Collectively, these findings may explain our current data regarding *sigS* upregulation in both the

presence of cell-envelope targeting chemicals, as well as the absence of genes involved in cell-envelope stability (Shaw *et al.*, 2008).

Additionally, an insertion was identified in SACOL0682 encoding a Na+/H+ antiporter, as well as in SACOL1392, which is a predicted sodium:alanine symporter belonging to a family that functions to import L-alanine and Na^+ into the cell (Kamata *et al.*, 1992; MacLeod and MacLeod, 1992). As alanine is an important component of teichoic acids and other cell envelope constituents, the absence of this transporter may limit the availability of this amino acid within the cell, possibly hampering cell wall turnover or increasing pressure for *de novo* alanine biosynthesis. An additional connection between amino acid biosynthesis and *sigS* expression was also identified through insertion into SACOL1429, involved in the biosynthesis of the amino acid, aspartate. Insertion in SACOL0168 (*argJ*), which encodes a glutamate N-acetyltransferase was also identified. In *Bacillus stearothermophilus* it is involved in catalyzing the reaction between glutamate and acetylglutamate during arginine biosynthesis, and between glutamate and aceytlornithine in ornithine biosynthesis (Sakanyan *et al.*, 1993). As *argJ* is encoded on a transcript upstream of *argB*, an acetylglutamate kinase, it is possible that the transposon insertion into *argJ* leads to polar effects on *argB*; however, both genes encode proteins involved in the same pathway. These findings suggest that σ^S may be involved in amino acid metabolism. Additionally, we have shown earlier in this work that *sigS* expression is increased during growth in amino acid limiting media. As such, these findings may explain our earlier study that details an importance for functional σ^S during extended survival under starvation conditions (Shaw *et al.*, 2008). Mutation of SACOL0513

encoding GltC, a transcriptional regulator, led to increased expression of *sigS*. In the closely related organism, *B. subtilis*, GltC is a member of the LysR family of transcriptional activators, and serves to upregulate *gltA* and *gltB*, genes encoding glutamate synthase, further supporting a role for σ^S in amino acid biosynthesis (Bohannon and Sonenshein, 1989; Henikoff *et al.*, 1988; Schell, 1993). Disruption of SACOL1393, which encodes a putative LicT transcriptional antiterminator raised the level of *sigS* transcript in the cell. In the previous chapter, we identify a potential hairpin located 5' of the *sigS* coding region, as such this may play a role in regulating termination of the *sigS* transcript. Another regulator possibly involved in the expression of *sigS* is SACOL2086, belonging to the TenA family of transcriptional regulators, which was also identified in the screen. Collectively, this further supports a role for σ^S in amino acid biosynthesis and elucidates a number of potential *sigS* transcriptional regulators.

As discussed earlier, ECF sigma factors are subject to post translational regulation typically through a complicated proteolytic cascade involving cleavage of a membrane spanning anti-sigma factor. Through the use of our transposon screen, we identified two membrane proteins that potentially play a role in the regulation of *sigS*. The first of which, Msa, was previously determined to play a role in modulating expression of SarA, a global regulatory protein involved in virulence determinant expression (Sambanthamoorthy *et al.*, 2006). Interestingly, Msa contains three membrane spanning regions and when mutated leads to increased expression of *sigS*. As such, this protein may serve as a potential anti-sigma factor for σ^S, whereby disruption of it would lead to an increase in free σ^S within the cell, and subsequently an increase in its expression

through autoregulation. Additionally, a membrane spanning, C-terminal protease was also identified in our screen as potentially regulating *sigS* expression. This serine protease contains a conserved DegS domain, and, in *E. coli*, DegS initiates the proteolytic cascade to free the ECF sigma factor RpoE during the response to heat shock (Grigorova *et al.*, 2004). As such, this protease may serve in the regulatory pathway of σ^S.

CHAPTER 5:

FUNCTIONAL ANALYSIS OF σ^S

Note To Reader. Portions of these results have been previously published (Miller *et al.*, 2012; Shaw *et al.*, 2008) and are utilized with permission of the publisher (Appendix 1).

Background. ECF sigma factors often respond to detrimental environmental conditions brought on by a variety of stresses, ranging from heat shock to oxidative stress. During this response, the sigma factor binds core-RNAP and redirects the complex to upregulate genes that would otherwise remain inactive. These genes comprise a specific regulon, and subsequently help the cell combat stress (Helmann, 2002). In *B. subtilis*, the ECF sigma factor, σ^W, is expressed during growth in the presence of cell wall targeting antibiotics such as vancomycin; thus mutation of this gene leads to decreased survival during growth in the presence of these antibiotics. The reason for this is that σ^W is no longer able to direct transcription of its regulon, which includes *pbpE* (a penicillin-binding protein involved in cell wall biosynthesis) and *fosB* (a fosfomycin resistance enzyme), which help protect the cell during growth under these conditions (Cao *et al.*, 2001; Huang *et al.*, 1999). In *S. coelicolor*, σ^R responds to diamide and subsequently upregulates genes involved in both thiol metabolism, as well as those genes whose products produce the major thiol buffer, mycothiol (Newton *et al.*, 1996; Paget *et al.*,

1998; Paget *et al.*, 2001b; Park and Roe, 2008). In the context of pathogenic organisms, and survival within the harsh environment of the host, the regulon of a number of ECF sigma factors include genes important for disease causation and virulence (Bashyam and Hasnain, 2004). For example, the ECF sigma factor AlgU of *P. aeruginosa* upregulates the alginate synthesis gene cluster, including *algD*, the first in a 12-gene operon that encodes GDP mannose dehydrogenase, an enzyme involved in the conversion of GDP mannose into GDP mannuronate, the precursor for alginate polymerization. Activation of these alginate biosynthesis genes by AlgU subsequently produces the mucoid capsule (Hershberger *et al.*, 1995; Martin *et al.*, 1993; Yu *et al.*, 1995), which is important for full protection of the bacterium from antibiotics, oxidative stress and factors of the immune system. These mucoid enclosed bacteria can persist in human lungs, and cause high mortality rates in patients suffering from conditions such as cystic fibrosis (Govan and Harris, 1986). Furthermore, RpoE in *S. typhimurium*, amongst other genes, upregulates *htrA*, which is an extracellular protease important in virulence. As a result, mutations in RpoE decrease invasion of, and replication in, macrophages and epithelial cells (Chatfield *et al.*, 1992; Humphreys *et al.*, 1999; Johnson *et al.*, 1991). In the context of this study, we have shown previously, and herein, that expression of the lone ECF sigma factor of *S. aureus*, *sigS*, is minimal under standard conditions, except in the highly mutated laboratory strain RN4220. We were, however, able to identify a number of conditions that led to upregulation of *sigS*, including exposure to DNA damaging agents and cell wall destabilizing antimicrobial compounds; as well as challenge by components of the innate immune system. As such, the first objective of this aim is to analyze the sensitivity of *sigS* mutants to those chemicals shown to induce its expression.

Additionally, we will perform microarray analysis in order to elucidate the σ^S regulon in an effort to understand how it aids in circumventing stress. We have also previously demonstrated a role for σ^S in systemic *S. aureus* infections (Shaw *et al.*, 2008); therefore, we will continue to explore the involvement of this factor in the pathogenesis of *S. aureus* and investigate the mechanisms by which it contributes to disease causation.

Results

RN4220 *sigS* Mutants Have a Growth Defect Upon Exit From Stationary Phase. Examination of the *sigS* mutant was first conducted through growth curve analysis under standard conditions in order to determine whether loss of this gene leads to a defect in growth. Analysis was carried out in the 8325-4, SH1000 and USA300 LAC backgrounds and OD_{600} readings were taken every hour for 6 hours, and again at hour 24. As displayed in Figure 37A, mutation of σ^S led to no notable defects in growth across each of the backgrounds examined. These findings are perhaps not surprising as we have previously demonstrated that expression of *sigS* is minimal in these strains under the conditions examined. We did, however, demonstrate that *sigS* is expressed under standard conditions in the highly mutated laboratory strain, RN4220. Therefore, we repeated our analysis using a *sigS* mutant in this background. Mutation of σ^S in RN4220 resulted in a significant growth defect, which occurs as stationary phase cells are introduced into new growth media, prior to exponential phase (Figure 37B). At hour 2 we noted a 4.4-fold decrease in optical density of the *sigS* mutant compared to the wild-type, which peaks at hour 3 with a fold change of 5.3. This trend continues through hour

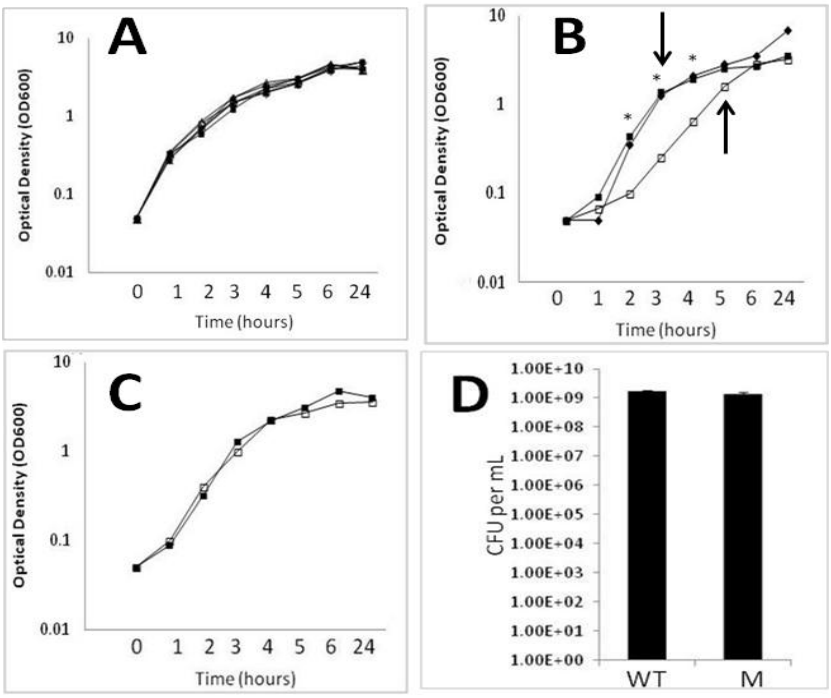

Figure 37. RN4220 *sigS* Mutants Have a Growth Defect Upon Exit From Stationary Phase. (A) Optical density readings (OD_{600}) of the wild-type (filled symbols) and *sigS* mutant strains (empty symbols) were taken every hour for 6 hours, and again at 24 h. Growth curve analysis was performed in 8325-4 (■), SH1000 (♦) and USA300 LAC (▲) backgrounds during growth at 37°C with shaking in TSB. **(B)** RN4220 wild-type (■), *sigS* mutant (□) and the *sigS* complemented strain (♦) were grown at 37°C with shaking in TSB, with growth monitored via OD_{600} every hour for 6 h, and again at 24 h. Growth curves are representative of at least three independent experiments that showed less than 10 % variability. * = $p<0.05$ using a Student *t* test and indicate significant difference in growth between the *sigS* mutant and its parental and complemented strains. **(C)** RN4220 (■) and its *sigS* mutant (□) were sub-cultured from mid exponential phase cells (denoted by arrows in B) and grown at 37°C with shaking in fresh TSB, with growth monitored via OD_{600} every hour for 6 h, and again at 24 h. Growth curves are representative of at least three independent experiments that showed less than 10 % variability. **(D)** Viability (CFU/mL) of stationary phase cells was determined via serial dilution of the RN4220 wild-type (WT) and *sigS* mutant (M) strains after growth at 37°C with shaking in TSB for 24 h. Adapted from Miller *et al*., 2012.

4 with a fold decrease of 3; however by hour 5, growth of the *sigS* mutant is comparable to that of the parent strain. Growth of the *sigS* complement strain was comparable to that of the wild-type at all time points, as displayed in Figure 37B. This is particularly interesting as *sigS* expression peaks at hour 3 in RN4220, which corresponds to the time point at which we observe the highest fold decrease in growth for the mutant strain. This deficiency seemingly occurs only upon the introduction of stationary phase cells into fresh media, as RN4220 *sigS* mutant cells sub-cultured from mid exponential phase display growth rates comparable to that of the parent strain, as displayed in Figure 37C. Examination of the viability of stationary phase RN4220 *sigS* mutant cells compared to the wild-type reveals equivalent CFU per mL values after 24 h of growth (Figure 37D), suggesting that the observed growth defect was not a result of fewer viable inoculating cells for the *sigS* mutant. Collectively this suggests that σ^S has a role upon exit from stationary phase in RN4220.

Disk Diffusion Analysis Reveals Similar Sensitivities Between the σ^S Mutant and Wild-Type Strains. In order to determine the conditions under which σ^S aids in the protection of the cell, we performed disk diffusion analysis using a variety of stress inducing compounds. This assay was carried out as a rapid means to assess a variety of chemicals in order to determine their effect on the σ^S mutant compared to the wild-type. TSA plates were overlayed with top agar seeded with either the wild-type or σ^S mutant strains. Sterile filter disks were placed on the solidified agar and 10 µl of appropriate chemical added. The conditions tested include those chemicals shown to increase *sigS* expression based on our transcriptional analysis, and are displayed in Table 7. The

Table 7. Stress Compounds Used in Disk Diffusion Analysis.

Stress	Agent	Concentration[c]	8325-4[a]		SH1000[a]		USA300 LAC[a]	
			WT[b]	M[b]	WT[b]	M[b]	WT[b]	M[b]
Acid	Hydrochloric Acid	6 M	16	16	12	12	13	13
	Phosphoric Acid	10 M	28	28	16	17	17	17
	Formic Acid	12 M	27	28	13	16	24	23
	Acetic Acid	1 M	6	6	6	6	6	6
	Sulphuric Acid	12 M	25	26	25	21	24	24
	Nitric Acid	6 M	13	13	30	28	15	16
	Trichloroacetic Acid	12 M	14	14	12	13	16	16
Alkali	Sodium Hydroxide	3 M	11	11	9	9	8	8
Osmotic	Sodium Chloride	1 M	6	6	6	6	6	6
	Glucose	1 M	6	6	6	6	6	6
Alcohol	Ethanol	100%	6	6	6	6	6	6
	Methanol	100%	6	6	6	6	6	6
	Isopropanol	100%	6	6	6	6	6	6
Detergent	Sodium Dodecyl Sulfate	10%	20	21	18	18	16	16
	Triton X-100	1%	6	6	6	6	6	6
	Tween-20	1%	6	6	6	6	6	6
	N-lauroyl Sarcosine	1%	20	20	15	15	18	17
Oxidative	Hydrogen Peroxide	30%	50	51	45	43	47	47
	Methyl Viologen	2 M	8	6	30	27	25	24
	Menadione	1%	35	35	27	27	24	24
	Pyrogallol	4 mg	11	11	6	6	9	9
Nitrosative	Sodium Nitroprusside	1M	6	6	8	8	6	6
DNA Damage	Methyl Methanesulfonate	50 mM	13	13	6	6	9	9
Antibiotics	Penicillin G	20 µg	50	50	45	45	47	47
	Chloramphenicol	50 µg	36	36	34	35	34	34
	Phosphomycin	20 µg	20	20	30	27	25	26
	Vancomycin	20 µg	16	15	7	7	15	14
	Spectinomycin	50 µg	20	20	12	13	11	11
	Ampicillin	1 mg	47	50	40	40	11	11
	Erythromycin	50 µg	33	33	35	35	35	35
	Lincomycin	250 µg	36	36	35	35	35	35
	Kanamycin	500 µg	27	27	23	23	22	22
	Neomycin	500 µg	25	25	24	24	23	23
	Puromycin	250 µg	27	27	24	25	18	18
	Mupirocin	20 µg	37	37	36	36	34	34
Disulfide	Diamide	500 mM	13	12	15	17	11	11
Miscellaneous	Berberine Chloride	128 µg	11	11	12	12	11	11
	Peracetic Acid	4.2 M	39	40	38	40	30	31

[a]Numbers represent the diameter of the zone of inhibition. All numbers displayed are in millimeters (mm).
[b]Analysis was performed utilizing both wild-type (WT) and *sigS* mutant strains (M).
[c]Numbers displayed are the final concentration added to the filter disk.

resulting zones of inhibition were measured and analyzed for an increase or decrease in diameter, indicating sensitivity or resistance to the chemical respectively (Figure 38). Disk diffusion analysis was carried out in the 8325-4, SH1000 and USA300 LAC backgrounds and subsequently revealed no notable difference in the susceptibility of the *sigS* mutants to the tested chemicals across each of the backgrounds examined (Table 7). These findings indicate that there seems to be little to no requirement for σ^S in the conditions tested; however, as these analyses were conducted during stationary phase, examination of earlier growth phases may be required to elicit a response. Furthermore, we find in our laboratory that these assays are far more qualitative rather than quantitative in nature, and were therefore used as an initial screening method.

***S. aureus sigS* Mutants are Sensitive to DNA Damage.** Thus far we have demonstrated that chemicals known to induce DNA damage stress as well as genes/proteins known to mediate this response, strongly impact *sigS* transcription. Beyond the growth defect observed for the RN4220 *sigS* mutant, we have yet to determine whether these inducing conditions lead to notably impaired growth phenotypes. We next sought to perform death-curve kill studies to examine the viability of *sigS* mutants during exposure to lethal concentrations of these agents in strains 8325-4, SH1000 and USA300. Therefore, we performed assays in liquid media, exposing exponentially growing cultures to 5X the MIC of select chemical stressors, and monitoring their viability over time. This was initially performed by spot plating 5 µl aliquots of 10^{-1} through 10^{-6} dilutions onto TSA, both prior to and 30 min post exposure of cells to 25 mM MMS. We found that under these conditions, there was a decline in mutant cell viability across strains 8325-4,

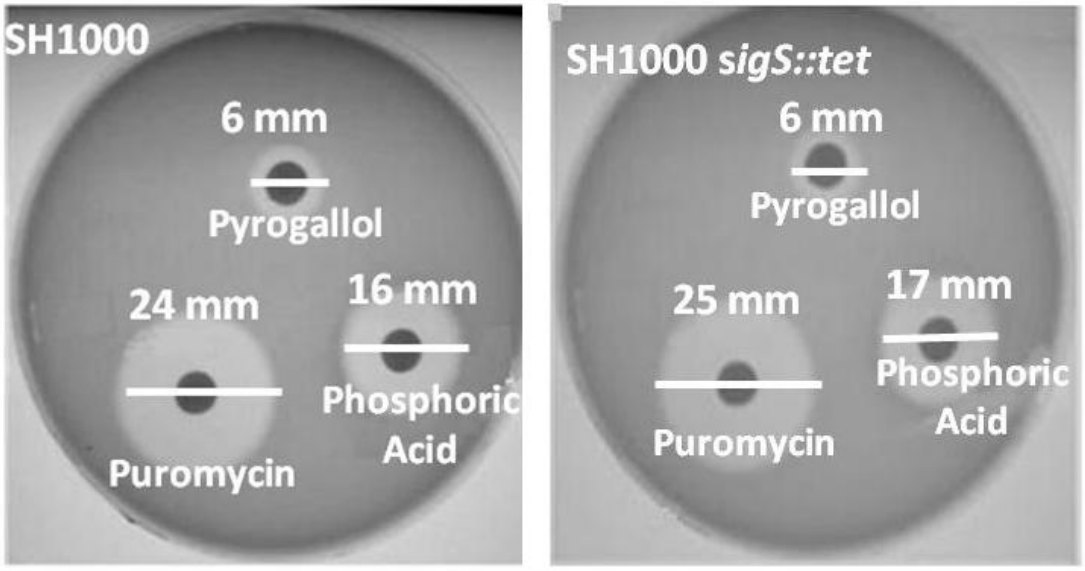

Figure 38. Disk Diffusion Analysis Reveals no Discernible Differences in Growth Upon *sigS* Deletion. Disk diffusion assays were carried out to determine stress conditions that lead to variations in the survivability of the σ^S mutant compared to the wild-type strain in the 8325-4, SH1000 and USA300 LAC backgrounds, SH1000, which is representative, is displayed here. TSA plates were overlayed with top agar seeded with either SH1000 wild-type or SH1000 σ^S mutant strains. Sterile filter disks were placed on the solidified agar and 10 µl of appropriate chemical added. The resulting zones of inhibition were measured in mm, and analyzed for an increase or decrease in diameter indicating sensitivity or resistance to the chemical, respectively. This data is representative of at least three independent experiments that showed less than 10 % variability.

SH1000 and USA300 LAC (Figure 39). We then repeated this analysis in the USA300 LAC background, and performed CFU per mL determinations both pre- and post exposure in order to quantify this decrease in viability (Figure 40A). Upon examination, we recovered 3.33-fold less *sigS* mutant cells compared to the wild-type, following exposure to this agent. Complementation of the σ^S mutation reduced the observed growth impairment significantly, although not completely to that of wild-type levels. This lack of full complementation is likely attributed to the instability of the plasmid in the presence of DNA damaging conditions, as suggested by others previously (Bouanchaud *et al.*, 1968; Hashimoto *et al.*, 1964; Nakamura, 1990; Salgo *et al.*, 1995; Voureka, 1952; Willetts, 1967). Our previous investigations into the downstream gene, *ecfX* as a potential anti-sigma factor led to the finding that it likely acts as a positive activator of *sigS*. Therefore, if *ecfX* fulfills such a function, then any phenotypes observed for the *sigS* mutant should be reproducible in the *ecfX* mutant strain as well. As such, our analysis included evaluation of a strain bearing a mutation in *ecfX* in an effort to validate our previous transcriptional findings. We found that 30 min post exposure to MMS, we did observe a decrease in the ability of the *ecfX* mutant to survive compared to the wild-type, however, the decrease was only 1.8 fold, and thus not as substantial as that observed for *sigS* alone. Complementation of *ecfX* resulted in a fold decrease of 1.1 in sensitivity, again possibly owing to plasmid instability described earlier.

In order to determine whether the role of σ^S was limited to protection against DNA alkylation (as induced by MMS), we next examined the ability of the USA300 σ^S mutant to survive exposure to agents that induce other types of DNA damage. As such, analysis

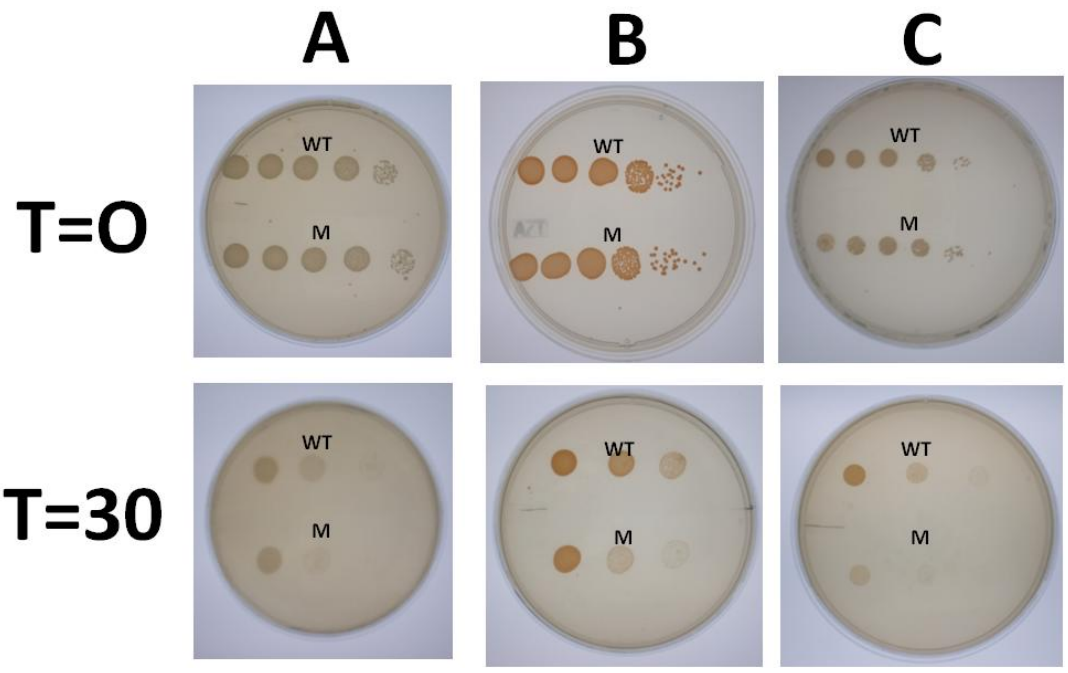

Figure 39. *sigS* Mutants are Sensitive to DNA Alkylation Damage. The wild-type (WT) and *sigS* mutant (M) strains were analyzed for viability in the presence of the DNA damage inducing agent, MMS (25 mM). Spot plating of 5 µl aliquots of dilutions 10^{-1} through 10^{-6} on TSA was performed both pre- and post-exposure. Analysis was conducted using **(A)** 8325-4, **(B)** SH1000 and **(C)** USA300 LAC.

was carried out for oxidative stress resulting from the addition of 5 mM H_2O_2. Following a 5 min exposure to this agent we observed 3.9- and 1.6-fold decreases in *sigS* and *ecfX* mutant viability, respectively, compared to the parent strain (Figure 40B); which was completely complementable in both cases. We next used 5 mM of the DNA intercalating agent ethidium bromide (EtBr), and found that the *sigS* mutant displayed a 2.1–fold decrease in viability, while the *ecfX* mutant resulted in a 1.6-fold decrease compare to the wild-type strain after 15 min of exposure (Figure 40C). We again saw that complementation was able to abrogate these affects; however, in the case of the *sigS* mutant it was not completely to levels of the wild-type strain. This is likely attributed to the ability of EtBr to cure plasmids upon exposure, as observed by others previously (Bouanchaud *et al.,* 1968). In order to determine if σ^S mediates protection against UV induced lesions and double strand breaks, we compared the survivability of the wild-type strain and its *sigS* and *ecfX* mutants in response to this stress. Exponentially growing cultures were serially diluted on TSA and subjected to UV at a dosage of 4,000 μJ cm^{-2}. Exposure at this level resulted in a 2.1- decrease in viability for the *sigS* mutant, whilst there was only a negligible decrease for the *ecfX* mutant (Figure 40D). Complementation in this assay is not possible because of plasmid instability, as we observed >83% loss upon exposure (data not shown). These assays were repeated in the presence of other DNA damage stressors, listed in Table 8, to ensure that the observed effects were due to the specific action of the drug on the cell, and not a result of an indirect effect of the chosen chemical. To this end, we repeated the spot plating method as described before with an alkylating agent (in addition to MMS): ethyl methanesulfonate. We also used the intercalating agent acridine orange, and the UV mimetic 4-nitroquinoline-1-oxide. All of

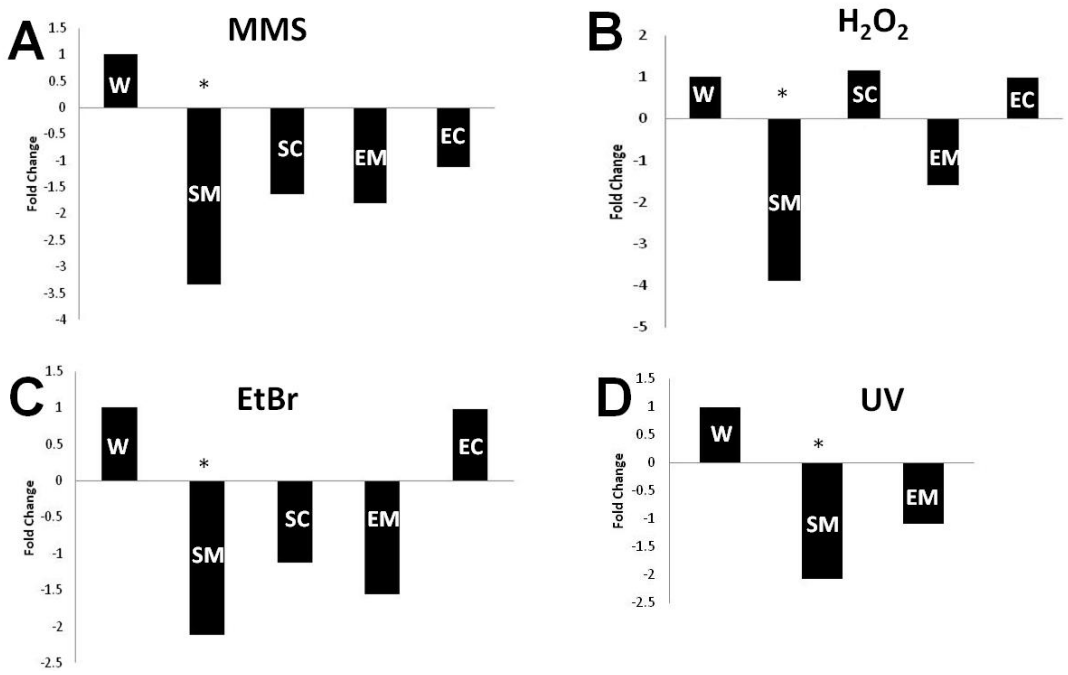

Figure 40. *sigS* Mutants are Sensitive to a Variety of DNA Damage-Inducing Stresses. The USA300 LAC wild-type (W), *sigS* mutant (SM), *sigS* complement strain (SC), *ecfX* mutant (EM), and *ecfX* complement strain (EC) were analyzed for viability in the presence of DNA damage inducing stressors. CFU counts were determined both pre- and post-exposure, and the survivability determined. The data is presented as fold change relative to the wild-type strain and is representative of at least three independent experiments that showed less than 10 % variability. (**A**) 30 min exposure to 25 mM MMS (**B**) 5 min exposure to 150 mM H_2O_2 (**C**) 15 min exposure to 5 mM EtBr (**D**) exposed to UV at 4,000 μJ per cm^2. * = $p<0.05$ using a Student *t* test. Adapted from Miller *et al*., 2012.

Table 8. DNA Damage Inducing Chemicals Utilized for Death Curve Analysis.

Stress	Agent	Concentration
Alkylates DNA	Methyl Methanesulfonate (MMS)	25 mM
	Ethyl Methanesulfonate	25 mM
	Nitrosoguanidine	0.04 ng mL^{-1}
Oxidative Damage	Hydrogen Peroxide (H_2O_2)	150 mM
Intercalating Agents	Ethidium Bromide (EtBr)	5 mM
	Acridine Orange	100 mM
Causes Double Strand Breaks and Lesions	Ultraviolet Light (UV)	4,000 µJ cm^{-2}
	4-nitroquinoline-1-oxide	450 mM

these agents resulted in sensitivities similar to that displayed for the conditions above (Figure 41).

Competitive Growth Analysis of the σ^S Mutant Against the Wild-Type. Thus far, we have demonstrated that a growth defect exists for the *sigS* mutant strain compared to the wild-type in the RN4220 background. Additionally, we see increased sensitivity of the *sigS* mutant during growth in lethal concentrations of a range of DNA damaging agents. With this in mind, we next sought to examine whether the mutant would survive when grown together with the wild-type. To this end, coculture studies comparing the viability of the *sigS* mutant to its parental strain were undertaken, both under standard conditions, and in the presence of chemical stressors. These assays were performed by taking exponentially growing cultures of both the wild-type and *sigS* mutant, and inoculating them into fresh media at a 1:1 ratio. This ratio was established by inoculating the media with both the wild-type and *sigS* mutant at an OD_{600} of 0.05. To ensure accuracy of this ratio, CFU per mL was determined for both the wild-type and *sigS* mutant at hour 0 via serial dilution and plating on both TSA, which allows the growth of both wild-type and the *sigS* mutant, and TSA containing tetracycline, which allows for growth of only the *sigS* mutant. The cultures were allowed to grow under the respective conditions, at which point viability was determined through serial dilution and plating as described above. As demonstrated in Figure 42, evaluation after a period of 24 hours resulted in a 3.6-fold decrease (ratio of 1:0.28) in viable σ^S mutant cells compared to the wild-type. This finding was further exacerbated after 7 days of growth, resulting in a 25-fold decrease (ratio of 1:0.04) in mutant cell viability. Following this, both strains were subjected to

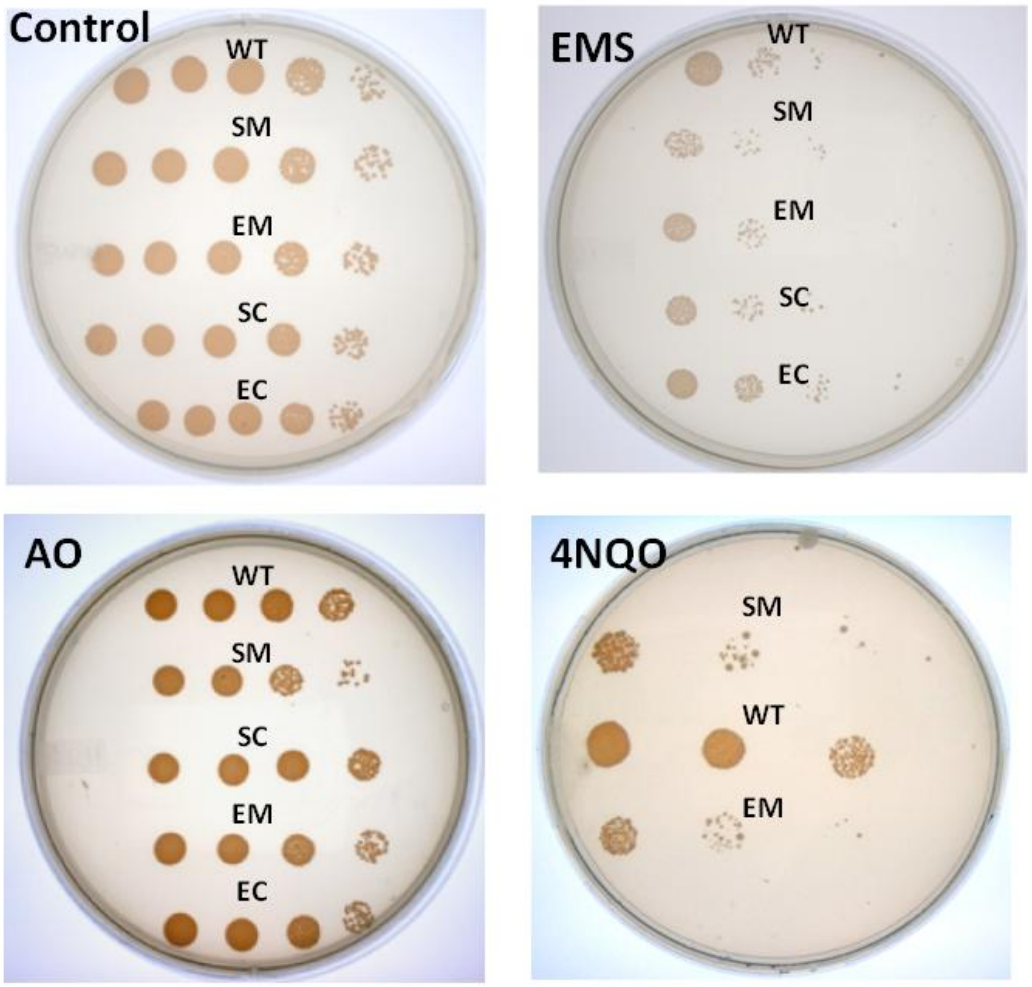

Figure 41. *sigS* Mutants are Sensitive to a Variety of DNA Damage-Inducing Stresses. The wild-type (WT), *sigS* (SM) and *ecfX* (EM) mutant strains along with the *sigS* and *ecfX* complements (SC and SE) were analyzed for viability in the presence of DNA damage inducing agents. Spot plating of 5 µl aliquots of dilutions 10^{-1} through 10^{-6} on TSA was performed both pre- and post-exposure. Analysis was performed: **(Control)** prior to exposure, **(EMS)** 30 min post exposure to 25 mM ethyl methanesulfonate (EMS), **(AO)** 1 h post exposure to 100 mM acridine orange (AO), and **(4NQO)** 1 h post exposure to 450 mM 4-nitroquinoline-1-oxide (4NQO).

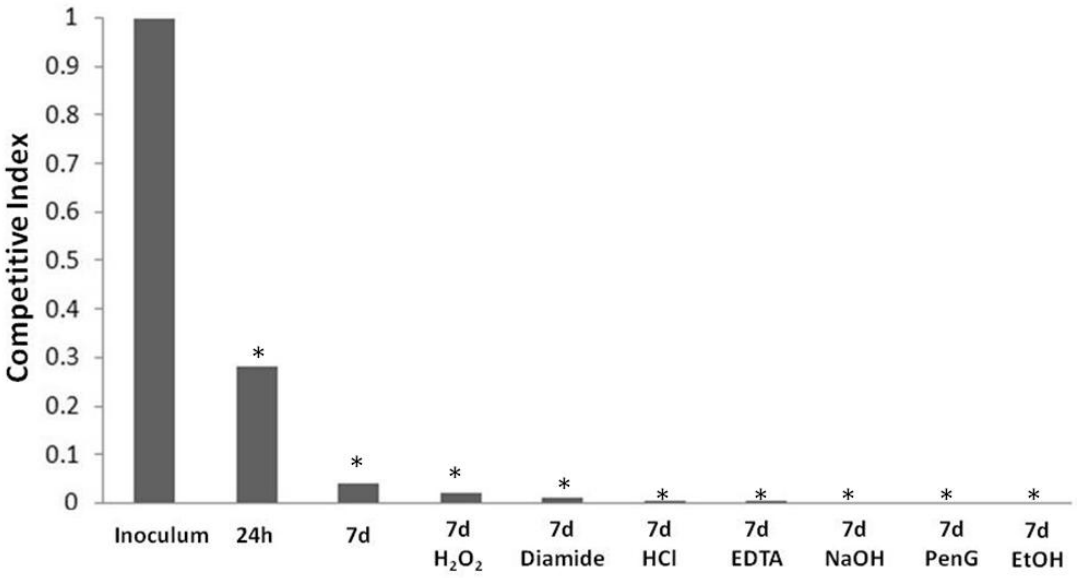

Figure 42. Competitive Growth Analysis of the *sigS* Mutant. SH1000 and its *sigS* mutant were cocultured in TSB for a period of 24 hour (24h) or 7 days (7d). Additionally, cocultures were performed in TSB containing subinhibitory concentrations of: hydrogen peroxide (1 mM), diamide (1.5 mM), HCl (10 mM), NaOH (10 mM), EDTA (0.1 mM), penicillin G (0.01 µg ml^{-1}) or ethanol (5%). The competitive index (CI) was determined for each strain after the respective growth periods, and represents the relative proportion of the two strains after inoculation at a 1:1 ratio. Data is indicative of at least 3 independent cultures that showed less than 10 % variability (Shaw *et al*., 2008).

similar testing, however sub-MIC concentrations of a variety of stress chemicals were also included in the growth media. In doing so, it was found that upon exposure to the oxidative stress chemicals hydrogen peroxide (1mM) and diamide (1.5 mM) for 7 days, a fold decrease of 50 (1:0.02) and 100 (1:0.01), respectively, was observed. When subjected to growth in 10 mM HCl, 0.1 mM EDTA or 10 mM NaOH an even greater reduction in *sigS* mutant survivability was observed, with fold decreases of 200 (1:0.005), 333.3 (1:0.003) and 1666 (1:0.0006), respectively. Finally, and most notably, we were unable to recover any viable *sigS* mutant cells after 7 days in the presence of the cell wall affecting chemicals Penicillin G (10 ng ml^{-1}) and ethanol (5%). These findings demonstrate a drastic decrease in the ability of *sigS* mutant to outcompete its parental strain, indicating that it likely contributes to starvation survival, and overall fitness of the cell.

Exploring the Role of σ^S Using Microarray Analysis. Thus far we have demonstrated that expression of *sigS* is significantly increased following DNA damage stress, cell wall destabilizing conditions and challenge by components of the innate immune system. We have shown that mutation of genes/proteins involved in DNA damage repair pathways, transport, regulation, membrane stability and amino acid biosynthesis also enhances *sigS* transcription. Further to this, we present evidence to suggest a role for σ^S in the protection of *S. aureus* against DNA damage created by a number of different chemicals/conditions. In order to understand the mechanistic contribution of σ^S to these processes, we next performed transcriptomic analysis to determine which genes are controlled by this regulator. This was undertaken in the RN4220 background, as *sigS* is

expressed in this strain under standard conditions. As noted earlier, the RN4220 *sigS* mutant displays an increased lag phase during growth in TSB. Therefore, in order to correct for this, 1 ml of overnight cultures of the wild-type and mutant strains was used to inoculate fresh TSB and allowed to grow to an OD_{600} of 1.0. This was achieved more rapidly by the wild-type strain (3 h), than the mutant (5 h). These were then used to inoculate fresh TSB to an OD_{600} of 0.05, before being allowed to grow for 3 h (a window of maximum *sigS* expression). After this time, both strains were found to grow equally well; therefore RNA was extracted from these samples, and used for microarray analysis. These studies were performed in triplicate for both the wild type and *sigS* mutant using the A-AFFY-147 Affymetrix GeneChip *S. aureus* Genome Array. Genes that demonstrated a 2-fold or greater variation in expression, and were deemed significant by *t*-test ($p \leq 0.05$) analysis, were submitted to the NIAID DAVID bioinformatic resource for pathway and gene ontology analysis (Table 9). In total, 52 genes were identified with altered expression of 2-fold or greater and statistical significance of $p \leq 0.05$. Surprisingly, 46 of these were negatively regulated by *sigS* and only 6 were positively affected by it. Genes found to be upregulated in the *sigS* mutant include those whose products form the cell envelope, as well as a number known to be involved in virulence (Table 10). Of the cell envelope genes that were induced, a number of lipoproteins and capsule genes appear to be increased in the absence of σ^S. Additionally, *lacA, lacB* and *lacD* of the lactose operon are increased in the mutant, with fold changes of 3.4-, 5.9- and 3.5-fold respectively. Furthermore, there is a 4.5-fold increase in transcription of the staphyloxanthin biosynthesis protein, which forms the carotenoid pigment that protects *S. aureus* from reactive oxygen species through its antioxidant properties (Clauditz *et al.*,

2006; Liu *et al.*, 2005). Of note, there was a 3.6-fold increase in the thermonuclease precursor, *nuc*, which, when cleaved and activated, forms NucB (a membrane protein) and NucA (a secreted protein). Both of these enzymes participate in the hydrolysis of nucleic acids, possibly serving as a method for nucleotide salvage from the extracellular milieu (Cuatrecasas *et al.*, 1967; Davis *et al.*, 1977; Okabayashi and Mizuno, 1974). An increase was observed in a number of *agr* regulated virulence determinants including a truncated beta-hemolysin as well as secreted proteases *splB, splC, aur, sspA* and *sspB*. We were only able to identify a decrease in 6 genes in the *sigS* mutant background, 3 of which are hypothetical proteins (Table 11). Two of these, SAV1031 and SAV2430, displayed fold decreases of 3.2 and 2.5, respectively, in the mutant. These are both hypothetical proteins that appear only in *S. aureus* and contain no conserved domains. The third, SACOL2433 demonstrated a decrease of 2.1-fold, and is homologous to a putative lipoprotein in *S. capitis*. A 2.5-fold decrease in the fermentative protein D-lactate dehydrogenase gene, SACOL2535, was also identified in the mutant strain. A fold decrease of 2.0 was observed for SACOL0799, which encodes a transferrin receptor. As the name suggests, this protein acts to recognize and bind transferrin, which is a human iron-transporting molecule, in an effort to scavenge iron from the environment during infection (Modun *et al.*, 1998). Finally, a 4.0-fold decrease was found for SACOL1729, encoding a threonyl-tRNA synthetase. Such extensive negative regulation by σ^S is unlikely to be the result of direct influence on these loci, as σ factors only mediate positive-regulation directly; therefore it suggests indirect control through other factors. When analyzing the complete gene set, no regulatory elements were immediately apparent, possibly suggesting that this finding is mediated via small RNAs. Given that

Table 9. Ontology Table of Genes Affected by σ^S Identified via Microarray Analysis.

Down-Regulated Ontologies	Number of Significant Genes[a]	Total Number of Genes[b]
Purine Biosynthesis	0	12
Hypothetical Proteins	3	3
Central Intermediary Metabolism	1	1
Cell Envelope	1	1
Protein Synthesis	1	1
Total	**6**	**18**
Up-Regulated Ontologies		
Virulence	17	23
Cell Envelope	13	16
Hypothetical Proteins	7	14
Transporters	4	4
Energy Metabolism	3	3
Regulators	1	1
DNA Metabolism	1	1
Total	**46**	**62**

[a]Statistical significance is based on a Student *t* test with a cut-off of $p \leq 0.05$
[b]Genes listed include all that have a change of 2-fold or greater.

Table 10. Genes Repressed by σ^S, Identified by Microarray Analysis.

Gene Category	ORF no.[a]	Description	Gene	Fold up regulation[b]
Virulence (17)	SAV1939	truncated beta-hemolysin	*hlb*	4.93
	0275	essB superfamily:type VII secretion virulence protein EssB		4.79
	0270	staphyloxanthin biosynthesis protein, putative		4.53
	0278	esXB superfamily: virulence factor		4.34
	2003	phospholipase C	*hlb*	3.89
	0271	ESAT-6/Esx family secreted protein EsxA/YukE		3.53
	1864	serine protease SplF, putative		3.42
	0272	type VII secretion protein EsaA		3.11
	0276	diarrheal toxin	*yukA*	3.00
	1868	serine protease SplB	*splB*	2.94
	1865	serine protease SplE, putative		2.85
	1057	V8 Protease	*sspA*	2.78
	2659	zinc metalloproteinase aureolysin	*aur*	2.63
	1867	serine protease SplC	*splC*	2.54
	0277	Virulence protein EsaC		2.39
	0273	Virulence protein EssA		2.30
	1056	cysteine protease precursor SspB	*sspB1*	2.15
Cell Envelope (13)	1825	N-acetylmuramoyl-L-alanine amidase, family 4		6.51
	1532	homologous to lipoprotein		4.60
	0139	capsular polysaccharide synthesis enzyme Cap5D	*cap5D*	4.22
	1533	lipoprotein, putative		3.89
	0608	sdrC protein (cell adhesion)	*sdrC*	3.83
	1531	lipoprotein, putative		3.78
	0080	staphylococcus tandem lipoprotein		3.55
	0851	lipoprotein, putative		3.53
	0137	capsular polysaccharide biosynthesis protein Cap5B	*cap5B*	3.04
	0299	lipoprotein, putative		2.93
	0082	staphylococcus tandem lipoprotein		2.92
	0484	Staphylococcus tandem lipoprotein		2.17

Table 10. Continued.

Gene Category	ORF no.[a]	Description	Gene	Fold up regulation[b]
	0083	staphylococcal tandem lipoprotein		2.16
Hypothetical Proteins (7)	1826	Hypothetical protein (No conserved domains)		15.92
	0850	Hypothetical protein (No conserved domains)		3.65
	2338	Hypothetical protein (Probable membrane protein)		3.27
	1529	Hypothetical protein (No conserved domains)		3.19
	0643	Hypothetical protein (Putative conserved transposase domain)		2.66
	0645	Hypothetical protein (Putative membrane protein)		2.11
	0267	Hypothetical protein (Putative exported protein)		2.03
Transporters (4)	2483	transporter, putative		2.53
	0265	ABC-2 type transport system permease		2.36
	0620	osmoprotectant proline transporter	*proP*	2.10
	0264	ABC transporter, ATP-binding protein		2.08
Energy Metabolism (3)	2185	galactose-6-phosphate isomerase, LacB subunit	*lacB*	5.91
	2183	tagatose 1,6-diphosphate aldolase	*lacD*	3.45
	2186	galactose-6-phosphate isomerase, LacA subunit	*lacA*	3.38
Regulator (1)	2070	sensor histidine kinase KdpD	*kdpD*	2.04
DNA Metabolism (1)	0860	Thermonuclease precursor	*nuc*	3.55

[a]ORF IDs are derived from the *S. aureus* COL genome unless otherwise stated.
[b]Fold change is of the *sigS* mutant relative to the wild-type strain.

Table 11. Genes Activated by σ^S, Identified by Microarray Analysis.

Gene Category	ORF no.[a]	Description	Gene	Fold Down-Regulation[b]
Hypothetical Protein (3)	SAV1031	Hypothetical protein		-3.24
	SAV2430	Hypothetical protein		-2.48
	2433	Hypothetical protein		-2.10
Central Intermediary Metabolism (1)	2535	D-isomer specific 2-hydroxyacid dehydrogenase family protein		-2.48
Cell Wall (1)	0799	Transferrin receptor		-2.03
Protein Synthesis (1)	1729	Threonyl-tRNA synthetase	*thrS*	-4.02

[a]ORF IDs are derived from the *S. aureus* COL genome unless otherwise stated.
[b]Fold change is of the *sigS* mutant relative to the wild-type strain.

many of these elements are encoded within existing ORFs, or in intergenic regions (Geissmann *et al.*, 2009; Vogel and Wagner, 2007; Waters and Storz, 2009), their influence would be difficult to detect in this analysis.

In *S. aureus* σ^S Regulates Transcription of the Purine Biosynthesis Pathway. None of the genes contained within the microarray analysis obviously account for the increased susceptibility of *sigS* mutants to DNA damage. In an attempt to gain greater insight into this, we examined the microarray data in more detail. In doing so, we identified 16 genes upregulated in the mutant strain, and 12 downregulated genes, in addition to those that are statistically significant, and discussed previously. Of the 16 newly identified upregulated genes, 6 of which are associated with the virulence of *S. aureus*, as well as 3 genes encoding cell-envelope associated proteins (Table 12). These virulence associated genes include *agrB, agrD* and *argC* with fold increases of 3.1, 2.4 and 2.3 respectively. We also observed 6.0- and 2.7-fold increases in the *agr* regulated virulence determinants *hld* and *splA* respectively. Of the cell-envelope associated genes, we saw fold increases of 5.4, 3.0 and 2.5 in a putative lipoprotein, *cap8E* and *cap5G*, respectively. The only genes that were identified as being downregulated in the *sigS* mutant after relaxing the statistical significance cut off were the genes involved in purine biosynthesis. Due to variation among replicate samples, these genes were not deemed statistically significant using the cut-off criteria imposed. Specifically, as listed in Table 13, *purQ* was decreased 3.37-fold, along with a number of other genes from the same locus, including *purE*, *purC* and *purF*, with fold changes of 3.09, 3.33 and 2.34 respectively. Furthermore, *purA*, located distally on the chromosome from this locus, displayed a 2.94-

Table 12. Genes Repressed by σ^S Identified by Microarray Analysis That are Not Statistically Significant[b].

Gene Category	ORF no.[a]	Description	Gene	Fold up regulation[c]
Virulence (23)	2022	delta-hemolysin	*hld*	6.00
	0276	diarrheal toxin	*yukA*	4.62
	2023	accessory gene regulator protein B	*agrB*	3.14
	1869	serine protease SplA	*splA*	2.65
	2024	accessory gene regulator protein D	*agrD*	2.41
	2025	accessory gene regulator protein C	*argC2*	2.27
Cell Envelope (16)	1225	putative lipoprotein		5.43
	0140	capsular polysaccharide synthesis enzyme	*cap8E*	3.02
	0142	capsular polysaccharide synthesis enzyme	*cap5G*	2.48
Hypothetical Proteins (14)	0274	Hypothetical protein		6.42
	2602	Hypothetical protein		5.82
	0300	Hypothetical protein		2.76
	0849	Hypothetical protein		2.25
	0266	Hypothetical protein		2.20
	0489	Hypothetical protein		2.02
	2294	Hypothetical protein		2.01

[a]ORF IDs are derived from the *S. aureus* COL genome unless otherwise stated.
[b]Statistical significance is based on Student *t* test with a cut-off of $p \leq 0.05$
[c]Fold change is of the *sigS* mutant relative to the wild-type strain.

Table 13. Genes Activated by σ^S Identified by Microarray Analysis That are Not Statistically Significant[b].

Gene Category	ORF no.[a]	Description	Gene	Fold down regulation[c]
Purines, pyrimidines, nucleosides, and nucleotides metabolism (12)	1077	Phosphoribosylformylglycinamindine synthase I	*purQ*	-3.37
	1075	Phosphoribosylaminoimidazole-succinocarboxamide synthase	*purC*	-3.33
	1073	Phosphoribosylaminoimidazole carboxylase,catalytic subunit	*purE*	-3.09
	0018	Adenylosuccinate synthetase	*purA*	-2.94
	1076	Phosphoribosylformylglycinamidine synthase	*purS*	-2.82
	1078	Phosphoribosylformylglycinamidine synthase II	*purL*	-2.52
	1080	Phosphoribosylformylglycinamidine cyclo-ligase	*purM*	-2.47
	1074	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	*purK*	-2.35
	1079	Amidophosphoribosyltransferase	*purF*	-2.34
	1081	Phosphoribosylglycinamide formyltransferase	*purN*	-2.28
	2242	Xanthine/uracil permease family protein		-2.09
	1082	Phosphoribosylaminoimidazolecarboxamide formyltransferase	*purH*	-2.05

[a]ORF IDs are derived from the *S. aureus* COL genome unless otherwise stated.
[b]Statistical significance is based on Student *t* test with a cut-off of $p \leq 0.05$
[c]Fold change is of the *sigS* mutant relative to the wild-type strain.

fold down-regulation in the mutant strain. To investigate this further, and determine if this affect was biologically meaningful, the role of σ^S in purine biosynthesis regulation was explored using qRT-PCR. Accordingly, we examined transcription of the four loci encoding *de novo* purine biosynthesis pathway genes: *purEKCSQLFMNHD*, *purA*, *purB* and *guaBA*, the chromosomal locations of which are depicted in Figure 43. As such, we carried out qRT-PCR analysis on *purE*, *purC* and *purD*, which resulted in 2.3-, 9.2- and 2.0-fold decreases, respectively, in the RN4220 σ^S mutant after 3 hours of growth (Figure 44). Transcription of *purA*, *purB*, *guaB* and *guaA* resulted in 2.0-, 4.6-, 2.1- and 4.6-fold decreases, respectively, in the mutant strain.

To verify that our findings were not specific to RN4220, studies were also carried out in USA300 LAC. When the same genes were profiled, we again observed a significant decrease in transcription in the σ^S mutant. Specifically, *purE*, *purC* and *purD* transcription resulted in 13.9-, 7.6- and 4.4-fold decreases, respectively, in the σ^S mutant (Figure 45). Transcription of *purA*, *purB*, *guaB* and *guaA* resulted in 2.5-, 8.8-, 5.4- and 5.3-fold decreases, respectively, in the mutant strain. We also repeated these analyses in the context of the downstream gene, *ecfX*, to assess its impact on the expression of purine biosynthesis genes. This was performed in an *ecfX* mutant in the USA300 LAC background, revealing there to be negligible decreases in the expression of the *pur* and *gua* genes compared to that of the wild-type strain; certainly not to the same levels as the *sigS* mutant (Figure 46). Collectively, this data supports our microarray analysis, and suggests a role for σ^S in the response to DNA damage via regulation of *de novo* purine biosynthesis.

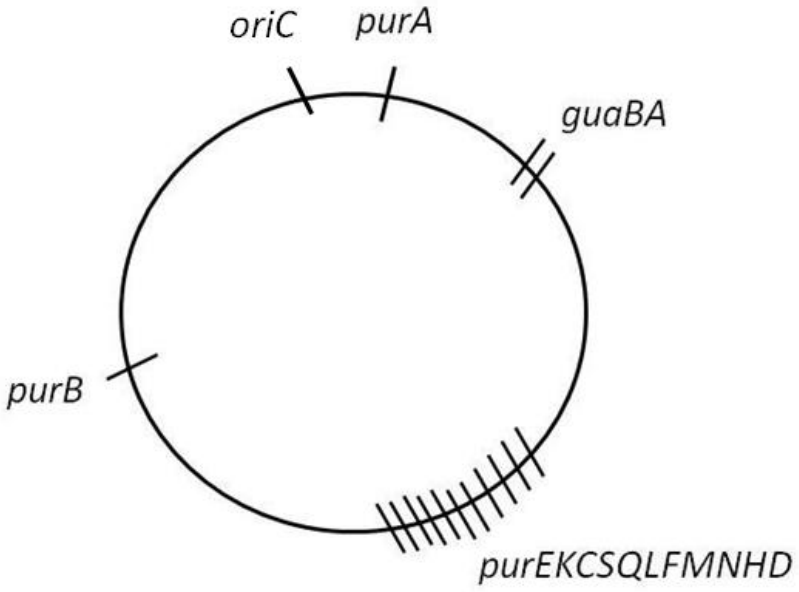

Figure 43. Chromosomal Locations of the Purine Biosynthesis Genes in *S. aureus*. The genes comprising the purine biosynthesis pathway are located at 4 distinct genetic loci throughout the *S. aureus* genome. *oriC* is shown for orientation.

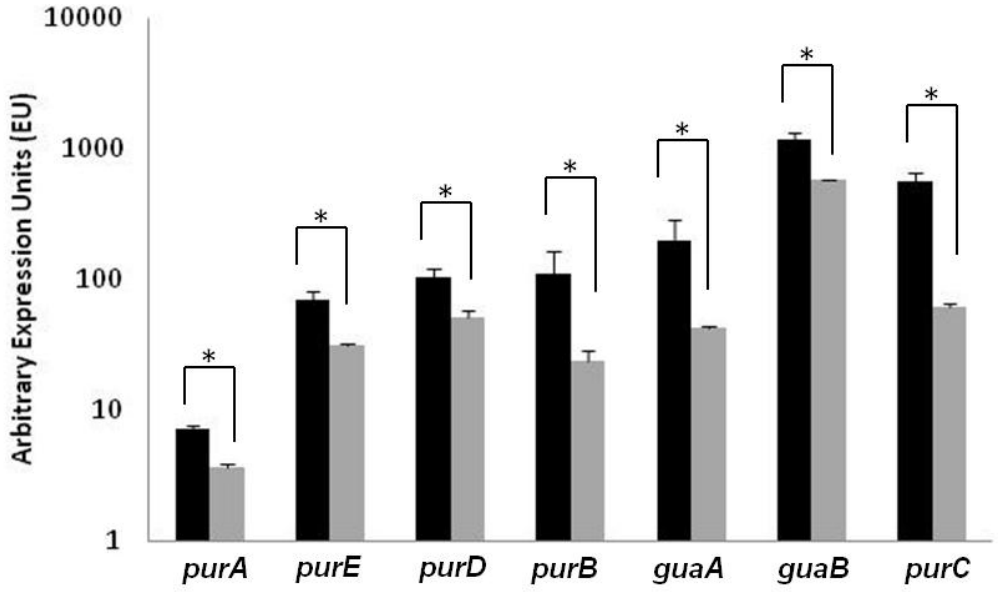

Figure 44. σ^S Regulates Expression of *de novo* Purine Biosynthesis Pathway Genes in RN4220. Quantitative real-time PCR analysis of RN4220 (black) and its *sigS* mutant (grey) was performed with cultures grown in TSB at 37°C for 3 h. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, * = $p<0.05$ using a Student *t* test.

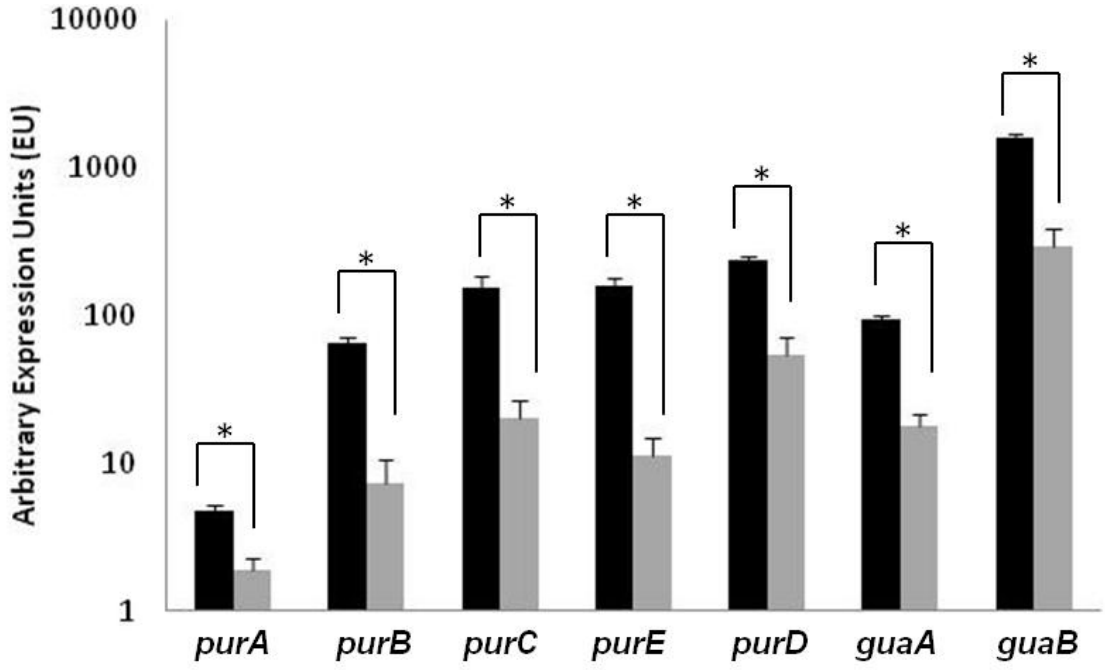

Figure 45. The Regulation of Purine Biosynthesis Genes by σ^S is Conserved Across *S. aureus* Strains. Quantitative real-time PCR analysis of USA300 wild-type (black) and its *sigS* mutant (grey) was performed with cultures grown in TSB at 37°C for 3 h. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, * = $p<0.05$ using a Student *t* test.

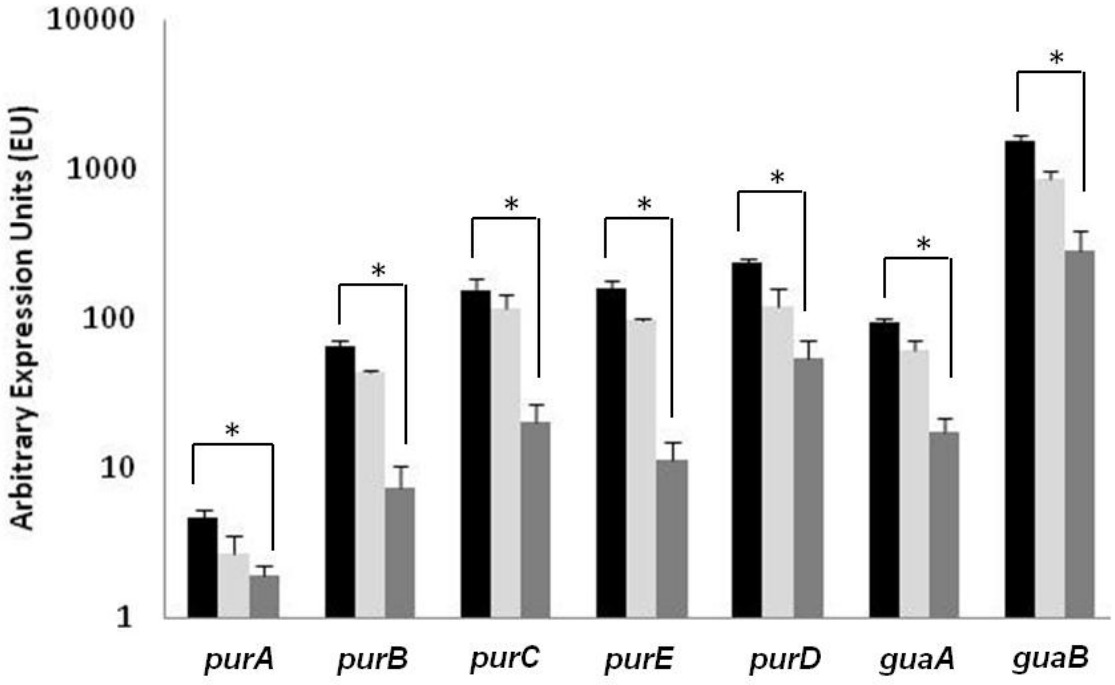

Figure 46. Transcription of the Purine Biosynthesis Genes is Unaffected by Mutation of *ecfX*. Quantitative real-time PCR analysis of USA300 wild-type (black), and its *ecfX* (light grey) and *sigS* (dark grey) mutants, was performed with cultures grown in TSB at 37^{o}C for 3 h. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, * = $p<0.05$ using a Student *t* test.

Purine Biosynthetic Activity is Decreased in σ^S Mutant Strains. We have presented evidence to suggest a role for σ^S in the upregulation of purine biosynthesis genes at the transcriptional level. As such, we next investigated whether these affects translate into alterations in enzymatic function. Accordingly, we performed activity based profiling for PurA (adenylosuccinate synthetase), which facilitates one of the later steps in adenosine generation (Figure 47A). Crude extracts of a USA300 LAC wild-type, its *sigS* mutant and complemented strain were used to measure the rate of formation of adenylosuccinate (sAMP). When assayed, we observed a 3.7-fold decrease in activity of PurA in the *sigS* mutant compared to its parent strain (Figure 47B). This change in activity was fully restored upon complementation. These findings specifically show that the decrease in transcription observed for purine biosynthesis genes correlates to altered enzymatic activity within the cell.

Deletion of σ^S Results in Decreased Levels of Both ADP and GDP in the Mutant Strain. Our transcriptional profiling and enzymatic activity assays strongly suggest that σ^S impacts the DNA damage response of *S. aureus* via regulation of the purine biosynthesis pathway. With this in mind, we sought to examine the levels of purines in the cell for strains bearing a mutation in σ^S. Total intracellular fractions were collected from cultures of the USA300 LAC wild-type and σ^S mutant strains grown as previously described for both the transcriptional and enzymatic analysis. Samples were deproteinated through acid precipitation, and the nucleotide concentrations of the resulting supernatants determined by HPLC using a Hypersil SAX column (4.6 × 250mm, Thermo Hypersil-Keystone, Bellefonte, PA) equipped with a guard column. Nucleotides were eluted using a discontinuous gradient at a constant flow rate of 1.0 ml

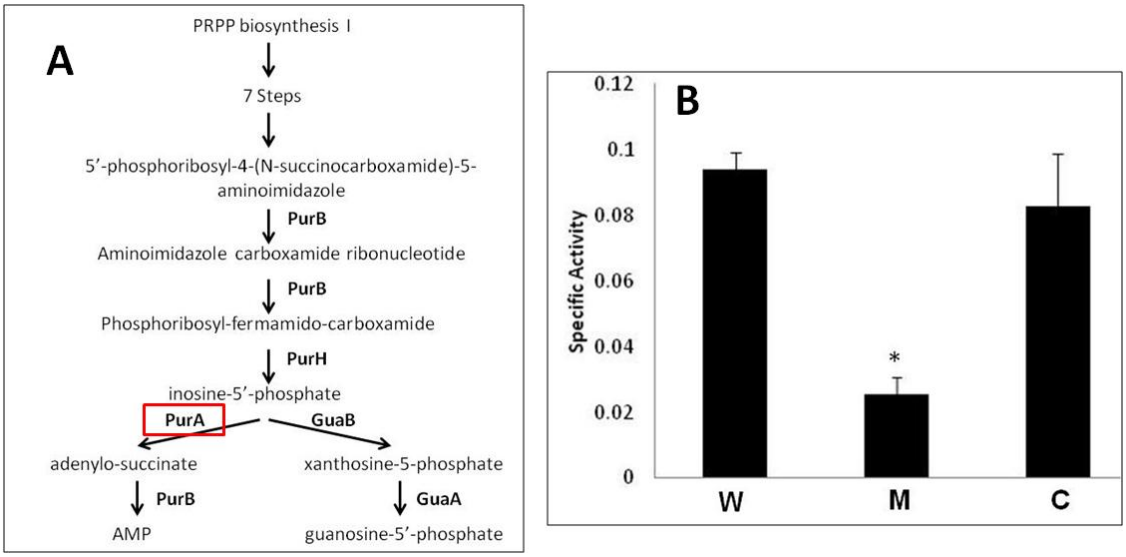

Figure 47. Adenylosuccinate Synthetase Activity is Decreased in a *sigS* Mutant. (A) The purine biosynthesis pathway. The conversion of inosine-5'-phosphate to adenylo-succinate by the enzyme adenylosuccinate synthetase is highlighted in red. **(B)** The USA300 LAC wild-type (W), its *sigS* mutant (M) and complement strain (C) were grown for 3 h in TSB. Bacterial cell extracts were generated, and PurA activity assays performed. One unit is defined as the amount of enzyme required to utilize or release one nanomole of substrate or product per minute. Specific activity is expressed as units of activity per mg of protein. Data presented is representative of at least three independent experiments with error bars presented as +/- SEM , * = $p<0.001$ using a Student *t* test.

min^{-1} with absorbance recorded at 254 nm. The nucleotide concentrations were determined from the peak areas, and standard curves from known concentrations of HPLC nucleotide markers. As displayed in Figure 48A-B, the resulting spectrums showed notable differences between the wild-type and σ^S mutants, particularly during elution between 5 and 10 minutes. Quantification of these differences in peaks through comparison to the HPLC nucleotide standards led to the finding that σ^S mutants display a decrease in the concentration of both ADP and GDP in the cytoplasm, with fold decreases of 2.0 and 6.2 respectively (Figure 48C). This suggests that the observed decreases in transcription as well as a decrease in enzymatic activity of the purine biosynthesis proteins, leads to decreases in the intracellular concentration of purine nucleotides.

Proteomic Analysis of the σ^S Mutant During DNA Damage Induced Stress. We next sought to further explore the role of σ^S through examination of the cytoplasmic proteome of the mutant against its parental strain. This was performed using strain 8325-4 in the presence of MMS, which resulted in the highest level of *sigS* expression. These conditions were selected to further supplement our transcriptomic analysis, with the aim of both validating the microarray and further elucidating components of the σ^S regulon. Thus, intracellular proteins were harvested from cultures of 8325-4 grown for 5 h in the presence of sub-MIC concentrations (2.5 mM) of MMS. Our analysis revealed 52 proteins to be negatively regulated by σ^S, and 95 to be positively affected. Of those proteins negatively regulated by σ^S, we observed a number involved in protein synthesis and modification as well as cell envelope associated ontologies. As displayed in Table 14, two fold increases were observed for SACOL1946 and SACOL2125, encoding a

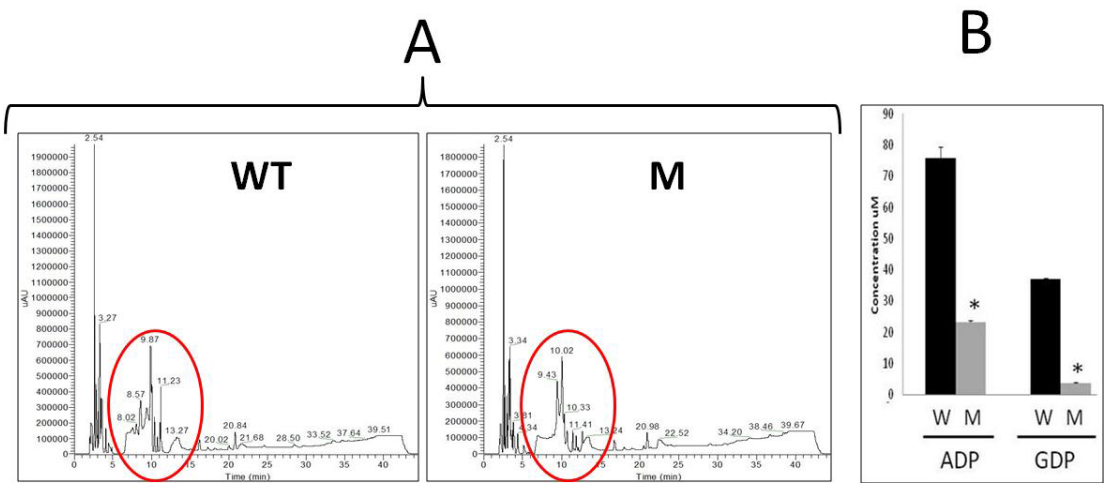

Figure 48. σ^S Deletion Results in Decreased Levels of Both ADP and GDP in the Mutant Strain. (A) Total intracellular components were collected from cultures of the USA300 LAC wild-type (WT) or the σ^S mutant (M) strains grown in TSB to hour 3. Samples were deproteinated and the nucleotide concentrations of the resulting supernatants determined by HPLC. Nucleotides were eluted using a discontinuous gradient at a constant flow rate of 1.0 ml min^{-1} with absorbance recorded at 254 nm. Differences in peak areas indicate alterations in the nucleotide pools. These differences found between the wild-type and *sigS* mutant strains are noted by red circles. **(B)** Nucleotide concentrations were determined for both the USA300 LAC wild-type (W) and σ^S mutant (M) by comparison to peaks from standard curves generated with HPLC nucleotide markers. Displayed are the resulting concentrations for purines ADP and GDP. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM, * = $p<0.05$ using a Student *t* test.

Table 14. Proteins Negatively Influenced by σ^S Identified by Proteomic Analysis.

Description	ORF ID	Protein	Fold Increase[a]
DNA metabolism: replication, recombination and repair			
Thermonuclease family protein	SACOL1357		2
Probable endonuclease 4	SACOL1614	Nfo	3
DNA polymerase III, delta subunit	SACOL1643	HolA	6
Protein Synthesis/Modifications			
Foldase protein	SACOL0544	PrsA	2
Putative zinc metalloprotease	SACOL1281	RseP	3
Methionine aminopeptidase	SACOL1946	Map	2
10 kDa chaperonin	SACOL2017	GroS	3.5
Peptidase, M20/M25/M40 family	SACOL2125		2
50S ribosomal protein L17	SACOL2212	RplQ	2
30S ribosomal protein S19	SACOL2235	RpsS	3
50S ribosomal protein L3	SACOL2239	RplC	4
Energy Metabolism			
Indole-3-pyruvate decarboxylase	SACOL0173	IpdC	2
FMN-dependent NADH-azoreductase	SACOL0190	AzoR	2.3
Cytochrome d ubiquinol oxidase, subunit I	SACOL1094	CydA	2.5
Polyprenyl synthetase	SACOL1510		3
Glucose-6-phosphate 1-dehydrogenase	SACOL1549	Zwf	2.3
Alpha-glucosidase	SACOL1551	MalA	2
glycine dehydrogenase	SACOL1594	GcvPA	9
Chorismate mutase	SACOL1787		2.7
ATP synthase subunit alpha	SACOL2097	AtpA	2.2
ATP synthase subunit b	SACOL2099	AtpF	2.3
Cell Division			
Putative septation protein	SACOL0541	SpoVG	4
Septation ring formation regulator ezrA	SACOL1767	EzrA	2.2
Regulators			
Response regulator protein vraR	SACOL1942	VraR	2
Anti-sigma-B factor antagonist	SACOL2056	RsbV	2.5
Transcriptional regulator, AraC family	SACOL2378		3
Cellular Detoxification			
Alkyl hydroperoxide reductase subunit C	SACOL0452	AhpC	2.3
Unknown Function/Hypothetical Proteins			
Hypothetical Protein	SACOL0003		2
YycI protein	SACOL0022	YycL	3
aminotransferase, class II	SACOL0596		5

Table 14. Continued.

Description	ORF ID	Protein	Fold Increase[a]
Hypothetical Protein	SACOL0669		2
GTP cyclohydrolase	SACOL0789	FolE2	8
Hypothetical Protein	SACOL0984		2
Hypothetical Protein	SACOL1089		2
DegV domain-containing protein	SACOL1460		2
Aminotransferase, class V	SACOL1677		2
Hypothetical Protein	SACOL1973		2
Hypothetical Protein	SACOL2020		3
Probable DEAD-box ATP-dependent RNA helicase	SACOL2072		2
Hypothetical Protein	SACOL2307		3
Hypothetical Protein	SACOL2338		4
Hypothetical Protein	SACOL2447		2
Isochorismatase family protein	SACOL2667		2
Biosynthesis of Cofactors			
Glutamate-1-semialdehyde 2,1-aminomutase 1	SACOL1714	HemL1	2
Biotin synthase	SACOL2426	BioB	2
Cell Envelope			
Serine-aspartate repeat-containing protein C (cell adhesion)	SACOL0608	SdrC	3.3
Penicillin-binding protein 1	SACOL1194	Pbp1	2
Glutamine synthetase	SACOL1329	GlnA	3.6
Aminoacyltransferase	SACOL1410	FemA	2
Penicillin-binding protein 2	SACOL1490	Pbp2	2.4
N-acetylmuramoyl-L-alanine amidase	SACOL1687	Sle1	3
Transporters			
Osmoprotectant ABC transporter, ATP-binding protein, putative	SACOL0781		2

[a]Fold increase refers to the amount of protein in the *sigS* mutant relative to the wild-type

methionine aminopeptidase and a M20/M25/M40 family peptidase respectively. Most notable, was an increase of 3-fold observed for SACOL1281, encoding a zinc metalloprotease demonstrating homology to RseP of *B. subtilis*; the site-2 membrane protease involved in activation of ECF sigma factor σ^E. We observed a 3.3-fold increase in SACOL0608, encoding SdrC, a cell adhesion protein also identified as being 3.8-fold upregulated in our microarray analysis. Fold increases of 2 and 2.4 were also observed for the penicillin-binding proteins Pbp1 and Pbp2 respectively. An osmoprotectant ABC transporter, SACOL0781 displayed a 2-fold increase, which is comparable to that of SACOL0620, which encodes an osmoprotectant proline transporter indentified in our microarray analysis. Further proteins found to be increased in the *sigS* mutant compared to the wild-type include SACOL1357, a thermonuclease family protein involved in the degradation and turnover of DNA, which displayed a 2-fold increase. Of those proteins decreased in the *sigS* mutant, many were involved in cell wall metabolism, including a D-alanine – D-alanine ligase involved in peptidoglycan biosynthesis, which was 4-fold less abundant in the mutant strain (Table 15). In addition, we observed decreases in DdlA, MurF, MurC and FemX, all of which are involved in peptidoglycan synthesis, and were 4-, 2-, 2- and 2.3-fold decreases respectively. There was also a 2.5-fold decrease in the teichoic acid biosynthesis protein SACOL0238, as well as a 3-fold decrease in DltD, which is involved in biosynthesis of surface polysaccharides and lipopolysaccharides, and contributes to the overall net charge of the cell wall (Peschel *et al.*, 1999). These findings corroborate our earlier transcriptional analyses, which demonstrate that *sigS* expression is increased upon exposure to cell wall destabilizing antimicrobial agents.

Table 15. Proteins Positively Influenced by σ^S Identified by Proteomic Analysis.

Description	ORF ID	Protein	Fold Decrease[a]
DNA metabolism: replication, recombination and repair			
DNA gyrase subunit B	SACOL0005	GyrB	5
DNA polymerase III, gamma and tau subunits	SACOL0520	DnaX	3
DNA replication Initiation-control protein	SACOL0528	YabA	5
Exoribonuclease, VacB/RNase II family	SACOL0846		2
MutS2 protein	SACOL1154	MutS2	2
UvrABC system protein C	SACOL1157	UvrC	3
DNA mismatch repair protein	SACOL1315	MutS	2
Nuclease sbcCD subunit C	SACOL1381	SbcC	5
Nuclease sbcCD subunit D	SACOL1382	SbcD	2
DNA topoisomerase 4 subunit A	SACOL1390	ParC	2
5'-3' exonuclease, putative	SACOL1479		2
Helicase, putative, RecD/TraA family	SACOL1674		2
DNA polymerase I	SACOL1737	PolA	2.25
DNA ligase	SACOL1965	LigA	2.25
DNA mistmatch repair MutS family protein	SACOL2037		2
Chromosome partioning protein	SACOL2735	ParB	4
Nucleotide Biosynthesis			
Phosphopentomutase	SACOL0124	DeoB	2.5
Thymidylate kinase	SACOL0524	Tmk	3
Deoxynucleoside kinase family protein	SACOL0603		4
Pyridine nucleotide-disulfide oxidoreductase	SACOL0640		4
nucleotide-binding protein	SACOL0830		3
Phosphoribosylformylglycinamidine synthase	SACOL1077	PurQ	4
Phosphoribosylformylglycinamidine synthase 2	SACOL1078	PurL	5
Phosphoribosylformylglycinamidine cyclo-ligase	SACOL1080	PurM	2
Bifunctional purine biosynthesis protein purH	SACOL1082	PurH	2
Phosphoribosylamine--glycine ligase	SACOL1083	PurD	3
Pyridine nucleotide-disulfide oxidoreductase	SACOL1520		2.3
Adenylosuccinate lyase	SACOL1969	PurB	2.8
NH(3)-dependent NAD(+) synthetase	SACOL1974	NadE	2.5

Table 15. Continued.

Description	ORF ID	Protein	Fold Decrease[a]
Protein Synthesis/Modifications			
Seryl-tRNA synthetase	SACOL0009	SerS	2
50S ribosomal protein L9	SACOL0015	RplI	3
Heat induced protein	SACOL0457	YflT	2
30S ribosomal protein S15	SACOL1292	RpsO	2.5
Elongation factor P	SACOL1587	Efp	2
Chaperone protein dnaJ	SACOL1636	DnaJ	2.5
Peptidase, U32 family	SACOL1667		2
GTPase	SACOL1699	Obg	4
Aminopeptidase PepS	SACOL1937	PepS	2
30S ribosomal protein S8	SACOL2225	RpsH	2.3
30S ribosomal protein S17	SACOL2230	RpsQ	3.5
50S ribosomal protein L23	SACOL2237	RplW	2
Iron sulfur cluster assembly protein			
FeS assembly ATPase	SACOL0914	SufC	3.5
FeS assembly protein SufD	SACOL0915	SufD	3
putative FeS assembly protein	SACOL0918		4
Cell Wall Metabolism			
Teichoic acid biosynthesis protein, putative	SACOL0238		2.5
DltD protein	SACOL0938	DltD	3
UDP-N-acetylmuramate--L-alanine ligase	SACOL1790	MurC	2
UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	SACOL2073	MurF	2
D-alanine--D-alanine ligase	SACOL2074	DdlA	4
Aminoacyltransferase femX	SACOL2253	FemX	2.25
Cell Wall Associated			
Hypothetical protein (putative lipoprotein)	SACOL1306		2
TPR repeat lipoprotein	SACOL1503		3
Cellular Detoxification			
Arsenate reductase, putative	SACOL0876		3.3
Glutathione peroxidase homolog	SACOL1325	BsaA	5
Disulphide isomerase	SACOL1558		3
Amino Acid Biosynthesis			
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	SACOL0428	MetE	2
Homoserine dehydrogenase	SACOL1362	Hom	2

Table 15. Continued.

Description	ORF ID	Protein	Fold Decrease[a]
Diaminopimelate decarboxylase	SACOL1435	LysA	3.25
Pyrroline-5-carboxylate reductase	SACOL1546	ProC	3
2-oxoisovalerate dehydrogenase, E1 component, beta subunit	SACOL1561		3.3
Aminotransferase, putative	SACOL2000		3
Energy Metabolism			
acyl-CoA synthetase	SACOL0164		3
Triosephosphate isomerase	SACOL0840	TpiA	2
Protein nagD homolog	SACOL0931		2.3
Glycerol kinase	SACOL1320	GlpK	2
Aminomethyltransferase	SACOL1595	GcvT	2
6-phosphofructokinase	SACOL1746	PfkA	2
Putative aldehyde dehydrogenase SACOL2114	SACOL2114		3
Glucosamine--fructose-6-phosphate aminotransferase	SACOL2145	GlmS	2.7
Zinc-type alcohol dehydrogenase-like protein	SACOL2177		2
Putative NAD(P)H nitroreductase	SACOL2534		2
Regulators			
Transcriptional regulatory protein	SACOL1535	SrrA	2
N utilization substance protein B homolog	SACOL1569	NusB	2
Transcriptional regulator, putative	SACOL1904		2
Serine-protein kinase rsbW	SACOL2055	RsbW	2
RNA-binding protein	SACOL2133		4
Unknown Function/Hypothetical Proteins			
Nitroreductase family protein	SACOL0874		2
Hypothetical protein (No conserved domains)	SACOL1258		3
B-lactamase	SACOL1870		3
Acetyltransferase, GNAT family	SACOL2532		2
Biosynthesis of Cofactors			
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase 2	SACOL0240		6
Folylpolyglutamate synthase/dihydrofolate synthase	SACOL1709	FolC	2
Delta-aminolevulinic acid dehydratase	SACOL1715	HemB	3
Pyridoxamine 5'-phosphate oxidase;	SACOL2379		3
Fatty Acid and Phospholipid Metabolism			

Table 15. Continued.

Description	ORF ID	Protein	Fold Decrease[a]
Carboxylesterase	SACOL0845	Est	2
Enoyl-(Acyl-carrier-protein) reductase	SACOL1016	FabI	5
Phosphate acyltransferase	SACOL1243	PlsX	4
Diacylglycerol kinase	SACOL1626	DgkA	2.5
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	SACOL1748	AccD	3
Adaptation to Atypical Conditions (Stringent Response)			
Guanosine polyphosphate pyrophosphohydrolases/synthetases	SACOL1689	RelA2	3
Cell Division			
Cell division protein ftsA	SACOL1198	FtsA	2.5
Cell division protein FtsY, putative	SACOL1251	FtsY	3
DNA translocase ftsK	SACOL1295	FtsK	2

[a]Fold decrease refers to the amount of protein in the *sigS* mutant relative to the wild-type

A large number of proteins positively influenced by σ^S were in the DNA metabolism, recombination and repair, and nucleotide biosynthesis ontologies. There are 15 proteins involved in DNA replication, recombination and repair that were identified by proteomic analysis as being decreased in the σ^S mutant compared to the parent. We found a decrease in the bi-component exonuclease SbcDC, with fold decreases of 2 and 5 for SbcD and SbcD, respectively. A decrease was also observed for a putative 5'-3' exonuclease, which demonstrated a fold change of 2. These exonucleases were also identified in our transposon screen, mutations in which led to increased expression of *sigS*. A fold decrease of 2 was observed for both the DNA mismatch repair proteins MutS and MutS2, while the observed decrease in the UvrC protein of the nucleotide excision repair protein was 3-fold. Sensitivity of the σ^S mutant was demonstrated during challenge by alkylating agents and UV radiation, both of which result in damage that can be repaired by MutS and UvrC respectively. A 5-fold decrease was observed for the DNA gyrase subunit B, the target of nalidixic acid and ciprofloxacin; antibiotics identified in our transcriptional analysis as leading to the upregulation of *sigS* expression. Collectively, this suggests a role for σ^S in the regulation of genes whose products help protect against and repair DNA damage.

We also identified 13 proteins involved in nucleotide biosynthesis as being decreased in the σ^S mutant. Included in this are the purine biosynthesis proteins PurL, PurQ, PurM, PurD, PurH and PurB, which demonstrated fold decreases of 5, 4, 2, 3, 2 and 2.8 respectively. This is comparable to that of our microarray analysis, where we observed 2.5, 3.4, 2.5 and 2.1- fold decreases for PurL, PurQ, PurM and PurH respectively. These

findings supplement our microarray analysis and further support a role for σ^S in the upregulation of purine biosynthesis genes in response to DNA damaging conditions.

Exploring the Importance of σ^S During *S. aureus* infection. We have previously established a role for σ^S in the pathogenesis of *S. aureus* using a murine model of septic arthritis. Specifically, we noted that mice infected with the σ^S mutant displayed decreased weight loss, bacterial load in the kidneys and mortality, which demonstrates a role for σ^S in systemic infections (Shaw *et al.*, 2008). Transcriptional analysis demonstrates a significant increase in expression of *sigS* across both laboratory and clinical strains of *S. aureus* post exposure to pig serum, as well as following phagocytosis by RAW 264.7 murine macrophage-like cells. Therefore, we sought to further examine the role of σ^S in combating the host immune system, and in the pathogenesis of *S. aureus* localized infections.

In *S. aureus*, σ^S Aids in Protection During Interaction with Components of the Innate Immune System. We first sought to determine the importance of σ^S during challenge by components of the innate immune system. This was first performed using whole human blood and the USA300 wild-type, *sigS* and *ecfX* mutants, and their complemented strains. As such, exponentially growing cells were inoculated into whole human blood, and incubated for 4 hours. After this time we recovered 2.1- and 1.3-fold less viable cells of the *sigS* and *ecfX* mutants, compared to the wild-type strain (Figure 49A). Complementation analysis of both mutants restored viability to levels similar to that of the wild-type. Following this, we also conducted macrophage survival assays,

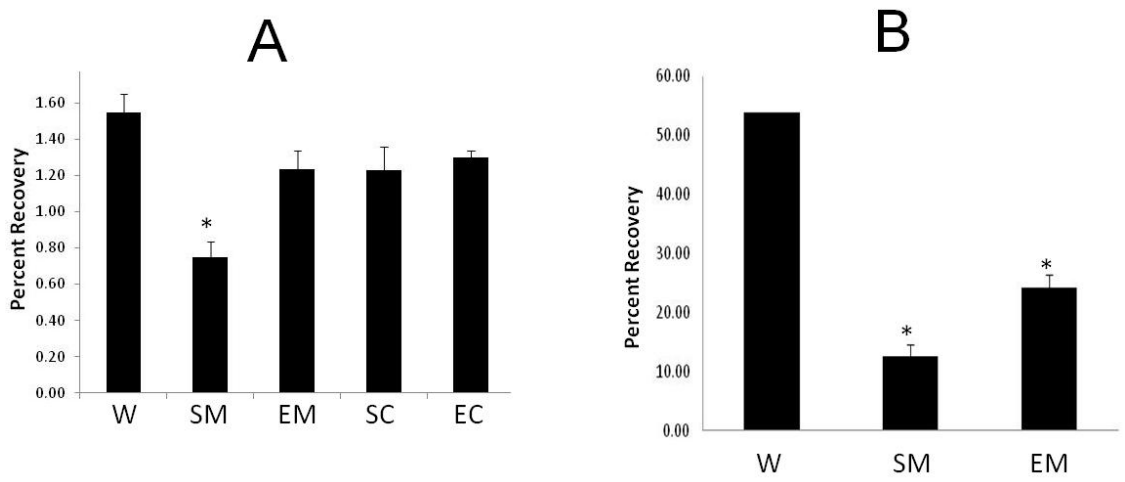

Figure 49. σ^S Aids in Protection of the *S. aureus* Cell During Interaction with Components of the Innate Immune System. (A) The USA300 wild-type (W), *sigS* mutant (SM) and *ecfX* mutant (EM) along with *sigS* complement (SC) and *ecfX* complement (EC) strains, were analyzed for viability 4 h post-exposure to whole human blood. (**B**) The USA300 wild-type (W), *sigS* mutant (SM) and *ecfX* mutant (EM) strains were analyzed for viability 24 h post phagocytosis by 264.7 RAW murine macrophage-like cells. CFU counts were determined both pre- and post-exposure, and the percent survival determined. Error bars are shown as +/- SEM, *= $p<0.05$ using a Student *t* test. Adapted from Miller *et al*., 2012.

again in the USA300 background, to assess the ability of the *sigS* mutant to persist upon phagocytosis. At 24 h post-phagocytosis by RAW 264.7 cells, we observed a 4.5- and 2.2-fold decrease in survivability of the *sigS* and *ecfX* mutant compared to the parent, respectively (Figure 49B). Complementation was not possibly due to loss of plasmid, which is likely explained by significantly instability of the vector during phagocytosis, which we routinely observe when performing this assay. Collectively, these findings support our earlier work, which indicates an important role for σ^S in the virulence of *S. aureus*.

In *S. aureus* σ^S is Required for Full Virulence in Localized Infections. Given our findings regarding the importance of σ^S in systemic models of infection, we set out to determine if this is true for virulence in localized infections as well. As such, 10, 5-wk old female SKH1-E nude mice were injected subcutaneously between the scapula with an inoculum of 1 X 10^8 cells of either SH1000 wild-type, or its *sigS* mutant. After 6 days of infection, the mice were euthanized and abscesses harvested. When determining the bacterial load per abscess, our studies revealed a reduction in the infectious capacity of the σ^S mutant. We were able to recover an average of 8.38 x 10^7 CFU per abscess or 84% of the mutant strain compared to a 6.61 x 10^8 CFU per abscess, or a 661% recovery, of the wild-type (Figure 50). This indicates that σ^S plays an important role in both localized and systemic *S. aureus* infections.

Discussion. As discussed previously, RN4220 contains a number of mutations throughout its genome that suggest it is perhaps far more prone to DNA damage than

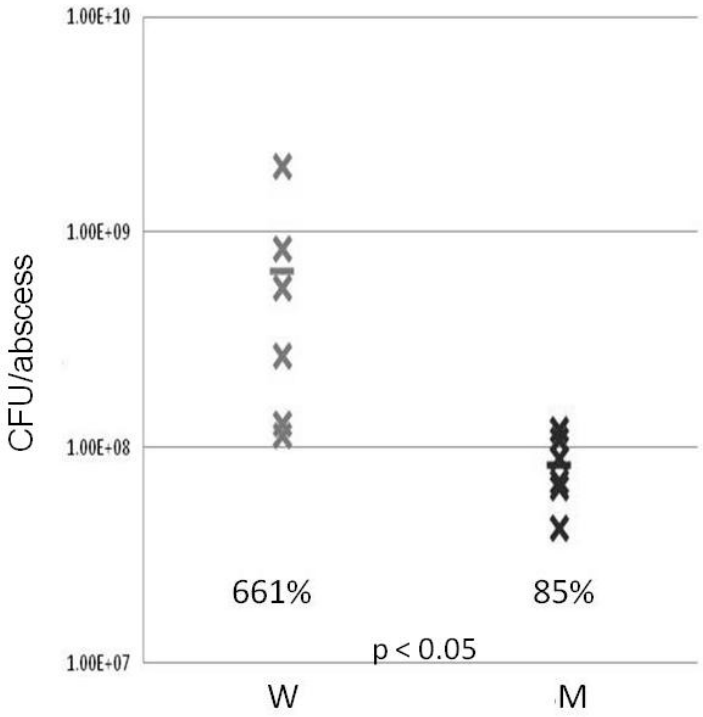

Figure 50. σ^S is Required for Full Virulence in Localized Infections of *S. aureus.* Five week old, female SKH1-E nude-mice were infected with $1x10^8$ CFU ml^{-1} of either the SH1000 wild-type (W) or *sigS* mutant (M) strains between the scapula. Mice were sacrificed and abscesses collected 6 days post infection. The data is presented as total bacterial load per abscess, with vertical bars representing sample mean and the average percent recovery listed. The statistical significance of bacterial recovery was evaluated using a Student *t* test with a 5% confidence limit.

other wild-type strains as a result of mutated and non-functional repair pathways. We have proposed this as an explanation as to why *sigS* is upregulated in RN4220 at levels greater than other *S. aureus* wild-type strains. Herein we show a significant growth defect for the *sigS* mutant in the RN4220 background as cells transition from stationary phase to exponential phase. We show that the viability of RN4220 *sigS* mutant cells are comparable to that of the wild-type during late stationary phase; however, upon introduction to ample nutrients the *sigS* mutant cells stall far longer than the parent strain before reaching exponential phase. This suggests that mutation of *sigS* coupled with the existing mutations in this strain leads to a decrease in the ability of the cell to sense and respond to the changing environment during exit from stationary phase. Previous reports demonstrate that an accumulation of iron occurs during lag phase, which increases sensitivity of the cells to oxidative stress (Rolfe *et al.*, 2012). Additionally, an increase occurs in nucleotide metabolism during lag phase as cells prepare for growth and division during exponential phase (Rolfe *et al.*, 2012). As such, it is likely that lag phase cells are more prone to DNA damage and subsequently require the use of DNA damage repair pathways and nucleotide precursors; both of which seem to require a functional σ^S. This suggests that upon entering into exponential phase, the RN4220 *sigS* mutant is decreased in its ability to both repair DNA damage and rapidly synthesize nucleotides, resulting in an inability to survive and replicate as efficiently as the wild-type. Indeed, this contention is further supported by the observation that in addition to the DNA damage repair pathways, RN4220 also carries a mutation in orotate phosphoribosyltransferase, which is involved in pyrimidine biosynthesis. This mutation also occurs in the NCTC8325 lineage strains 8325-4, as well as SH1000 (O'Neill, 2010); therefore, this

mutation alone is unlikely to lead to the observed growth defect of the *sigS* mutant. However, the additive effect of multiple mutations in DNA damage repair genes, as well as a decreased ability to synthesize DNA precursors, may lead to a greater need for σ^S in RN4220.

Following on from our transcription studies we also performed phenotype profiling by first utilizing disk diffusion analysis. We found that even under conditions leading to *sigS* expression, we observed no notable differences in the viability of the *sigS* mutant compared to the parent strain. This indicates that under the conditions tested, the σ^S mutant is equally well adapted as the parent strain. However, while this assay proves to be a valuable initial screen, it is also commonly qualitative, based on our findings. Furthermore, disk diffusion methods only allow end point analysis, thus phenotypic differences occurring during early growth phases may be overlooked. The RN4220 σ^S mutant growth defect occurs only during exponential phase, and by hour 6 growth seemingly reverts to that of the wild-type. Therefore, comprehensive analysis requires examination of time points prior to stationary growth, the single time point observed during disk diffusion analysis.

As such, we performed death curve analysis with exponentially growing cells exposed to lethal concentrations of DNA damage agents and found that σ^S mutants have increased sensitivity to a broad range of DNA damage inducing stresses. This data contradicts our previous findings from the disk diffusion assay, which showed no difference in the zone of inhibition of the mutant versus the wild-type. As stated earlier, this observation

indicates that the affect may be growth phase dependant, and therefore would not be seen with disk diffusions. Similar to MICs, disk diffusions allow only for analysis of stationary phase cultures, while the kill assays presented herein allow for examination of multiple times points during exposure. The range of DNA damage agents that *sigS* mutants are susceptible to include alkylating and intercalating agents, reactive oxygen species and UV induced damage; each of which leads to the activation of specific and distinct repair pathways. When we analyzed transcription of a number of DNA repair pathway genes in both *sigS* mutants and *S. aureus* wild-types, we observed no alterations in expression (Figure 51). However, during transcriptomic profiling we did identify decreased transcription for genes involved in *de novo* purine biosynthesis. Following challenge by DNA damage agents, there may be increased pressure for the cell to synthesize purines as the nucleotide pool is being depleted by activated repair pathways. As *de novo* purine biosynthesis appears to be σ^S dependent, this would explain why *sigS* is induced following DNA-damage, and why *sigS* mutants have impaired survival under such stress. Collectively these findings corroborate our transcriptional analysis, and suggest a role for σ^S in the protection of *S. aureus* to general DNA damage stress, as opposed to one specific repair pathway.

We show in our coculture studies, where the parent and mutant strains were grown together under a variety of conditions, that the *sigS* mutant is important for survival and fitness. When the SH1000 *sigS* mutant was forced to compete with its parental strain, it displayed significantly reduced abilities for growth and proliferation. This phenotype was only exacerbated during prolonged growth periods (7 days), or in the presence of

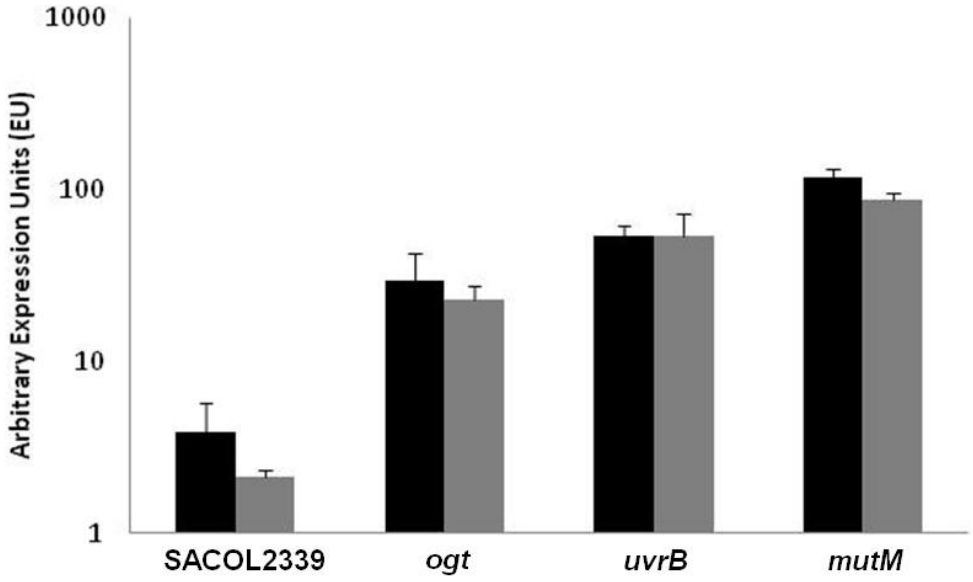

Figure 51. Expression of a Variety of DNA Repair Pathway Genes are Not Altered in the σ^S Mutant. Quantitative real-time PCR analysis of RN4220 (black) and its *sigS* mutant (grey) was performed with cultures grown in TSB at 37^{o}C with shaking for 3h. The data presented is from at least 3 independent experiments. Error bars are shown as +/- SEM.

external stressors. Of those external stressors examined, a number have been implicated in leading to DNA damage, including hydrogen peroxide, diamide and acid/alkali stress. This supports both our previous transcriptional analysis, as well as our phenotypic analysis, which reveals a role for σ^S in the protection of *S. aureus* against DNA damage stress. We demonstrate a decreased ability of the *sigS* mutant to survive against the wild-type in the presence of EDTA, a chelating agent that acts to sequester metals. This connects well with our transcriptional analysis, which shows that *sigS* transcription is increased during growth in metal limiting media. As discussed earlier, this is likely a result of a decrease in cell wall stability in the absence of divalent cations. The decreased ability of the *sigS* mutant to survive against the parent strain in the presence of the cell wall targeting compounds, Penicillin G and ethanol, further supports our previous transcriptional analyses, which shows *sigS* transcription to be increased during exposure to cell wall destabilizing chemicals. Collectively, this suggests that *sigS* is advantageous to *S. aureus* both during standard growth conditions, as well as during times of starvation and/or stress.

Microarray analysis revealed increases in the lactose fermentation genes, *lacA*, *lacB* and *lacD* in the absence of σ^S. This connects well with previous findings that show mutation of the lactose repressor, *lacR*, leads to increased expression of *sigS*. This would suggest that *sigS* responds to carbohydrate limitation, and may provide additional explanation as to why *sigS* mutants are less able to survive under starvation conditions. We performed carbohydrate utilization assays on the RN4220 and USA300 LAC *sigS* mutants against the parent strains and found no notable differences in the ability to ferment lactose or any

other carbohydrate, including xylose, D-glucosamin HCl, mannose, ribose, galactose, fructose, raffinose and trehalose (Figure 52). This analysis is similar to our disk diffusion assays, in that it only provides end point data when cells are deep into stationary phase. As such, there may be differences in the rapidity at which the *sigS* mutant utilizes these carbohydrates during earlier growth phases. However, as the genes in the lactose operon are increased in the *sigS* mutant, this would suggest that they are not direct targets of this regulator, and thus carbohydrate utilization may not be the major role for σ^S in *S. aureus* cells.

Another increase found in the *sigS* mutant through microarray analysis is the gene encoding staphyloxanthin. This protein is a carotenoid pigment that gives *S. aureus* its characteristic golden color, which would suggest that in a *sigS* mutant pigmentation is increased. More importantly, this protein acts as an antioxidant to help protect the cell against oxidative stress (Clauditz *et al.*, 2006). These findings support our transcriptional and phenotypic analyses, which show both *sigS* expression, and *sigS* mutant sensitivity, to be increased during exposure to hydrogen peroxide. Further to this, previous reports demonstrate a correlation between staphyloxanthin production and purine biosynthesis and as such corroborate our findings that σ^S is involved in *de novo* nucleotide synthesis (Lan *et al.*, 2010). There was also an increase in genes encoding cell envelope associated proteins, such capsule (which helps inhibit phagocytosis), and the serine aspartate rich adhesion protein, SdrC. It has been shown in *E. coli*, that post exposure to antibiotics, capsular genes are increased; and we have previously shown that expression of *sigS* is increased under these conditions (Drayson *et al.*, 2011). This suggests that the cell may

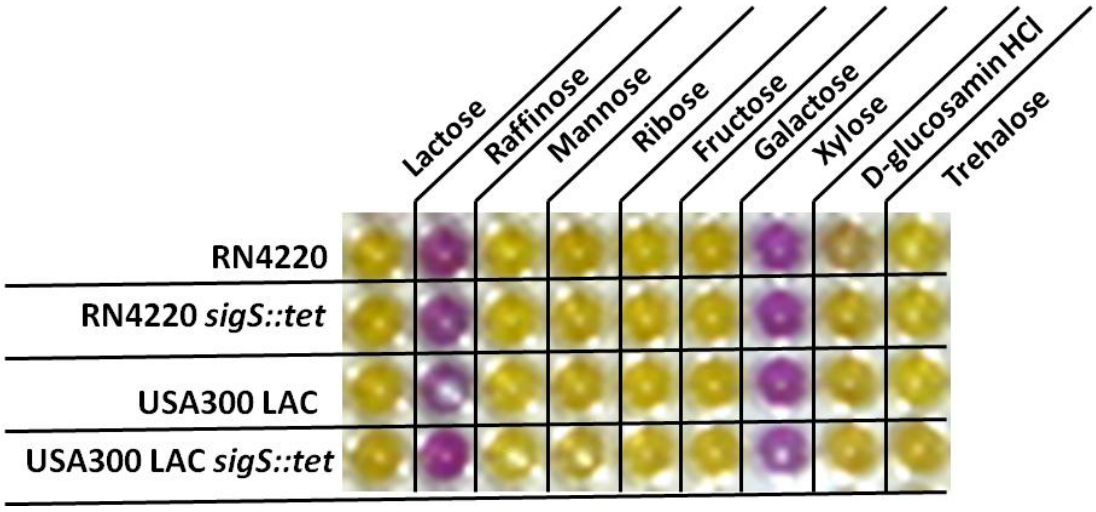

Figure 52. Carbohydrate Utilization Does Not Appear to be Mediated by σ^S. The RN4220 and USA300 LAC wild-type and *sigS* mutant strains were grown in purple indicator broth containing different carbohydrates at a concentration of 1%. Transition of the media from purple to yellow indicates that fermentation has occurred.

compensate for a loss of σ^S by increasing capsule production, which may serve as a barrier to external stress, such as antibiotics. We also noted that an increase in transcription of the gene encoding the cell adhesion protein, SdrC, corresponds to increased clumping of the RN4220 *sigS* mutant during growth in rich media at 37^{o}C (Figure 53). This increase in adhesion may aid in protection of the cell from antibiotics in the absence of σ^S, as previous work demonstrates that exposure to antibiotics alters the cells adhesive capacity (Beachey *et al.*, 1981). We further see an increase in a number of secreted virulence determinants in the mutant strain, including both hemolysins and proteases, possibly as a means to acquire nutrients from the surrounding environment in order to enhance survival in the absence of σ^S. We also discovered an increase in the thermonuclease precursor, *nuc*, which may serve to degrade DNA and RNA in the extracellular milieu in an effort to circumvent nucleotide depletion.

This corresponds to the decrease we find across the purine biosynthesis genes in the absence of σ^S, suggesting that they are likely targets of regulation by σ^S, and by extension that it plays a role in *de novo* nucleotide synthesis. This is confirmed in multiple *S. aureus* backgrounds through qRT-PCR analysis of each of the transcripts encoding purine biosynthesis genes located throughout the genome. These findings were further confirmed at the enzymatic level through observation of decreased activity of the purine biosynthesis enzyme, adenylosuccinate synthetase, which catalyzes the conversion of inosine 5' phosphate to adenylosuccinate in the generation of adenosine. We also show a decrease in the levels of ADP and GDP in the cell in the absence of σ^S, which, taken

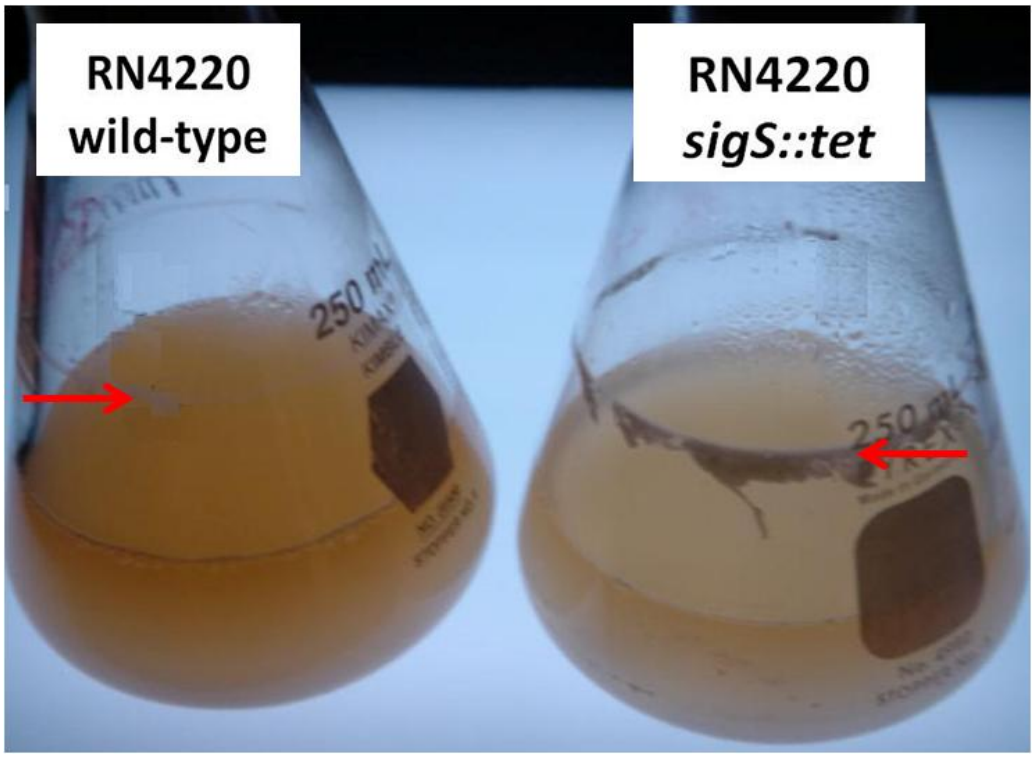

Figure 53. The Absence of σ^S Leads to Increased Clumping in RN4220. The RN4220 wild-type and *sigS* mutant strains were grown in complex liquid media at 37°C with shaking overnight. The flasks shown here display an increase in the accumulation or clumping of cells in the *sigS* mutant on the sides of the flasks, as denoted by the red arrows. These findings are repeatedly observed.

together with our transcriptional and enzymatic analyses, further confirms a role for this regulator in *S. aureus* purine biosynthesis.

Proteomic analysis of the 8325-4 *sigS* mutant compared to the wild-type strain grown in the presence of MMS demonstrates a number of proteins altered in abundance. The zinc metalloprotease, RseP, was increased in the *sigS* mutant, and is an intramembrane protease demonstrating homology to RseP of *E. coli*. In this organism, it acts as the site-2-protease involved in the cleavage of the RpoE anti-sigma factor (Akiyama *et al.*, 2004). As such, our findings suggest that RseP of *S. aureus* may serve in the σ^S regulatory cascade. In such a scenario, upon deletion of *sigS*, the cell would attempt to increase RseP levels in order to generate more free and active σ^S protein. Another protein increased in this analysis was a putative thermonuclease family protein, possibly involved in degradation of DNA and RNA to provide nucleotide precursors. This corroborates our microarray analysis, which shows in the absence of σ^S the cell requires increases in transcription of the thermonuclease, *nuc*, possibly as a means for nucleotide salvage. Our earlier finding that mutation of *sigS* leads to increased clumping of the cells is also supported by our proteomic analysis. Specifically, we identify the adhesion protein, SdrC as being increased in the *sigS* mutant background, confirming our microarray analysis, which also identified *sdrC* as being increased.

Of those proteins decreased in the *sigS* mutant, a number are involved in cell wall metabolism. The first of these is DdlA, or the D-alanine – D-alanine ligase, which produces the cell wall peptidoglycan precursor, D-alanine – D-alanine, and is vital for

cell wall biosynthesis (Walsh, 1989). Additionally, the product of the gene co-transcribed with DdlA, MurF, was also identified as being decreased in a *sigS* mutant. This protein is involved in cell wall biosynthesis, and functions by attaching the D-alanine – D-alanine motif to the UDP-N-acetylmuramic acid tripeptide (Sobral *et al.*, 2006). Both DdlA and MurF are involved in resistance to certain cell wall targeting antimicrobials (Sobral *et al.*, 2003). Specifically, previous reports demonstrate that increased susceptibility of *S. aureus* to β-lactam antibiotics is directly related to impairment of DdlA (Meziane-Cherif *et al.*, 2010). Correlatively, we show an increase in expression of *sigS* post exposure to the β-lactam antibiotics ampicillin and oxacillin. Further to this, we show increased susceptibility of the *sigS* mutant during coculture analysis to the presence of penicillin G, another β-lactam, which may also be explained by this decrease in DdlA and MurF in the *sigS* mutant. The observed *sigS* expression and *sigS* mutant sensitivity to cell wall targeting antibiotics may also be a result of a decrease in the UDP-N-acetylmuramic acid:L-alanine ligase, MurC (Kurokawa *et al.*, 2008), and the peptidyltransferase, FemX, important for catalyzing the formation of the interpeptide bridge (Schneider *et al.*, 2004). This decrease in FemX in the *sigS* mutant correlates well with our findings from our transposon analysis, which reveals an insertion in another peptidyltransferase, FemB, leads to increased expression of *sigS*. Collectively, this supports our transcriptional and phenotypic studies and suggests a role for σ^S in the response to extracytoplasmic stimuli. Indeed, preliminary analysis in our laboratory reveals increased sensitivity of the *sigS* mutant to cell wall targeting antimicrobial agents (Burda and Shaw unpublished observations).

Importantly, a decrease was observed in the proteomics studies for proteins involved in DNA metabolism, replication, recombination and repair; including the DNA mismatch repair proteins MutS and MutS2. MutS was also identified in our transposon screen, disruption of which leads to increased expression of *sigS*, and also a mutator phenotype in *S. aureus* (O'Neill, 2010), meaning that strains acquire random SNPs at a higher frequency. In *E. coli*, MutS has a role in preventing homologous recombination; however, in *S. aureus* it appears to only have a minor role in this process (Matic *et al.*, 1994; Prunier and Leclercq, 2005; Rayssiguier *et al.*, 1989). Further to this, we identified a decrease in the levels of the bicomponent exonuclease SbcDC. As stated previously, in *E. coli*, this exonuclease functions to repair DNA and maintain genome stability through cleavage of DNA hairpins caused by intrastrand base pairing of palindromic DNA (Chalker *et al.*, 1988; Connelly *et al.*, 1998; Connelly *et al.*, 1999; Leach and Stahl, 1983). Its function in *S. aureus* is not completely understood; however, it has been documented to be induced by the SOS response, and has a role in protection against DNA damage created by UV irradiation (Chen *et al.*, 2007). SACOL1479, which encodes another 5'-3' exonuclease was also decreased in the *sigS* mutant. While little is known about this protein, it has been demonstrated that *E.coli* deficient in 5'-3' exonucleases are more susceptible to DNA damage induced by UV exposure (Chase and Masker, 1977). Therefore, it is plausible that this 5'-3' exonuclease and SbcC may be necessary to repair DNA in *S. aureus* that has been damaged by UV irradiation. This further corroborates our finding that *sigS* mutants are sensitive to damage by UV light; and we have previously identified disruptions in *sbcC*, and the 5'-3' exonuclease, as leading to increased expression of *sigS*. This increase in expression of *sigS* in exonuclease mutants,

as described during our previous transposon analysis, may be explained by an increased need for purines, as some exonucleases play a role in DNA and mRNA recycling as a part of purine salvage pathways that are induced following *de novo* inhibition (Datta and Niyogi, 1975; Wakamatsu *et al.*, 2011). This suggests that in addition to the purine biosynthesis genes, the *sbcDC* operon may be controlled by σ^S, further supporting a role for it in both DNA damage repair and *de novo* purine synthesis.

Of major significance, we observed a decrease in the levels of proteins involved in purine biosynthesis upon proteomic analysis. These findings are unsurprising as we have shown transcription of these genes to be decreased in the *sigS* mutant. Further to this, we have also performed enzyme activity assays that corroborate both of these findings. The levels of nucleotides in the cells were also tested, and found to be deficient in the pools of ADP and GDP in the *sigS* mutant. Collectively, this affirms the role we have established herein for σ^S in *de novo* synthesis of purines.

Phenotypically, we show that σ^S is important for survival during growth in whole human blood, and following phagocytosis. This supports our earlier work, which shows strong upregulation of *sigS* expression (regardless of background) under these same conditions, and reveals a major requirement for σ^S during virulence (Shaw *et al.*, 2008). Previous reports demonstrate an increase in the purine biosynthesis genes during growth of *S. aureus* in both human serum and blood (Malachowa and DeLeo, 2011). As we demonstrate a requirement for σ^S for full activity of the purine biosynthesis genes, this may explain our expression analysis during growth in serum, as well as our observation

that *sigS* mutants display decreased survival during growth in human blood. Furthermore, studies have shown that adenosine synthesis is utilized by *S. aureus* as a method for evading host immune responses (Thammavongsa *et al.*, 2009). Reports also demonstrate the importance of a functional purine biosynthesis pathway for full virulence in *S. aureus*, as well as a number of other pathogenic organisms, including *Bacillus anthracis, Mycobacterium tuberculosis, Brucella abortus* and *Salmonella typhimurium* (Alcantara *et al.*, 2004; Crawford *et al.*, 1996; Jackson *et al.*, 1999; Jenkins *et al.*, 2011; Lan *et al.*, 2010; McFarland and Stocker, 1987; O'Callaghan *et al.*, 1988; Samant *et al.*, 2008). Moreover, it has been observed that pathogenic organisms such as *Burkholderia* spp., *B. abortus* and *Vibrio cholerae* defective in DNA damage repair pathways are attenuated in virulence, underscoring that pathogenic bacteria are bombarded by DNA damaging conditions during infection (Cuccui *et al.*, 2007; Davies *et al.*, 2011; Rolfe *et al.*, 2012; Yeager *et al.*, 2001). Together this suggests that, upon entry into the host, bacterial pathogens are faced with an array of DNA damaging conditions, which requires extensive use of repair pathways, and an increased need for nucleotide building blocks. As free nucleotide pools are very low within the human host, *de novo* synthesis becomes of paramount importance. Given that, at least in the case of purines, this is under the control of σ^S in *S. aureus*, this likely goes some way to explaining the avirulence of *sigS* mutants.

CHAPTER 6:

FINAL DISCUSSION

Discussion. Herein we present characterization of the lone extracytoplasmic function (ECF) sigma factor, σ^S, of *Staphylococcus aureus*. Specifically, we demonstrate conditions under which *sigS* is expressed and we have identified potential members of the σ^S regulatory cascade; as well as potential members of the σ^S regulon. Promoter mapping of the *sigS* locus reveals four discrete transcriptional start sites. Promoters, P1, P3 and P4 appear to be under the control of the housekeeping σ factor, σ^A (Figure 54A) while P2 is autoregulated by σ^S (Figure 54B). Through transcriptional analysis, we demonstrate that expression of *sigS* is minimal under standard conditions in the majority of *S. aureus* strains. This likely results in baseline expression from one or a combination of promoters, as we are able to detect transcript in the SH1000 background under standard conditions during our initial 5' RACE analysis. As is typical of many members of the ECF subfamily, any σ^S protein produced under these conditions is likely sequestered by an anti-sigma factor and held inactive. Upon the introduction of a stress, such as cell wall disruption or DNA damage, σ^S would be freed from the anti-sigma factor and allowed to upregulated the genes in its regulon, including its own P2 promoter. We have identified herein a numbers of stressors that bring about increased expression of *sigS*, which fall under two major classes. The first of which are considered a typical response of ECF

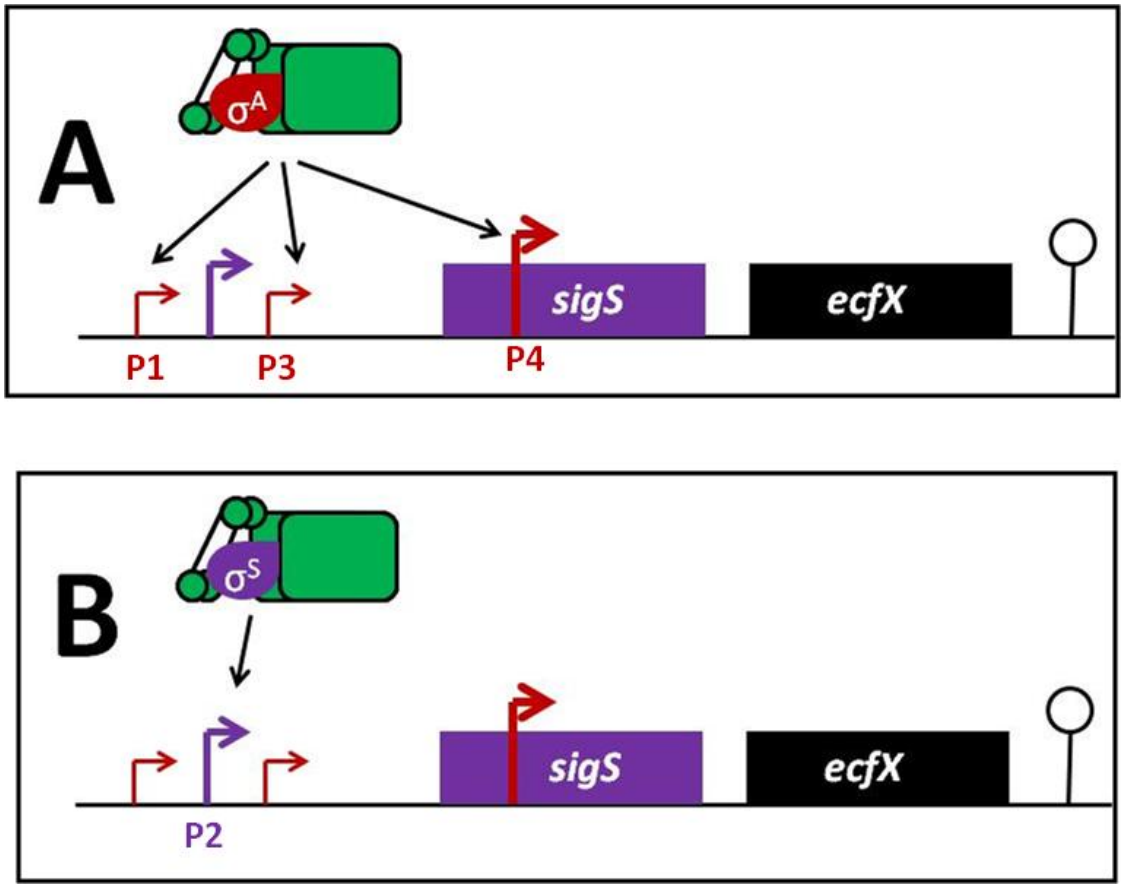

Figure 54. Depiction of *sigS* Promoter Recognition. (A) The *sigS* promoters P1, P3 and P4 are under the control of σ^A. (B) The *sigS* promoter P2 is under the control of σ^S.

sigma factors, and include those chemicals that lead to cell wall destabilization such as oxacillin, ampicillin, cefotaxime, SDS and phosphomycin. The second group is distinct from that of classical ECF sigma factor responses, and is made up of compounds that induce DNA damage stress, including MMS, EMS, ciprofloxacin and hydrogen peroxide. Furthermore, we were able to establish that expression of *sigS* is strongly induced during challenge by components of the innate immune system, likely as a result of exposure to a combination of cell wall destabilizing conditions and DNA damage stress.

Although the specific pathways for σ^S regulation are still unclear, we suggest that at least two signal transduction mechanisms may exist, as σ^S responds to both extracytoplasmic stress (cell wall targeting antibiotics) and cytoplasmic stress (DNA damage); which are typically signaled in different ways. Interestingly, this is true for RpoE of *E. coli*, which was originally identified as being triggered by misfolded proteins in the periplasm or outer membrane through regulation by RseA, a transmembrane anti-sigma factor (Dartigalongue *et al.*, 2001). Evidence exists to suggest that RpoE also responds to nutritional stress in response to ppGpp in a RseA-independent manner (Costanzo and Ades, 2006). The signaling mechanisms employed by ECF sigma factors that respond to extracytoplasmic stress involve negative regulation by a membrane bound anti-sigma factor, while those responding to cytoplasmic stresses function via soluble, cytoplasmically located anti-sigma factors. Upon examination of the *sigS* locus, two candidate anti-sigma factors were identified. The σ^S locus, displayed in Figure 55, contains a downstream gene *ecfX* located 112bp away, as well as a transcriptionally divergent gene, *ecfY*. This gene clustering is conserved across all sequenced strains of

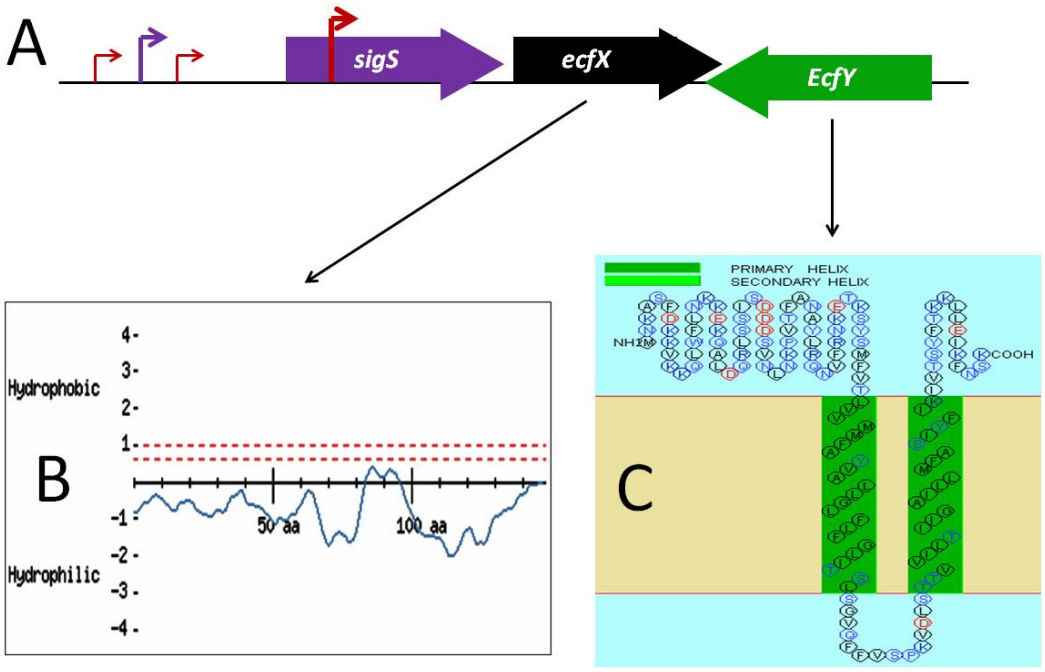

Figure 55. Hydrophobicity Plots of the Putative Anti-Sigma Factors in the *sigS* Locus. (A) Depicted is the *sigS* locus, detailing the downstream genes *ecfX* and *ecfY*, which present candidate anti-sigma factors. Transmembrane domains were determined from protein sequence utilizing a Kyte-Doolittle hydrophobicity plot **(B)** EcfX or a SOSUI membrane topology map **(C)** EcfY; however, only EcfY displays a transmembrane domain.

the Staphylococci. Hydrophobicity plots indicate a membrane spanning region within EcfY; however, no such region exists in EcfX (Figure 55). While neither EcfY nor EcfX fit the classic model, individually they each have characteristics of membrane bound and cytoplasmic anti-σ factors, respectively. To investigate this, we began by examining whether *ecfX* was cotranscribed with *sigS*. Our data shows potential transcriptional linkage of *ecfX* with *sigS*; thus if it functions as an anti-σ factor, then its inactivation should result in free σ^S protein, and upregulation of the *sigS* gene via autoregulation. Our data herein actually demonstrates a requirement for a functional EcfX for expression of *sigS*, suggesting that this gene acts as a positive activator. These findings appear counterintuitive as the general mechanism previously established for ECF σ factors include negative regulation by a downstream co-transcribed gene (Helmann, 2002). We sought to further corroborate these findings through phenotypic analysis, with the hypothesis that if *ecfX* fulfills such a function, then any phenotypes observed for the *sigS* mutant should be reproducible in the *ecfX* mutant strain as well. Indeed, strains bearing mutation of *ecfX* displayed a decreased ability to survive compared to the wild-type; however, this decrease was not as substantial as that observed for *sigS* alone. In addition to providing validation of our earlier findings, this also confirms that the sensitivity observed for the *sigS* mutant strains is not a result of polar effects on the downstream gene, *ecfX*. If this were the case, the *sigS* mutant phenotypes would be mirrored in the *ecfX* mutant strains. Further to this, our initial transcriptional analysis suggests stronger activity of the internal promoter, which likely generates *ecfX* transcript. One might suggest that response of the internal promoter to DNA damage and cell wall destabilizing conditions leads to the upregulation of *ecfX* to circumvent the stress. However, if this

were indeed true, a mutation in *ecfX* alone would lead to decreased survival during exposure to these stress conditions, which, as we demonstrate herein, is not the case. As mutations in *ecfX* do not demonstrate phenotypes to the full extent as that seen with *sigS* mutants, this suggests that activity of σ^S is diminished only slightly in the absence of *ecfX*. Bioinformatic analysis of EcfX reveals a ComK-like domain located in its C-terminus (Figure 56). In *B. subtilis*, ComK acts as a DNA-binding protein involved in the upregulation of itself and other competence genes (van Sinderen *et al.*, 1995). Therefore, EcfX may act as a DNA-binding protein in a manner akin to that of ComK, which would suggest novel control of σ^S. Further to this, ComK of *B. subtilis* associates with ClpC and MecA in a complex, indicating that it also possesses protein-binding ability (Dubnau *et al.*, 2002; Nakano *et al.*, 2002; Turgay *et al.*, 1997; Turgay *et al.*, 1998). As the exact residues required for DNA-binding and protein-protein interaction are still unknown for ComK, it is not clear whether EcfX possess homology to the DNA binding domain or the protein-protein interaction domain. This suggests that EcfX may interact with σ^S at the protein level, possibly through stabilization of the σ^S-RNAP complex. This is supported by the fact that in the ancient Staphylococcal species, *S. carnosus*, σ^S and EcfX are actually a single gene/protein, suggesting that they function together in the cell towards a common goal (Figure 57).

As we have shown that EcfX is not acting as an anti-sigma factor, we currently believe that the membrane associated protein, EcfY, may serve as the σ^S anti-sigma factor. Of note, examples exist that demonstrate mutation in the anti-sigma factor of ECF-sigma factors can have a toxic affect on the cell, due to hyperactivity of the σ factor. For

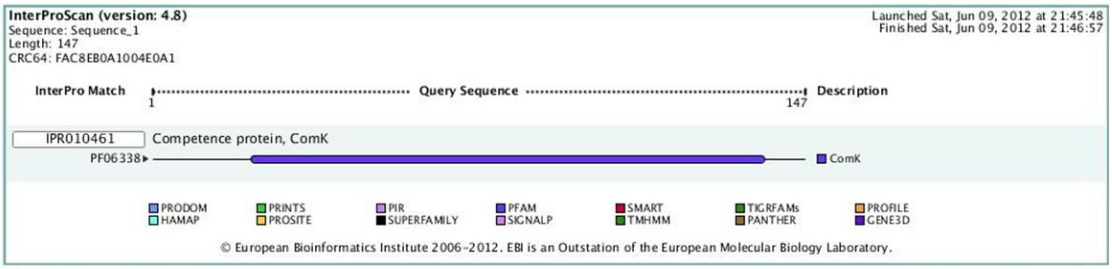

Figure 56. Bioinformatic Analysis of EcfX Reveals a ComK-like Domain. A domain search was performed on EcfX through analysis with InterPro Scan, revealing a ComK-like domain.

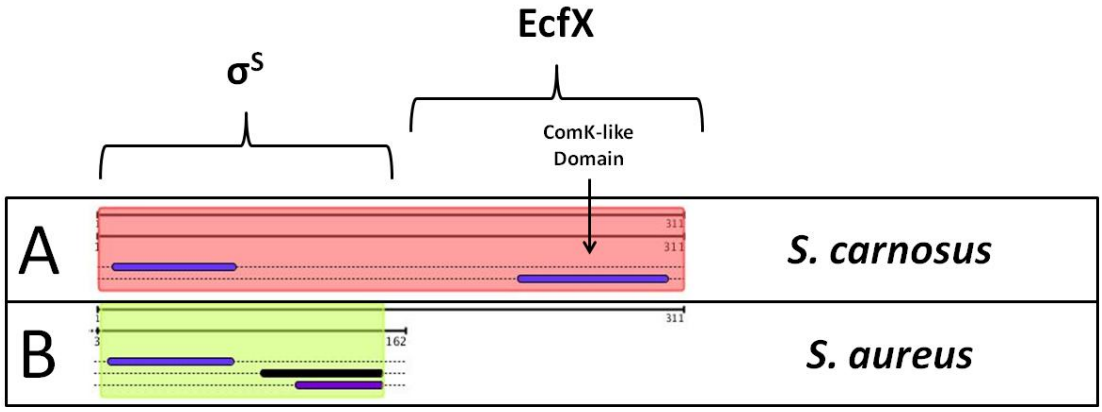

Figure 57. Bioinformatic Analysis of σ^S in *S. carnosus* Reveals a Single Gene/Protein Containing Both σ^S and EcfX. BLAST analysis was performed on σ^S through InterPro. (A) *S. carnosus* reveals a single protein containing both σ^S and EcfX, while (B) σ^S and EcfX are individual proteins in *S. aureus*.

example, the anti-sigma factor of RpoE2 in *Sinorhizobium meliloti* can only be mutated if RpoE2 is mutated as well; this was confirmed via introduction of a multicopy plasmid containing *rpoE2*, which produced only slow-growing colonies (Sauviac *et al.*, 2007). This is further corroborated by the inability to overexpress the ECF sigma factor RpoE from *E. coli*, or its *Pseudomonas aeruginosa* homologue AlgU, due to toxicity (Nitta *et al.*, 2000; Rezuchova and Kormanec, 2001; Schurr *et al.*, 1996). This may, therefore, explain why we did not identify any insertion in *ecfY* during our transposon screen. This contention is further corroborated by the observation that, no transposon insertions were obtained for *ecfY* in a recently generated ordered transposon library for *S. aureus* strain USA300 (The Nebraska Center for Staphylococcal Research). Indeed, preliminary analysis in our laboratory suggests that *S. aureus* cells are remarkably recalcitrant to overexpression of *sigS*.

We have demonstrated that expression of *sigS* under standard conditions can be achieved by random mutation throughout the genome, as observed for the laboratory strain RN4220, and our NTG generated SH1000 strains. Additionally, this can be accomplished by individual transposon mutation in select genes, which led to the identification of a number of candidate σ^S regulators, including a potential anti-sigma factor and a site-1-protease. The former is Msa, which was previously determined to play a role in modulating expression of SarA, a global regulatory protein involved in virulence determinant expression (Sambanthamoorthy *et al.*, 2006). Msa contains three membrane spanning regions, and, when mutated, leads to increased expression of *sigS*. As such, this protein may serve as a potential anti-sigma factor for σ^S, whereby disruption of it would

lead to an increase in free σ^S within the cell, and subsequently an increase in its expression through autoregulation. A membrane spanning, C-terminal protease, CtpA, was also identified in our screen as potentially regulating *sigS* expression. This serine protease demonstrates homology to DegS of *E. coli*, which initiates the proteolytic cascade to free the ECF sigma factor RpoE during the response to heat shock (Grigorova *et al.*, 2004). Like DegS, CtpA contains one transmembrane domain, a prc or c-terminal peptidase domain, and a PDZ signaling/protein-interaction domain. Of note, an additional putative site-1-protease denoted PrsW has been identified via *in silico* analysis by our group. This putative protease demonstrates homology to PrsW of *B. subtilis*, which acts to cleave the σ^W anti-sigma factor, RsiW, in response to antimicrobial peptides and cell envelope stress (Ellermeier and Losick, 2006). Current work suggests that the PrsW homologue is the σ^S site-1-protease, as a number of phenotypes are conserved between the *prsW* and *sigS* mutants (Krute and Shaw, unpublished observations). Therefore, one might suggest activation of σ^S can occur via multiple site-1-proteases, including both CtpA and PrsW, as depicted in Figure 58. An example of such a scenario comes from CtpB of *B. subtilis*, which acts alongside a second protease, SpoIV, to bring about activation of the sporulation sigma factor, σ^K (Pan *et al.*, 2003). Typically, ECF-sigma factor activation proceeds, following site-1- cleavage, by site-2-proteolysis of the anti-sigma factor. In the case of σ^S, we have identified a candidate S2P, the zinc metalloprotease, RseP, which was increased in the *sigS* mutant during our proteomic analysis. RseP is an intramembrane protease demonstrating homology to RseP of *E. coli* and *B. subtilis*, both of which act as the site-2-protease involved in the cleavage of the RpoE and σ^W anti-sigma factors, respectively (Akiyama *et al.*, 2004). Similar to

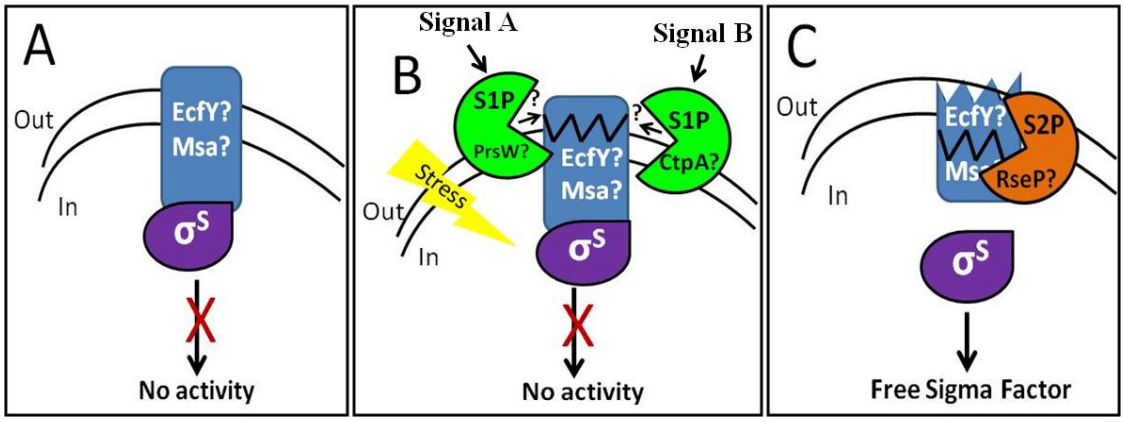

Figure 58. The Putative Proteolytic Cascade Leading to σ^S Activation. (A) During times of noninduction, σ^S is sequestered by an anti-sigma factor; putatively either Msa or EcfY. **(B)** Upon the introduction of stress (DNA damage or cell wall destabilization), the site-1-protease (S1P) cleaves the anti-sigma factor at the C-terminus. Candidate proteases involved in this cleavage event include PrsW and CtpA. Additionally, the signals that PrsW and CtpA receive to activate σ^S may be different; however, this also has yet to be confirmed. **(C)** The putative site-2-protease (S2P) RseP then cleaves the anti-sigma factor at the N-terminus, releasing σ^S.

that observed for EcfY, RseP is also considered a putative essential gene, as it too was not identified in the ordered transposon library; which is confirmed by preliminary work in our laboratory (Krute and Shaw, unpublished observations). As such, our findings suggest that RseP of *S. aureus* may serve in the σ^S regulatory cascade as depicted in Figure 58).

Transcriptomic and proteomic analysis of the *sigS* mutant has revealed potential genes under its control. These include the cell wall metabolism genes, *femX* and *ddlA*, which may go some way to explaining the increase in *sigS* expression observed during exposure to cell wall destabilizing conditions. Importantly, preliminary analysis in our laboratory also reveals increased sensitivity of the *sigS* mutant to cell wall targeting antimicrobial agents (Burda and Shaw, unpublished observations). Further to this, we identify the purine biosynthesis genes *purA, purB, guaBA* and *purEKCSQLFMNHD*, as well as the exonucleases *sbcDC* and SACOL1479, as targets for σ^S activity. These genes are believed to aid in the response to DNA damage, which may explain why *sigS* expression is increased under such conditions. Analysis of the region 5' of the start codon for these genes led to the identification of putative *sigS* recognized promoters, displaying the consensus cAAAgt -12 bp- taTCA, as displayed in Table 16. Interestingly, the putative recognition sites appear to differ slightly from the consensus identified in the *sigS* operon, which may be explained by the observation that σ^E of *E. coli* will recognize promoter sequences from the genes under its control that are far more divergent than its general consensus (Dartigalongue *et al.*, 2001). It has been suggested that this organism can afford these deviations as it only encodes two ECF σ factors; whereas other organisms

Table 16. Putative Promoter Elements of σ^S Regulon Genes.

Gene[a]	ORF ID	Operon size	Function	-35	-10	Spacing[b]	5'UTR[c]
sigS	SACOL1827	2	Sigma Factor	**CAAAGT**	**TATCA**	12	134
sbcD	SACOL1381	2	Exonuclease	a**AAAG**g	**TAT**t**A**	11	38
	SACOL1479	1	5'-3' Exonuclease	g**AAAGT**	at**TCA**	12	116
purA	SACOL0018	1	Purine Biosynthesis	g**AAAGT**	**T**g**TCA**	13	161
purB	SACOL1969	1	Purine Biosynthesis	t**AAAGT**	**T**ta**CA**	11	207
guaB	SACOL0460	2	Purine Biosynthesis	**CAAA**cg	**T**g**T**g**A**	10	43
purE	SACOL1073	11	Purine Biosynthesis	a**AAAG**a	a**A**a**CA**	9	109
femX	SACOL2253	1	Cell Wall Biosynthesis	g**AAA**ca	at**TCA**	13	158
ddlA	SACOL2074	2	Cell Wall Biosynthesis	**CAAAG**a	**TA**a**CA**	15	68

[a] The first gene in the operon is listed.

[b] The spacing listed represents the nucleotides located between the -10 and -35 elements.

[c] The 5' UTR is the distance from the -10 element to the start codon.

such as *B. subtilis* encoding a number of ECF σ factors, require highly conserved promoters in order to differentiate unique ECF regulons (Helmann, 2002). As σ^S is the only ECF σ factor encoded by *S. aureus*, it is possible that it can tolerate significant divergence from consensus in a manner similar to that of σ^E of *E. coli*.

We demonstrate here that *sigS* activity is increased when *S. aureus* is challenged by complement during growth in pig serum. Further to this, we also present evidence for *sigS* upregulation during *ex vivo* infection, as we demonstrate a strong upregulation (regardless of background) upon phagocytosis by murine macrophage-like cells. Phenotypically, we show that σ^S is important for survival during growth in whole human blood, and following phagocytosis. This supports our previous work, which reveals a major requirement for σ^S during virulence in septic infections (Shaw *et al.*, 2008). Furthermore, we provide evidence herein that σ^S is also important for virulence in localized infections as well. As stated earlier, our transcriptional analysis reveals expression of *sigS* during exposure to DNA damage stress; however, we show the σ^S regulon to potentially include purine biosynthesis genes. In support of this, it has been observed that pathogenic organisms such as *Burkholderia* spp., *B. abortus* and *Vibrio cholerae* defective in DNA damage repair pathways are attenuated in virulence, underscoring that pathogenic bacteria are bombarded by DNA damaging conditions during infection (Cuccui *et al.*, 2007; Davies *et al.*, 2011; Roux *et al.*, 2006; Yeager *et al.*, 2001). Following challenge by DNA damage agents, there may be increased pressure for the cell to synthesize purines as the nucleotide pool is being depleted by activated repair pathways. This is supported by the fact that previous reports demonstrate an increase in

purine biosynthesis gene expression during growth of *S. aureus* in both human serum and blood (Malachowa and DeLeo, 2011). As *de novo* purine biosynthesis appears to be σ^S dependent, this would explain why *sigS* is induced following DNA-damage, and why *sigS* mutants have impaired survival during infection. Furthermore, studies have shown that adenosine synthesis is utilized by *S. aureus* as a method for evading host immune responses (Thammavongsa *et al.*, 2009). Reports also demonstrate the importance of a functional purine biosynthesis pathway for full virulence in *S. aureus*, as well as a number of other pathogenic organisms, including *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Brucella abortus* and *Salmonella typhimurium* (Alcantara *et al.*, 2004; Crawford *et al.*, 1996; Jackson *et al.*, 1999; Jenkins *et al.*, 2011; Lan *et al.*, 2010; McFarland and Stocker, 1987; O'Callaghan *et al.*, 1988; Samant *et al.*, 2008). Together this suggests that, upon entry into the host, bacterial pathogens are faced with an array of DNA damaging conditions, which requires extensive use of repair pathways, and an increased need for nucleotide building blocks. As free nucleotide pools are very low within the human host, *de novo* synthesis becomes of paramount importance. Given that, at least in the case of purines, this is under the control of σ^S in *S. aureus*, this likely goes some way to explaining the avirulence of *sigS* mutants.

Future Directions. Our analysis of σ^S thus far has led to the identification of 4 unique promoters; however, it is unclear what transcript is generated from each. As such, a major future direction should be in defining the *sigS* and *ecfX* transcripts through the use of RNAseq or 3' RACE analysis. Due to the low level of expression of this locus, even under inducing conditions, the sensitivity of northern blot analysis has thus far proven too

weak to detect *sigS* transcript, and as such is perhaps not the method of choice for these purposes. Furthermore, the specific conditions under which each individual promoter responds is still unknown. Future work may involve determining which promoter(s) is/are responsible for the increase in expression observed during exposure to cell wall destabilization or DNA damage. As we have shown this to be difficult herein due to low levels of transcript generated from the individual promoters, the method of choice would likely be qRT-PCR, with various primers located 3' of each promoter. Preliminary analysis in our laboratory reveals a number of potential DNA-binding proteins that recognize the upstream *sigS* promoter region (Burda and Shaw, unpublished observations). These regulatory factors putatively influence the activity of the different *sigS* promoters, and as such their identification would provide novel insight into the regulation of σ^S. This identification could be accomplished through the use of pull down assays utilizing biotin tagged *sigS* promoter DNA and protein lysates. These lysates could be generated from cells expressing *sigS* so as to identify positive regulators, or cells harvested during periods of minimal *sigS* expression to identify negative regulators. The resulting proteins could then be studied via LC-MS analysis. We have also identified here potential members of the σ^S regulatory cascade, and as such a major focus moving forward should be to identify the roles for EcfX and EcfY. We show herein a role for EcfX in the positive activation of *sigS*; however, we have yet to elucidate whether this occurs through DNA-binding activity or protein-protein interactions. As such, protein-protein pull-down assays using purified σ^S and EcfX would be an ideal method for initial analysis. To begin to evaluate a role for EcfY in the regulation of σ^S, one may suggest performing qRT-PCR analysis of this gene in the presence of a mutation in *sigS*. The

idea behind this being that, as *sigS* and *ecfY* are not transcriptionally linked, transcription of the σ^S anti-sigma factor may be decreased to compensate for the mutation in *sigS* if it is acting as a negative regulator. Further, if EcfY is truly an essential gene due to the necessity to negatively regulate σ^S, then mutation of EcfY should be possible in a *sigS* mutant background. If a double knockout strain is possible, insertion of a plasmid containing a functional *sigS* gene under the control of an inducible promoter would verify this hypothesis, as induction should be lethal to the cell. Elucidation of the regulatory cascade controlling σ^S activity, including further evaluation of the potential S1P and S2P, PrsW, CtpA and RseP, becomes important for a complete understanding of σ^S function. An important initial analysis of these genes would include qRT-PCR of *sigS* in the presence of mutations in each potential regulator during induction conditions. Specifically, if these proteins participate in the proteolytic cascade leading to σ^S activation, and subsequent autoregulation, then in the absence of these proteins, *sigS* expression should be decreased.

Thus far our research has focused on identifying those regulators that positively affect *sigS* expression. As such, an additional area of study could be the utilization of a reverse transposon screen to identify regulators that negatively affect *sigS* expression. This would be accomplished by plating the existing 8325-4 *sigS-lacZ* transposon library on TSA containing 2.5 mM MMS and X-gal, which will result in the production of blue colonies due to *sigS* upregulation. However, any transposon insertions resulting in disruption of the activation of σ^S would be no longer blue, and could then be selected for insertion site identification. Herein we also identify potential σ^S target genes, including

the purine biosynthesis genes *purA, purB, guaBA* and *purEKCSQLFMNHD*; however, it is still unclear whether their observed decreases in transcription are a direct result of σ^S activity. Therefore, one could use transcription run off analysis as a means of investigating whether σ^S complexed with RNAP can direct transcription of these genes.

REFERENCES CITED

(1944) Discussion on Penicillin. *Proc R Soc Med* **37**: 101-112.

Adelberg, E.A., and Pittard, J. (1965) Chromosome Transfer in Bacterial Conjugation. *Bacteriol Rev* **29**: 161-172.

Ades, S.E., Connolly, L.E., Alba, B.M., and Gross, C.A. (1999) The Escherichia coli sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. *Genes Dev* **13**: 2449-2461.

Aiyar, S.E., Juang, Y.L., Helmann, J.D., and Dehaseth, P.L. (1994) Mutations in Sigma-Factor That Affect the Temperature-Dependence of Transcription from a Promoter, but Not from a Mismatch Bubble in Double-Stranded DNA. *Biochemistry* **33**: 11501-11506.

Akagi, S., Sugiyama, H., and Makino, H. (2008) [Infection and chronic kidney disease]. *Nihon Rinsho* **66**: 1794-1798.

Akiyama, Y., Kanehara, K., and Ito, K. (2004) RseP (YaeL), an Escherichia coli RIP protease, cleaves transmembrane sequences. *Embo Journal* **23**: 4434-4442.

Alba, B.M., Leeds, J.A., Onufryk, C., Lu, C.Z., and Gross, C.A. (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. *Genes Dev* **16**: 2156-2168.

Alcantara, R.B., Read, R.D., Valderas, M.W., Brown, T.D., and Roop, R.M., 2nd (2004) Intact purine biosynthesis pathways are required for wild-type virulence of Brucella abortus 2308 in the BALB/c mouse model. *Infect Immun* **72**: 4911-4917.

Alvarez-Martinez, C.E., Baldini, R.L., and Gomes, S.L. (2006) A Caulobacter crescentus extracytoplasmic function sigma factor mediating the response to oxidative stress in stationary phase. *J Bacteriol* **188**: 1835-1846.

Ando, M., Yoshimatsu, T., Ko, C., Converse, P.J., and Bishai, W.R. (2003) Deletion of Mycobacterium tuberculosis sigma factor E results in delayed time to death with bacterial persistence in the lungs of aerosol-infected mice. *Infect Immun* **71**: 7170-7172.

Anthony, L.C., and Burgess, R.R. (2002) Conformational flexibility in or sigma(70) region 2 during transcription initiation. *Journal of Biological Chemistry* **277**: 46433-46441.

Archer, G.L. (1998) Staphylococcus aureus: a well-armed pathogen. *Clin Infect Dis* **26**: 1179-1181.

Arthur, T.M., and Burgess, R.R. (1998) Localization of a sigma(70) binding site on the N terminus of the Escherichia coli RNA polymerase beta ' subunit. *Journal of Biological Chemistry* **273**: 31381-31387.

Arthur, T.M., Anthony, L.C., and Burgess, R.R. (2000) Mutational analysis of beta '(260-309), a sigma(70) binding site located on Escherichia coli core RNA polymerase. *Journal of Biological Chemistry* **275**: 23113-23119.

Bae, T., Banger, A.K., Wallace, A., Glass, E.M., Aslund, F., Schneewind, O., and Missiakas, D.M. (2004) Staphylococcus aureus virulence genes identified by bursa aurealis mutagenesis and nematode killing. *Proc Natl Acad Sci U S A* **101**: 12312-12317.

Baptiste, K.E., Williams, K., Willams, N.J., Wattret, A., Clegg, P.D., Dawson, S., Corkill, J.E., O'Neill, T., and Hart, C.A. (2005) Methicillin-resistant staphylococci in companion animals. *Emerg Infect Dis* **11**: 1942-1944.

Bashyam, M.D., and Hasnain, S.E. (2004) The extracytoplasmic function sigma factors: role in bacterial pathogenesis. *Infect Genet Evol* **4**: 301-308.

Bastiat, B., Sauviac, L., and Bruand, C. (2010) Dual control of Sinorhizobium meliloti RpoE2 sigma factor activity by two PhyR-type two-component response regulators. *J Bacteriol* **192**: 2255-2265.

Batchelor, F.R., Doyle, F.P., Nayler, J.H., and Rolinson, G.N. (1959) Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. *Nature* **183**: 257-258.

Bayer, M.G., Heinrichs, J.H., and Cheung, A.L. (1996) The molecular architecture of the sar locus in Staphylococcus aureus. *J Bacteriol* **178**: 4563-4570.

Beachey, E.H., Eisenstein, B.I., and Ofek, I. (1981) Sublethal concentrations of antibiotics and bacterial adhesion. *Ciba Found Symp* **80**: 288-305.

Begier, E.M., Frenette, K., Barrett, N.L., Mshar, P., Petit, S., Boxrud, D.J., Watkins-Colwell, K., Wheeler, S., Cebelinski, E.A., Glennen, A., Nguyen, D., and Hadler, J.L. (2004) A high-morbidity outbreak of methicillin-resistant Staphylococcus aureus among players on a college football team, facilitated by cosmetic body shaving and turf burns. *Clin Infect Dis* **39**: 1446-1453.

Beranek, D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat Res* **231**: 11-30.

Berends, E.T., Horswill, A.R., Haste, N.M., Monestier, M., Nizet, V., and von Kockritz-Blickwede, M. (2010) Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. *J Innate Immun* **2**: 576-586.

Berscheid, A., Sass, P., Weber-Lassalle, K., Cheung, A.L., and Bierbaum, G. (2012) Revisiting the genomes of the Staphylococcus aureus strains NCTC 8325 and RN4220. *Int J Med Microbiol*.

Beynon, J., Cannon, M., Buchanan-Wollaston, V., and Cannon, F. (1983) The nif promoters of Klebsiella pneumoniae have a characteristic primary structure. *Cell* **34**: 665-671.

Bhakdi, S., and Tranum-Jensen, J. (1991) Alpha-toxin of Staphylococcus aureus. *Microbiol Rev* **55**: 733-751.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B., and Projan, S. (2004) Microarray-based analysis of the Staphylococcus aureus sigmaB regulon. *J Bacteriol* **186**: 4085-4099.

Blumberg, P.M., and Strominger, J.L. (1972) Five penicillin-binding components occur in Bacillus subtilis membranes. *J Biol Chem* **247**: 8107-8113.

Bohannon, D.E., and Sonenshein, A.L. (1989) Positive regulation of glutamate biosynthesis in Bacillus subtilis. *J Bacteriol* **171**: 4718-4727.

Bone, R.C. (1994) Gram-positive organisms and sepsis. *Arch Intern Med* **154**: 26-34.

Boschi-Muller, S., Olry, A., Antoine, M., and Branlant, G. (2005) The enzymology and biochemistry of methionine sulfoxide reductases. *Biochim Biophys Acta* **1703**: 231-238.

Bouanchaud, D.H., Scavizzi, M.R., and Chabbert, Y.A. (1968) Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. *J Gen Microbiol* **54**: 417-425.

Boyle-Vavra, S., and Daum, R.S. (2007) Community-acquired methicillin-resistant Staphylococcus aureus: the role of Panton-Valentine leukocidin. *Lab Invest* **87**: 3-9.

Braun, V., Mahren, S., and Ogierman, M. (2003) Regulation of the FecI-type ECF sigma factor by transmembrane signalling. *Current Opinion in Microbiology* **6**: 173-180.

Brinkman, F.S., Schoofs, G., Hancock, R.E., and De Mot, R. (1999) Influence of a putative ECF sigma factor on expression of the major outer membrane protein,

OprF, in Pseudomonas aeruginosa and Pseudomonas fluorescens. *J Bacteriol* **181**: 4746-4754.

Bronner, S., Stoessel, P., Gravet, A., Monteil, H., and Prevost, G. (2000) Variable expressions of Staphylococcus aureus bicomponent leucotoxins semiquantified by competitive reverse transcription-PCR. *Appl Environ Microbiol* **66**: 3931-3938.

Brook, I. (2009) Role of methicillin-resistant Staphylococcus aureus in head and neck infections. *J Laryngol Otol* **123**: 1301-1307.

Brooks, B.E., and Buchanan, S.K. (2008) Signaling mechanisms for activation of extracytoplasmic function (ECF) sigma factors. *Biochim Biophys Acta* **1778**: 1930-1945.

Buck, M., Gallegos, M.T., Studholme, D.J., Guo, Y., and Gralla, J.D. (2000) The bacterial enhancer-dependent sigma(54) (sigma(N)) transcription factor. *J Bacteriol* **182**: 4129-4136.

Bunce, C., Wheeler, L., Reed, G., Musser, J., and Barg, N. (1992) Murine model of cutaneous infection with gram-positive cocci. *Infect Immun* **60**: 2636-2640.

Burgess, R.R., and Anthony, L. (2001) How sigma docks to RNA polymerase and what sigma does. *Current Opinion in Microbiology* **4**: 126-131.

Burns, P.A., Gordon, A.J., Kunsmann, K., and Glickman, B.W. (1988) Influence of neighboring base sequence on the distribution and repair of N-ethyl-N-nitrosourea-induced lesions in Escherichia coli. *Cancer Res* **48**: 4455-4458.

Campbell, E.A., Muzzin, O., Chlenov, M., Sun, J.L., Olson, C.A., Weinman, O., Trester-Zedlitz, M.L., and Darst, S.A. (2002) Structure of the bacterial RNA polymerase promoter specificity sigma subunit. *Mol Cell* **9**: 527-539.

Campbell, E.A., Greenwell, R., Anthony, J.R., Wang, S., Lim, L., Das, K., Sofia, H.J., Donohue, T.J., and Darst, S.A. (2007) A conserved structural module regulates transcriptional responses to diverse stress signals in bacteria. *Mol Cell* **27**: 793-805.

Cao, M., Bernat, B.A., Wang, Z., Armstrong, R.N., and Helmann, J.D. (2001) FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in Bacillus subtilis. *J Bacteriol* **183**: 2380-2383.

Cao, M., Kobel, P.A., Morshedi, M.M., Wu, M.F., Paddon, C., and Helmann, J.D. (2002a) Defining the Bacillus subtilis sigma(W) regulon: a comparative analysis of promoter consensus search, run-off transcription/macroarray analysis (ROMA), and transcriptional profiling approaches. *J Mol Biol* **316**: 443-457.

Cao, M., Wang, T., Ye, R., and Helmann, J.D. (2002b) Antibiotics that inhibit cell wall biosynthesis induce expression of the Bacillus subtilis sigma(W) and sigma(M) regulons. *Mol Microbiol* **45**: 1267-1276.

Carroll, R.K., Robison, T.M., Rivera, F.E., Davenport, J.E., Jonsson, I.M., Florczyk, D., Tarkowski, A., Potempa, J., Koziel, J., and Shaw, L.N. (2012) Identification of an intracellular M17 family leucine aminopeptidase that is required for virulence in Staphylococcus aureus. *Microbes Infect*.

Casewell, M.W., and Hill, R.L. (1986) The carrier state: methicillin-resistant Staphylococcus aureus. *J Antimicrob Chemother* **18 Suppl A**: 1-12.

Chaba, R., Grigorova, I.L., Flynn, J.M., Baker, T.A., and Gross, C.A. (2007) Design principles of the proteolytic cascade governing the sigma(E)-mediated envelope stress response in Escherichia coli: keys to graded, buffered, and rapid signal transduction. *Genes Dev* **21**: 124-136.

Chalker, A.F., Leach, D.R., and Lloyd, R.G. (1988) Escherichia coli sbcC mutants permit stable propagation of DNA replicons containing a long palindrome. *Gene* **71**: 201-205.

Chambers, H.F., Korzeniowski, O.M., and Sande, M.A. (1983) Staphylococcus aureus endocarditis: clinical manifestations in addicts and nonaddicts. *Medicine (Baltimore)* **62**: 170-177.

Chambers, H.F., and Miick, C. (1992) Characterization of penicillin-binding protein 2 of Staphylococcus aureus: deacylation reaction and identification of two penicillin-binding peptides. *Antimicrob Agents Chemother* **36**: 656-661.

Chan, P.F., and Foster, S.J. (1998) The role of environmental factors in the regulation of virulence-determinant expression in Staphylococcus aureus 8325-4. *Microbiology* **144 (Pt 9)**: 2469-2479.

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R., Brown, W.J., Cardo, D., and Fridkin, S.K. (2003) Infection with vancomycin-resistant Staphylococcus aureus containing the vanA resistance gene. *N Engl J Med* **348**: 1342-1347.

Chang, W., Small, D.A., Toghrol, F., and Bentley, W.E. (2006a) Global transcriptome analysis of Staphylococcus aureus response to hydrogen peroxide. *J Bacteriol* **188**: 1648-1659.

Chang, W., Toghrol, F., and Bentley, W.E. (2006b) Toxicogenomic response of Staphylococcus aureus to peracetic acid. *Environmental Science & Technology* **40**: 5124-5131.

Chase, J.W., and Masker, W.E. (1977) Deoxyribonucleic acid repair in Escherichia coli mutants deficient in the 5'----3' exonuclease activity of deoxyribonucleic acid polymerase I and exonuclease VII. *J Bacteriol* **130**: 667-675.

Chatfield, S.N., Strahan, K., Pickard, D., Charles, I.G., Hormaeche, C.E., and Dougan, G. (1992) Evaluation of Salmonella typhimurium strains harbouring defined mutations in htrA and aroA in the murine salmonellosis model. *Microb Pathog* **12**: 145-151.

Chen, Z., Luong, T.T., and Lee, C.Y. (2007) The sbcDC locus mediates repression of type 5 capsule production as part of the SOS response in Staphylococcus aureus. *J Bacteriol* **189**: 7343-7350.

Cheng, A.G., McAdow, M., Kim, H.K., Bae, T., Missiakas, D.M., and Schneewind, O. (2010) Contribution of coagulases towards Staphylococcus aureus disease and protective immunity. *PLoS Pathog* **6**: e1001036.

Cheung, A.L., Koomey, J.M., Butler, C.A., Projan, S.J., and Fischetti, V.A. (1992) Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr. *Proc Natl Acad Sci U S A* **89**: 6462-6466.

Cheung, A.L., Wolz, C., Yeaman, M.R., and Bayer, A.S. (1995) Insertional inactivation of a chromosomal locus that modulates expression of potential virulence determinants in Staphylococcus aureus. *J Bacteriol* **177**: 3220-3226.

Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H., and Xiong, Y.Q. (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. *FEMS Immunol Med Microbiol* **40**: 1-9.

Cheung, A.L., Nishina, K., and Manna, A.C. (2008) SarA of Staphylococcus aureus binds to the sarA promoter to regulate gene expression. *J Bacteriol* **190**: 2239-2243.

Chien, Y., Manna, A.C., Projan, S.J., and Cheung, A.L. (1999) SarA, a global regulator of virulence determinants in Staphylococcus aureus, binds to a conserved motif essential for sar-dependent gene regulation. *J Biol Chem* **274**: 37169-37176.

Cirz, R.T., Jones, M.B., Gingles, N.A., Minogue, T.D., Jarrahi, B., Peterson, S.N., and Romesberg, F.E. (2007) Complete and SOS-mediated response of Staphylococcus aureus to the antibiotic ciprofloxacin. *J Bacteriol* **189**: 531-539.

Clark, N.C., Weigel, L.M., Patel, J.B., and Tenover, F.C. (2005) Comparison of Tn1546-like elements in vancomycin-resistant Staphylococcus aureus isolates from Michigan and Pennsylvania. *Antimicrob Agents Chemother* **49**: 470-472.

Clarke, S.R., Harris, L.G., Richards, R.G., and Foster, S.J. (2002) Analysis of Ebh, a 1.1-megadalton cell wall-associated fibronectin-binding protein of Staphylococcus aureus. *Infect Immun* **70**: 6680-6687.

Clauditz, A., Resch, A., Wieland, K.P., Peschel, A., and Gotz, F. (2006) Staphyloxanthin plays a role in the fitness of Staphylococcus aureus and its ability to cope with oxidative stress. *Infect Immun* **74**: 4950-4953.

Connelly, J.C., Kirkham, L.A., and Leach, D.R. (1998) The SbcCD nuclease of Escherichia coli is a structural maintenance of chromosomes (SMC) family protein that cleaves hairpin DNA. *Proc Natl Acad Sci U S A* **95**: 7969-7974.

Connelly, J.C., de Leau, E.S., and Leach, D.R. (1999) DNA cleavage and degradation by the SbcCD protein complex from Escherichia coli. *Nucleic Acids Res* **27**: 1039-1046.

Costanzo, A., and Ades, S.E. (2006) Growth phase-dependent regulation of the extracytoplasmic stress factor, sigmaE, by guanosine 3',5'-bispyrophosphate (ppGpp). *J Bacteriol* **188**: 4627-4634.

Courvalin, P. (2006) Vancomycin resistance in gram-positive cocci. *Clin Infect Dis* **42 Suppl 1**: S25-34.

Craig, M.L., Tsodikov, O.V., McQuade, K.L., Schlax, P.E., Jr., Capp, M.W., Saecker, R.M., and Record, M.T., Jr. (1998) DNA footprints of the two kinetically significant intermediates in formation of an RNA polymerase-promoter open complex: evidence that interactions with start site and downstream DNA induce sequential conformational changes in polymerase and DNA. *J Mol Biol* **283**: 741-756.

Crawford, R.M., Van De Verg, L., Yuan, L., Hadfield, T.L., Warren, R.L., Drazek, E.S., Houng, H.H., Hammack, C., Sasala, K., Polsinelli, T., Thompson, J., and Hoover, D.L. (1996) Deletion of purE attenuates Brucella melitensis infection in mice. *Infect Immun* **64**: 2188-2192.

Cuatrecasas, P., Edelhoch, H., and Anfinsen, C.B. (1967) Fluorescence studies of the interaction of nucleotides with the active site of the nuclease of Staphylococcus aureus. *Proc Natl Acad Sci U S A* **58**: 2043-2050.

Cuccui, J., Easton, A., Chu, K.K., Bancroft, G.J., Oyston, P.C., Titball, R.W., and Wren, B.W. (2007) Development of signature-tagged mutagenesis in Burkholderia pseudomallei to identify genes important in survival and pathogenesis. *Infect Immun* **75**: 1186-1195.

da Silva, J.F., Koide, T., Gomes, S.L., and Marques, M.V. (2007) The single "Extracytoplasmic-Function sigma factor of Xylella fastidiosa is involved in the heat shock response and presents an unusual regulatory mechanism. *J Bacteriol* **189**: 551-560.

Dartigalongue, C., Missiakas, D., and Raina, S. (2001) Characterization of the Escherichia coli sigma E regulon. *J Biol Chem* **276**: 20866-20875.

Datta, A.K., and Niyogi, K. (1975) A novel oligoribonuclease of Escherichia coli. II. Mechanism of action. *J Biol Chem* **250**: 7313-7319.

Davies, B.W., Bogard, R.W., Dupes, N.M., Gerstenfeld, T.A., Simmons, L.A., and Mekalanos, J.J. (2011) DNA damage and reactive nitrogen species are barriers to Vibrio cholerae colonization of the infant mouse intestine. *PLoS Pathog* **7**: e1001295.

Davis, A., Moore, I.B., Parker, D.S., and Taniuchi, H. (1977) Nuclease B. A possible precursor of nuclease A, an extracellular nuclease of Staphylococcus aureus. *J Biol Chem* **252**: 6544-6553.

de Azavedo, J.C., Foster, T.J., Hartigan, P.J., Arbuthnott, J.P., O'Reilly, M., Kreiswirth, B.N., and Novick, R.P. (1985) Expression of the cloned toxic shock syndrome toxin 1 gene (tst) in vivo with a rabbit uterine model. *Infect Immun* **50**: 304-309.

de Neeling, A.J., van den Broek, M.J., Spalburg, E.C., van Santen-Verheuvel, M.G., Dam-Deisz, W.D., Boshuizen, H.C., van de Giessen, A.W., van Duijkeren, E., and Huijsdens, X.W. (2007) High prevalence of methicillin resistant Staphylococcus aureus in pigs. *Vet Microbiol* **122**: 366-372.

Deora, R., and Misra, T.K. (1995) Purification and characterization of DNA dependent RNA polymerase from Staphylococcus aureus. *Biochem Biophys Res Commun* **208**: 610-616.

Deora, R., and Misra, T.K. (1996) Characterization of the primary sigma factor of Staphylococcus aureus. *J Biol Chem* **271**: 21828-21834.

Deora, R., Tseng, T., and Misra, T.K. (1997a) Alternative transcription factor sigma(SB) of Staphylococcus aureus: Characterization and role in transcription of the global regulatory locus sar. *J Bacteriol* **179**: 6355-6359.

Deora, R., Tseng, T., and Misra, T.K. (1997b) Alternative transcription factor sigmaSB of Staphylococcus aureus: characterization and role in transcription of the global regulatory locus sar. *J Bacteriol* **179**: 6355-6359.

Diep, B.A., Gill, S.R., Chang, R.F., Phan, T.H., Chen, J.H., Davidson, M.G., Lin, F., Lin, J., Carleton, H.A., Mongodin, E.F., Sensabaugh, G.F., and Perdreau-Remington, F. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. *Lancet* **367**: 731-739.

Diep, B.A., Stone, G.G., Basuino, L., Graber, C.J., Miller, A., des Etages, S.A., Jones, A., Palazzolo-Ballance, A.M., Perdreau-Remington, F., Sensabaugh, G.F., DeLeo, F.R., and Chambers, H.F. (2008) The arginine catabolic mobile element and staphylococcal chromosomal cassette mec linkage: convergence of virulence and

resistance in the USA300 clone of methicillin-resistant Staphylococcus aureus. *J Infect Dis* **197**: 1523-1530.

Dominski, Z. (2007) Nucleases of the metallo-beta-lactamase family and their role in DNA and RNA metabolism. *Crit Rev Biochem Mol Biol* **42**: 67-93.

Dona, V., Rodrigue, S., Dainese, E., Palu, G., Gaudreau, L., Manganelli, R., and Provvedi, R. (2008) Evidence of complex transcriptional, translational, and posttranslational regulation of the extracytoplasmic function sigma factor sigmaE in Mycobacterium tuberculosis. *J Bacteriol* **190**: 5963-5971.

Dornbusch, K., Miller, G.H., Hare, R.S., and Shaw, K.J. (1990) Resistance to aminoglycoside antibiotics in gram-negative bacilli and staphylococci isolated from blood. Report from a European collaborative study. The ESGAR Study Group (European Study Group on Antibiotic Resistance). *J Antimicrob Chemother* **26**: 131-144.

Dossett, J.H., Kronvall, G., Williams, R.C., Jr., and Quie, P.G. (1969) Antiphagocytic effects of staphylococcal protein A. *J Immunol* **103**: 1405-1410.

Drapeau, G.R. (1978) Role of metalloprotease in activation of the precursor of staphylococcal protease. *J Bacteriol* **136**: 607-613.

Drayson, R., Leggat, T., and Wood, M. (2011) Increased Antibiotic Resistance Post-exposure to Sub-inhibitoryConcentrations is Independent of Capsular Polysaccharide Production in Escherichia coli. *JEMI* **15**.

Dubnau, E., Fontan, P., Manganelli, R., Soares-Appel, S., and Smith, I. (2002) Mycobacterium tuberculosis genes induced during infection of human macrophages. *Infect Immun* **70**: 2787-2795.

Dufour, Y.S., Landick, R., and Donohue, T.J. (2008) Organization and Evolution of the Biological Response to Singlet Oxygen Stress. *J Mol Biol* **383**: 713-730.

Durr, H., Korner, C., Muller, M., Hickmann, V., and Hopfner, K.P. (2005) X-ray structures of the Sulfolobus solfataricus SWI2/SNF2 ATPase core and its complex with DNA. *Cell* **121**: 363-373.

Ellermeier, C.D., and Losick, R. (2006) Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in Bacillus subtilis. *Genes Dev* **20**: 1911-1922.

Emori, T.G., and Gaynes, R.P. (1993) An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin Microbiol Rev* **6**: 428-442.

Enz, S., Mahren, S., Stroeher, U.H., and Braun, V. (2000) Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. *J Bacteriol* **182**: 637-646.

Erickson, J.W., and Gross, C.A. (1989) Identification of the sigma E subunit of Escherichia coli RNA polymerase: a second alternate sigma factor involved in high-temperature gene expression. *Genes Dev* **3**: 1462-1471.

Eriksen, K.R. (1961) [Nosocomial "Pyocyaneus"-infections]. *Ugeskr Laeger* **123**: 1377-1382.

Eschenburg, S., Priestman, M., and Schonbrunn, E. (2005) Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) is essential for product release. *J Biol Chem* **280**: 3757-3763.

Evers, S., Quintiliani, R., Jr., and Courvalin, P. (1996) Genetics of glycopeptide resistance in enterococci. *Microb Drug Resist* **2**: 219-223.

Farber, B.F., and Moellering, R.C., Jr. (1983) Retrospective study of the toxicity of preparations of vancomycin from 1974 to 1981. *Antimicrob Agents Chemother* **23**: 138-141.

Finck-Barbancon, V., Prevost, G., and Piemont, Y. (1991) Improved purification of leukocidin from Staphylococcus aureus and toxin distribution among hospital strains. *Research in Microbiology* **142**: 75-85.

Finks, J., Wells, E., Dyke, T.L., Husain, N., Plizga, L., Heddurshetti, R., Wilkins, M., Rudrik, J., Hageman, J., Patel, J., and Miller, C. (2009) Vancomycin-resistant Staphylococcus aureus, Michigan, USA, 2007. *Emerg Infect Dis* **15**: 943-945.

Fleury, B., Kelley, W.L., Lew, D., Gotz, F., Proctor, R.A., and Vaudaux, P. (2009) Transcriptomic and metabolic responses of Staphylococcus aureus exposed to supra-physiological temperatures. *BMC Microbiol* **9**: 76.

Flynn, J.M., Levchenko, I., Sauer, R.T., and Baker, T.A. (2004) Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. *Genes Dev* **18**: 2292-2301.

Ford, C.W., Hamel, J.C., Stapert, D., and Yancey, R.J. (1989) Establishment of an experimental model of a Staphylococcus aureus abscess in mice by use of dextran and gelatin microcarriers. *J Med Microbiol* **28**: 259-266.

Fournier, B., Klier, A., and Rapoport, G. (2001) The two-component system ArlS-ArlR is a regulator of virulence gene expression in Staphylococcus aureus. *Mol Microbiol* **41**: 247-261.

Fuda, C.C.S., Fisher, J.F., and Mobashery, S. (2005) beta-Lactam resistance in Staphylococcus aureus: the adaptive resistance of a plastic genome. *Cellular and Molecular Life Sciences* **62**: 2617-2633.

Garnier, F., Chainier, D., Walsh, T., Karlsson, A., Bolmstrom, A., Grelaud, C., Mounier, M., Denis, F., and Ploy, M.C. (2006) A 1 year surveillance study of glycopeptide-intermediate Staphylococcus aureus strains in a French hospital. *J Antimicrob Chemother* **57**: 146-149.

Geissmann, T., Chevalier, C., Cros, M.J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., Francois, P., Vandenesch, F., Gaspin, C., and Romby, P. (2009) A search for small noncoding RNAs in Staphylococcus aureus reveals a conserved sequence motif for regulation. *Nucleic Acids Res* **37**: 7239-7257.

Georgopapadakou, N.H., and Liu, F.Y. (1980a) Binding of beta-lactam antibiotics to penicillin-binding proteins of Staphylococcus aureus and Streptococcus faecalis: relation to antibacterial activity. *Antimicrob Agents Chemother* **18**: 834-836.

Georgopapadakou, N.H., and Liu, F.Y. (1980b) Penicillin-binding proteins in bacteria. *Antimicrob Agents Chemother* **18**: 148-157.

Gertz, S., Engelmann, S., Schmid, R., Ziebandt, A.K., Tischer, K., Scharf, C., Hacker, J., and Hecker, M. (2000) Characterization of the sigma(B) regulon in Staphylococcus aureus. *J Bacteriol* **182**: 6983-6991.

Giachino, P., Engelmann, S., and Bischoff, M. (2001) Sigma(B) activity depends on RsbU in Staphylococcus aureus. *J Bacteriol* **183**: 1843-1852.

Giraudo, A.T., Mansilla, C., Chan, A., Raspanti, C., and Nagel, R. (2003) Studies on the expression of regulatory locus sae in Staphylococcus aureus. *Current Microbiology* **46**: 246-250.

Goerke, C., Fluckiger, U., Steinhuber, A., Bisanzio, V., Ulrich, M., Bischoff, M., Patti, J.M., and Wolz, C. (2005) Role of Staphylococcus aureus global regulators sae and sigmaB in virulence gene expression during device-related infection. *Infect Immun* **73**: 3415-3421.

Goodson, M. (1989) Habituation to alkali in Escherichia coli. *Lett Appl Microbiol* **9**: 71-73.

Goundan, P.N., Mehrotra, A., Mani, D., and Varadarajan, I. (2010) Community acquired methicillin-resistant Staphylococcus aureus pneumonia leading to rhabdomyolysis: a case report. *Cases J* **3**: 61.

Govan, J.R., and Harris, G.S. (1986) Pseudomonas aeruginosa and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiol Sci* **3**: 302-308.

Griffith, R.S. (1981) Introduction to vancomycin. *Rev Infect Dis* **3 suppl**: S200-204.

Grigorova, I.L., Chaba, R., Zhong, H.J., Alba, B.M., Rhodius, V., Herman, C., and Gross, C.A. (2004) Fine-tuning of the Escherichia coli sigmaE envelope stress response relies on multiple mechanisms to inhibit signal-independent proteolysis of the transmembrane anti-sigma factor, RseA. *Genes Dev* **18**: 2686-2697.

Gruber, T.M., Markov, D., Sharp, M.M., Young, B.A., Lu, C.Z., Zhong, H.J., Artsimovitch, I., Geszvain, K.M., Arthur, T.M., Burgess, R.R., Landick, R., Severinov, K., and Gross, C.A. (2001) Binding of the initiation factor sigma(70) to core RNA polymerase is a multistep process. *Mol Cell* **8**: 21-31.

Gruber, T.M., and Gross, C.A. (2003) Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu Rev Microbiol* **57**: 441-466.

Guerout-Fleury, A.M., Shazand, K., Frandsen, N., and Stragier, P. (1995) Antibiotic-resistance cassettes for Bacillus subtilis. *Gene* **167**: 335-336.

Guinane, C.M., Cotter, P.D., Ross, R.P., and Hill, C. (2006) Contribution of penicillin-binding protein homologs to antibiotic resistance, cell morphology, and virulence of Listeria monocytogenes EGDe. *Antimicrob Agents Chemother* **50**: 2824-2828.

Gunesekere, I.C., Kahler, C.M., Ryan, C.S., Snyder, L.A., Saunders, N.J., Rood, J.I., and Davies, J.K. (2006) Ecf, an alternative sigma factor from Neisseria gonorrhoeae, controls expression of msrAB, which encodes methionine sulfoxide reductase. *J Bacteriol* **188**: 3463-3469.

Gunetileke, K.G., and Anwar, R.A. (1968) Biosynthesis of uridine diphospho-N-acetylmuramic acid. II. Purification and properties of pyruvate-uridine diphospho-N-acetylglucosamine transferase and characterization of uridine diphospho-N-acetylenopyruvylglucosamine. *J Biol Chem* **243**: 5770-5778.

Gurbay, A., Gonthier, B., Signorini-Allibe, N., Barret, L., Favier, A., and Hincal, F. (2006) Ciprofloxacin-induced DNA damage in primary culture of rat astrocytes and protection by Vitamin E. *Neurotoxicology* **27**: 6-10.

Hahn, M.Y., Raman, S., Anaya, M., and Husson, R.N. (2005) The Mycobacterium tuberculosis extracytoplasmic-function sigma factor SigL regulates polyketide synthases and secreted or membrane proteins and is required for virulence. *J Bacteriol* **187**: 7062-7071.

Halasa, T., Nielen, M., De Roos, A.P., Van Hoorne, R., de Jong, G., Lam, T.J., van Werven, T., and Hogeveen, H. (2009) Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. *J Dairy Sci* **92**: 599-606.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* **2**: 95-108.

Handwerger, S., Perlman, D.C., Altarac, D., and McAuliffe, V. (1992) Concomitant high-level vancomycin and penicillin resistance in clinical isolates of enterococci. *Clin Infect Dis* **14**: 655-661.

Hashimoto, H., Kono, K., and Mitsuhashi, S. (1964) Elimination of Penicillin Resistance of Staphylococcus Aureus by Treatment with Acriflavine. *J Bacteriol* **88**: 261-262.

Hawiger, J., Timmons, S., Strong, D.D., Cottrell, B.A., Riley, M., and Doolittle, R.F. (1982) Identification of a region of human fibrinogen interacting with staphylococcal clumping factor. *Biochemistry* **21**: 1407-1413.

Helmann, J.D., and Chamberlin, M.J. (1988) Structure and Function of Bacterial Sigma Factors. *Annual Review of Biochemistry* **57**: 839-872.

Helmann, J.D. (1995) Compilation and analysis of Bacillus subtilis sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res* **23**: 2351-2360.

Helmann, J.D. (2002) The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**: 47-110.

Helmann, J.D. (2006) Deciphering a complex genetic regulatory network: the Bacillus subtilis sigmaW protein and intrinsic resistance to antimicrobial compounds. *Sci Prog* **89**: 243-266.

Henikoff, S., Haughn, G.W., Calvo, J.M., and Wallace, J.C. (1988) A large family of bacterial activator proteins. *Proc Natl Acad Sci U S A* **85**: 6602-6606.

Henze, U., Sidow, T., Wecke, J., Labischinski, H., and Berger-Bachi, B. (1993) Influence of femB on methicillin resistance and peptidoglycan metabolism in Staphylococcus aureus. *J Bacteriol* **175**: 1612-1620.

Hersh, A.L., Chambers, H.F., Maselli, J.H., and Gonzales, R. (2008) National trends in ambulatory visits and antibiotic prescribing for skin and soft-tissue infections. *Arch Intern Med* **168**: 1585-1591.

Hershberger, C.D., Ye, R.W., Parsek, M.R., Xie, Z.D., and Chakrabarty, A.M. (1995) The algT (algU) gene of Pseudomonas aeruginosa, a key regulator involved in alginate biosynthesis, encodes an alternative sigma factor (sigma E). *Proc Natl Acad Sci U S A* **92**: 7941-7945.

Hewlett, A.L., Falk, P.S., Hughes, K.S., and Mayhall, C.G. (2009) Epidemiology of methicillin-resistant Staphylococcus aureus in a university medical center day care facility. *Infect Control Hosp Epidemiol* **30**: 985-992.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F.C. (1997) Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother* **40**: 135-136.

Hiramatsu, K. (2001) Vancomycin-resistant Staphylococcus aureus: a new model of antibiotic resistance. *Lancet Infect Dis* **1**: 147-155.

Hiratsu, K., Amemura, M., Nashimoto, H., Shinagawa, H., and Makino, K. (1995) The rpoE gene of Escherichia coli, which encodes sigma E, is essential for bacterial growth at high temperature. *J Bacteriol* **177**: 2918-2922.

Hoffee, P.A., May, R., and Robertson, B.D. (1978) Purine nucleoside phosphorylase from Salmonella typhimurium and rat liver. *Methods Enzymol* **51**: 517-524.

Hooper, D.C. (2002) Fluoroquinolone resistance among Gram-positive cocci. *Lancet Infect Dis* **2**: 530-538.

Horsburgh, M.J., Clements, M.O., Crossley, H., Ingham, E., and Foster, S.J. (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in Staphylococcus aureus. *Infect Immun* **69**: 3744-3754.

Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K., and Foster, S.J. (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. *J Bacteriol* **184**: 5457-5467.

Howden, B.P., Ward, P.B., Charles, P.G., Korman, T.M., Fuller, A., du Cros, P., Grabsch, E.A., Roberts, S.A., Robson, J., Read, K., Bak, N., Hurley, J., Johnson, P.D., Morris, A.J., Mayall, B.C., and Grayson, M.L. (2004) Treatment outcomes for serious infections caused by methicillin-resistant Staphylococcus aureus with reduced vancomycin susceptibility. *Clin Infect Dis* **38**: 521-528.

Howden, B.P., Davies, J.K., Johnson, P.D., Stinear, T.P., and Grayson, M.L. (2010) Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin Microbiol Rev* **23**: 99-139.

Huang, X., Gaballa, A., Cao, M., and Helmann, J.D. (1999) Identification of target promoters for the Bacillus subtilis extracytoplasmic function sigma factor, sigma W. *Mol Microbiol* **31**: 361-371.

Hughes, K.T., and Mathee, K. (1998) The anti-sigma factors. *Annu Rev Microbiol* **52**: 231-286.

Huijsdens, X.W., van Dijke, B.J., Spalburg, E., van Santen-Verheuvel, M.G., Heck, M.E., Pluister, G.N., Voss, A., Wannet, W.J., and de Neeling, A.J. (2006) Community-acquired MRSA and pig-farming. *Ann Clin Microbiol Antimicrob* **5**: 26.

Humphreys, S., Stevenson, A., Bacon, A., Weinhardt, A.B., and Roberts, M. (1999) The alternative sigma factor, sigmaE, is critically important for the virulence of Salmonella typhimurium. *Infect Immun* **67**: 1560-1568.

Imlay, J.A., Chin, S.M., and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **240**: 640-642.

Imlay, J.A., and Linn, S. (1988) DNA damage and oxygen radical toxicity. *Science* **240**: 1302-1309.

Ingavale, S.S., Van Wamel, W., and Cheung, A.L. (2003) Characterization of RAT, an autolysis regulator in Staphylococcus aureus. *Mol Microbiol* **48**: 1451-1466.

Iordanescu, S., and Surdeanu, M. (1976) Two restriction and modification systems in Staphylococcus aureus NCTC8325. *J Gen Microbiol* **96**: 277-281.

Ishihama, A. (2000) Functional modulation of Escherichia coli RNA polymerase. *Annu Rev Microbiol* **54**: 499-518.

Jackson, M., Phalen, S.W., Lagranderie, M., Ensergueix, D., Chavarot, P., Marchal, G., McMurray, D.N., Gicquel, B., and Guilhot, C. (1999) Persistence and protective efficacy of a Mycobacterium tuberculosis auxotroph vaccine. *Infect Immun* **67**: 2867-2873.

Janzon, L., Lofdahl, S., and Arvidson, S. (1986) Evidence for a Coordinate Transcriptional Control of Alpha-Toxin and Protein-a Synthesis in Staphylococcus-Aureus. *FEMS Microbiol Lett* **33**: 193-198.

Jenkins, A., Cote, C., Twenhafel, N., Merkel, T., Bozue, J., and Welkos, S. (2011) Role of purine biosynthesis in Bacillus anthracis pathogenesis and virulence. *Infect Immun* **79**: 153-166.

Ji, G.Y., Beavis, R.C., and Novick, R.P. (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc Natl Acad Sci U S A* **92**: 12055-12059.

Johnson, K., Charles, I., Dougan, G., Pickard, D., O'Gaora, P., Costa, G., Ali, T., Miller, I., and Hormaeche, C. (1991) The role of a stress-response protein in Salmonella typhimurium virulence. *Mol Microbiol* **5**: 401-407.

Jones, A.K., Fulcher, N.B., Balzer, G.J., Urbanowski, M.L., Pritchett, C.L., Schurr, M.J., Yahr, T.L., and Wolfgang, M.C. (2010) Activation of the Pseudomonas aeruginosa AlgU regulon through mucA mutation inhibits cyclic AMP/Vfr signaling. *J Bacteriol* **192**: 5709-5717.

Jones, A.L., Needham, R.H., and Rubens, C.E. (2003) The Delta subunit of RNA polymerase is required for virulence of Streptococcus agalactiae. *Infect Immun* **71**: 4011-4017.

Jonsson, I.M., Arvidson, S., Foster, S., and Tarkowski, A. (2004) Sigma factor B and RsbU are required for virulence in Staphylococcus aureus-induced arthritis and sepsis. *Infect Immun* **72**: 6106-6111.

Jonsson, K., Signas, C., Muller, H.P., and Lindberg, M. (1991) Two different genes encode fibronectin binding proteins in Staphylococcus aureus. The complete nucleotide sequence and characterization of the second gene. *Eur J Biochem* **202**: 1041-1048.

Joo, D.M., Ng, N., and Calendar, R. (1997) A sigma(32) mutant with a single amino acid change in the highly conserved region 2.2 exhibits reduced core RNA polymerase affinity. *Proc Natl Acad Sci U S A* **94**: 4907-4912.

Kamata, H., Akiyama, S., Morosawa, H., Ohta, T., Hamamoto, T., Kambe, T., Kagawa, Y., and Hirata, H. (1992) Primary structure of the alanine carrier protein of thermophilic bacterium PS3. *J Biol Chem* **267**: 21650-21655.

Kanehara, K., Ito, K., and Akiyama, Y. (2002) YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. *Genes Dev* **16**: 2147-2155.

Kantor, H.S., Temples, B., and Shaw, W.V. (1972) Staphylococcal delta hemolysin: purification and characterization. *Arch Biochem Biophys* **151**: 142-156.

Karlsson, A., Saravia-Otten, P., Tegmark, K., Morfeldt, E., and Arvidson, S. (2001) Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in Staphylococcus aureus sarA mutants due to up-regulation of extracellular proteases. *Infect Immun* **69**: 4742-4748.

Katayama, A., Fujita, N., and Ishihama, A. (2000) Mapping of subunit-subunit contact surfaces on the beta ' subunit of Escherichia coli RNA polymerase. *Journal of Biological Chemistry* **275**: 3583-3592.

Kavanaugh, J.S., Thoendel, M., and Horswill, A.R. (2007) A role for type I signal peptidase in Staphylococcus aureus quorum sensing. *Mol Microbiol* **65**: 780-798.

Kemp, E.H., Sammons, R.L., Moir, A., Sun, D., and Setlow, P. (1991) Analysis of transcriptional control of the gerD spore germination gene of Bacillus subtilis 168. *J Bacteriol* **173**: 4646-4652.

Kempker, R., Difrancesco, L., Martin-Gorgojo, A., and Franco-Paredes, C. (2009) Expanding spectrum of illness due to community-associated methicillin-resistant Staphylococcus aureus: a case report. *Cases J* **2**: 7437.

Kennedy, L.J., Moore, K., Jr., Caulfield, J.L., Tannenbaum, S.R., and Dedon, P.C. (1997) Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents. *Chem Res Toxicol* **10**: 386-392.

Khanna, T., Friendship, R., Dewey, C., and Weese, J.S. (2008) Methicillin resistant Staphylococcus aureus colonization in pigs and pig farmers. *Vet Microbiol* **128**: 298-303.

Kihm, D.J., Leyer, G.J., An, G.H., and Johnson, E.A. (1994) Sensitization of heat-treated Listeria monocytogenes to added lysozyme in milk. *Appl Environ Microbiol* **60**: 3854-3861.

Kim, M.S., Hahn, M.Y., Cho, Y., Cho, S.N., and Roe, J.H. (2009) Positive and negative feedback regulatory loops of thiol-oxidative stress response mediated by an unstable isoform of sigma(R) in actinomycetes. *Mol Microbiol* **73**: 815-825.

Kirby, W.M. (1944) Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant Staphylococci. *Science* **99**: 452-453.

Klein, E., Smith, D.L., and Laxminarayan, R. (2007) Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005. *Emerg Infect Dis* **13**: 1840-1846.

Klevens, R.M., Edwards, J.R., Tenover, F.C., McDonald, L.C., Horan, T., and Gaynes, R. (2006a) Changes in the epidemiology of methicillin-resistant Staphylococcus aureus in intensive care units in US hospitals, 1992-2003. *Clin Infect Dis* **42**: 389-391.

Klevens, R.M., Morrison, M.A., Fridkin, S.K., Reingold, A., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Fosheim, G., McDougal, L.K., and Tenover, F.C. (2006b) Community-associated methicillin-resistant Staphylococcus aureus and healthcare risk factors. *Emerg Infect Dis* **12**: 1991-1993.

Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Zell, E.R., Fosheim, G.E., McDougal, L.K., Carey, R.B., and Fridkin, S.K. (2007) Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *JAMA* **298**: 1763-1771.

Kolar, S.L., Nagarajan, V., Oszmiana, A., Rivera, F.E., Miller, H.K., Davenport, J.E., Riordan, J.T., Potempa, J., Barber, D.S., Koziel, J., Elasri, M.O., and Shaw, L.N. (2011) NsaRS is a cell-envelope-stress-sensing two-component system of Staphylococcus aureus. *Microbiology* **157**: 2206-2219.

Komatsu, Y. (1973) Adenosine uptake by isolated membrane vesicles from Escherichia coli K-12. *Biochim Biophys Acta* **330**: 206-221.

Koprivnjak, T., Mlakar, V., Swanson, L., Fournier, B., Peschel, A., and Weiss, J.P. (2006) Cation-induced transcriptional regulation of the dlt operon of Staphylococcus aureus. *J Bacteriol* **188**: 3622-3630.

Kornblum, J., Hartman, B.J., Novick, R.P., and Tomasz, A. (1986) Conversion of a homogeneously methicillin-resistant strain of Staphylococcus aureus to heterogeneous resistance by Tn551-mediated insertional inactivation. *Eur J Clin Microbiol* **5**: 714-718.

Kourbatova, E.V., Halvosa, J.S., King, M.D., Ray, S.M., White, N., and Blumberg, H.M. (2005) Emergence of community-associated methicillin-resistant Staphylococcus aureus USA 300 clone as a cause of health care-associated infections among patients with prosthetic joint infections. *Am J Infect Control* **33**: 385-391.

Kuehnert, M.J., Kruszon-Moran, D., Hill, H.A., McQuillan, G., McAllister, S.K., Fosheim, G., McDougal, L.K., Chaitram, J., Jensen, B., Fridkin, S.K., Killgore, G., and Tenover, F.C. (2006) Prevalence of Staphylococcus aureus nasal colonization in the United States, 2001-2002. *J Infect Dis* **193**: 172-179.

Kullik, I., and Giachino, P. (1997) The alternative sigma factor sigma(B) in Staphylococcus aureus: Regulation of the sigB operon in response to growth phase and heat shock. *Arch Microbiol* **167**: 151-159.

Kurokawa, K., Nishida, S., Ishibashi, M., Mizumura, H., Ueno, K., Yutsudo, T., Maki, H., Murakami, K., and Sekimizu, K. (2008) Staphylococcus aureus MurC participates in L-alanine recognition via histidine 343, a conserved motif in the shallow hydrophobic pocket. *J Biochem* **143**: 417-424.

Lan, L., Cheng, A., Dunman, P.M., Missiakas, D., and He, C. (2010) Golden pigment production and virulence gene expression are affected by metabolisms in Staphylococcus aureus. *J Bacteriol* **192**: 3068-3077.

Lancaster, J.R., Jr. (1997) A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* **1**: 18-30.

Lane, W.J., and Darst, S.A. (2006) The structural basis for promoter -35 element recognition by the group IV sigma factors. *PLoS Biol* **4**: e269.

Le Jeune, A., Torelli, R., Sanguinetti, M., Giard, J.C., Hartke, A., Auffray, Y., and Benachour, A. (2010a) The Extracytoplasmic Function Sigma Factor SigV Plays a Key Role in the Original Model of Lysozyme Resistance and Virulence of Enterococcus faecalis. *PLoS One* **5**.

Le Jeune, A., Torelli, R., Sanguinetti, M., Giard, J.C., Hartke, A., Auffray, Y., and Benachour, A. (2010b) The extracytoplasmic function sigma factor SigV plays a key role in the original model of lysozyme resistance and virulence of Enterococcus faecalis. *PLoS One* **5**: e9658.

Leach, D.R., and Stahl, F.W. (1983) Viability of lambda phages carrying a perfect palindrome in the absence of recombination nucleases. *Nature* **305**: 448-451.

LeBel, M. (1988) Ciprofloxacin: chemistry, mechanism of action, resistance, antimicrobial spectrum, pharmacokinetics, clinical trials, and adverse reactions. *Pharmacotherapy* **8**: 3-33.

Leoni, L., Orsi, N., de Lorenzo, V., and Visca, P. (2000) Functional analysis of PvdS, an iron starvation sigma factor of Pseudomonas aeruginosa. *J Bacteriol* **182**: 1481-1491.

Leski, T.A., and Tomasz, A. (2005) Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. *J Bacteriol* **187**: 1815-1824.

Levine, D.P. (2006) Vancomycin: a history. *Clin Infect Dis* **42 Suppl 1**: S5-12.

Liang, X., Zheng, L., Landwehr, C., Lunsford, D., Holmes, D., and Ji, Y. (2005) Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of Staphylococcus aureus. *J Bacteriol* **187**: 5486-5492.

Libman, H., and Arbeit, R.D. (1984) Complications associated with Staphylococcus aureus bacteremia. *Arch Intern Med* **144**: 541-545.

Lindahl, T., and Wood, R.D. (1999) Quality control by DNA repair. *Science* **286**: 1897-1905.

Lindsay, J.A., and Foster, S.J. (1999) Interactive regulatory pathways control virulence determinant production and stability in response to environmental conditions in Staphylococcus aureus. *Mol Gen Genet* **262**: 323-331.

Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J., and Nizet, V. (2005) Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* **202**: 209-215.

Llamas, M.A., van der Sar, A., Chu, B.C.H., Sparrius, M., Vogel, H.J., and Bitter, W. (2009) A Novel Extracytoplasmic Function (ECF) Sigma Factor Regulates Virulence in Pseudomonas aeruginosa. *PLoS Pathog* **5**.

Loffler, B., Hussain, M., Grundmeier, M., Bruck, M., Holzinger, D., Varga, G., Roth, J., Kahl, B.C., Proctor, R.A., and Peters, G. (2010) Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* **6**: e1000715.

Lonetto, M., Gribskov, M., and Gross, C.A. (1992) The Sigma-70 Family - Sequence Conservation and Evolutionary Relationships. *J Bacteriol* **174**: 3843-3849.

Lonetto, M.A., Brown, K.L., Rudd, K.E., and Buttner, M.J. (1994) Analysis of the Streptomyces coelicolor sigE gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci U S A* **91**: 7573-7577.

Lourenco, R.F., Kohler, C., and Gomes, S.L. (2011) A two-component system, an anti-sigma factor and two paralogous ECF sigma factors are involved in the control of general stress response in Caulobacter crescentus. *Mol Microbiol* **80**: 1598-1612.

Love, P.E., Lyle, M.J., and Yasbin, R.E. (1985) DNA-damage-inducible (din) loci are transcriptionally activated in competent Bacillus subtilis. *Proc Natl Acad Sci U S A* **82**: 6201-6205.

Lovett, C.M., Jr., Love, P.E., and Yasbin, R.E. (1989) Competence-specific induction of the Bacillus subtilis RecA protein analog: evidence for dual regulation of a recombination protein. *J Bacteriol* **171**: 2318-2322.

Lowther, W.T., Weissbach, H., Etienne, F., Brot, N., and Matthews, B.W. (2002) The mirrored methionine sulfoxide reductases of Neisseria gonorrhoeae pilB. *Nature Structural Biology* **9**: 348-352.

Luo, J.Y., Sharif, K.A., Jin, R.Z., Fujita, N., Ishihama, A., and Krakow, J.S. (1996) Molecular anatomy of the beta' subunit of the E-coli RNA polymerase: Identification of regions involved in polymerase assembly. *Genes to Cells* **1**: 819-827.

Luong, T.T., Newell, S.W., and Lee, C.Y. (2003) Mgr, a novel global regulator in Staphylococcus aureus. *J Bacteriol* **185**: 3703-3710.

MacLeod, P.R., and MacLeod, R.A. (1992) Identification and sequence of a Na(+)-linked gene from the marine bacterium Alteromonas haloplanktis which functionally complements the dagA gene of Escherichia coli. *Mol Microbiol* **6**: 2673-2681.

MacMicking, J., Xie, Q.W., and Nathan, C. (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* **15**: 323-350.

Mahren, S., Enz, S., and Braun, V. (2002) Functional interaction of region 4 of the extracytoplasmic function sigma factor FecI with the cytoplasmic portion of the FecR transmembrane protein of the Escherichia coli ferric citrate transport system. *J Bacteriol* **184**: 3704-3711.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci U S A* **95**: 12462-12467.

Malachowa, N., and DeLeo, F.R. (2011) Staphylococcus aureus survival in human blood. *Virulence* **2**: 567-569.

Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F.R., and Smith, I. (1999) Differential expression of 10 sigma factor genes in Mycobacterium tuberculosis. *Mol Microbiol* **31**: 715-724.

Manna, A., and Cheung, A.L. (2001) Characterization of sarR, a modulator of sar expression in Staphylococcus aureus. *Infect Immun* **69**: 885-896.

Manna, A.C., Ingavale, S.S., Maloney, M., van Wamel, W., and Cheung, A.L. (2004) Identification of sarV (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in Staphylococcus aureus. *J Bacteriol* **186**: 5267-5280.

Maor, Y., Rahav, G., Belausov, N., Ben-David, D., Smollan, G., and Keller, N. (2007) Prevalence and characteristics of heteroresistant vancomycin-intermediate Staphylococcus aureus bacteremia in a tertiary care center. *J Clin Microbiol* **45**: 1511-1514.

Maor, Y., Hagin, M., Belausov, N., Keller, N., Ben-David, D., and Rahav, G. (2009) Clinical features of heteroresistant vancomycin-intermediate Staphylococcus aureus bacteremia versus those of methicillin-resistant S. aureus bacteremia. *J Infect Dis* **199**: 619-624.

Maresso, A.W., and Schneewind, O. (2006) Iron acquisition and transport in Staphylococcus aureus. *Biometals* **19**: 193-203.

Marques, S.C., RezendeI, J.G., AlvesI, L.A., SilvaI, B.C., AlvesII, E., Ronaldo de AbreuI, L., and Piccoli, R.H. (2007) Formation of biofilms by Staphylococcus aureus on stainless steel and glass surfaces and its resistance to some selected chemical sanitizers. *Brazilian Journal of Microbiology* **38**.

Marquis, R.E. (1968) Salt-induced contraction of bacterial cell walls. *J Bacteriol* **95**: 775-781.

Martin, D.W., Schurr, M.J., Mudd, M.H., and Deretic, V. (1993) Differentiation of Pseudomonas aeruginosa into the alginate-producing form: inactivation of mucB causes conversion to mucoidy. *Mol Microbiol* **9**: 497-506.

Matic, I., Radman, M., and Rayssiguier, C. (1994) Structure of recombinants from conjugational crosses between Escherichia coli donor and mismatch-repair deficient Salmonella typhimurium recipients. *Genetics* **136**: 17-26.

Mazurakova, V., Sevcikova, B., Rezuchova, B., and Kormanec, J. (2006) Cascade of sigma factors in streptomycetes: identification of a new extracytoplasmic function sigma factor sigmaJ that is under the control of the stress-response sigma factor sigmaH in Streptomyces coelicolor A3(2). *Arch Microbiol* **186**: 435-446.

McAleese, F.M., Walsh, E.J., Sieprawska, M., Potempa, J., and Foster, T.J. (2001) Loss of clumping factor B fibrinogen binding activity by Staphylococcus aureus involves cessation of transcription, shedding and cleavage by metalloprotease. *J Biol Chem* **276**: 29969-29978.

McCracken, A., and Timms, P. (1999) Efficiency of transcription from promoter sequence variants in Lactobacillus is both strain and context dependent. *J Bacteriol* **181**: 6569-6572.

McDougal, L.K., Steward, C.D., Killgore, G.E., Chaitram, J.M., McAllister, S.K., and Tenover, F.C. (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: Establishing a national database. *J Clin Microbiol* **41**: 5113-5120.

McFarland, W.C., and Stocker, B.A. (1987) Effect of different purine auxotrophic mutations on mouse-virulence of a Vi-positive strain of Salmonella dublin and of two strains of Salmonella typhimurium. *Microb Pathog* **3**: 129-141.

McGavin, M.J., Zahradka, C., Rice, K., and Scott, J.E. (1997) Modification of the Staphylococcus aureus fibronectin binding phenotype by V8 protease. *Infect Immun* **65**: 2621-2628.

McNamara, P.J., Milligan-Monroe, K.C., Khalili, S., and Proctor, R.A. (2000) Identification, cloning, and initial characterization of rot, a locus encoding a regulator of virulence factor expression in Staphylococcus aureus. *J Bacteriol* **182**: 3197-3203.

Mecsas, J., Rouviere, P.E., Erickson, J.W., Donohue, T.J., and Gross, C.A. (1993) The activity of sigma E, an Escherichia coli heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev* **7**: 2618-2628.

Menzies, B.E. (2003) The role of fibronectin binding proteins in the pathogenesis of Staphylococcus aureus infections. *Curr Opin Infect Dis* **16**: 225-229.

Merighi, M., Majerczak, D.R., Stover, E.H., and Coplin, D.L. (2003) The HrpX/HrpY two-component system activates hrpS expression, the first step in the regulatory cascade controlling the Hrp regulon in Pantoea stewartii subsp. stewartii. *Mol Plant Microbe Interact* **16**: 238-248.

Merrick, M.J. (1983) Nitrogen control of the nif regulon in Klebsiella pneumoniae: involvement of the ntrA gene and analogies between ntrC and nifA. *EMBO J* **2**: 39-44.

Meziane-Cherif, D., Saul, F.A., Moubareck, C., Weber, P., Haouz, A., Courvalin, P., and Perichon, B. (2010) Molecular basis of vancomycin dependence in VanA-type Staphylococcus aureus VRSA-9. *J Bacteriol* **192**: 5465-5471.

Miller, H.K., Carroll, R.K., Burda, W.N., Krute, C.N., Davenport, J.E., and Shaw, L.N. (2012) The ECF Sigma Factor, sigmaS, Protects Against Both Cytoplasmic and Extracytoplasmic Stresses in Staphylococcus aureus. *J Bacteriol*.

Missiakas, D., Betton, J.M., and Raina, S. (1996a) New components of protein folding in extracytoplasmic compartments of Escherichia coli SurA, FkpA and Skp/OmpH. *Mol Microbiol* **21**: 871-884.

Missiakas, D., Schwager, F., Betton, J.M., Georgopoulos, C., and Raina, S. (1996b) Identification and characterization of HsIV HsIU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in Escherichia coli. *EMBO J* **15**: 6899-6909.

Missiakas, D., Mayer, M.P., Lemaire, M., Georgopoulos, C., and Raina, S. (1997) Modulation of the Escherichia coli sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol Microbiol* **24**: 355-371.

Miyazaki, E., Chen, J.M., Ko, C., and Bishai, W.R. (1999) The Staphylococcus aureus rsbW (orf159) gene encodes an anti-sigma factor of SigB. *J Bacteriol* **181**: 2846-2851.

Miyazaki, S., Matsumoto, Y., Sekimizu, K., and Kaito, C. (2012) Evaluation of Staphylococcus aureus virulence factors using a silkworm model. *FEMS Microbiol Lett* **326**: 116-124.

Modun, B., Evans, R.W., Joannou, C.L., and Williams, P. (1998) Receptor-mediated recognition and uptake of iron from human transferrin by Staphylococcus aureus and Staphylococcus epidermidis. *Infect Immun* **66**: 3591-3596.

Moellering, R.C., Jr. (2006) Vancomycin: a 50-year reassessment. *Clin Infect Dis* **42 Suppl 1**: S3-4.

Montgomery, C.P., Boyle-Vavra, S., and Daum, R.S. (2009) The arginine catabolic mobile element is not associated with enhanced virulence in experimental

invasive disease caused by the community-associated methicillin-resistant Staphylococcus aureus USA300 genetic background. *Infect Immun* **77**: 2650-2656.

Montgomery, K., Ryan, T.J., Krause, A., and Starkey, C. (2010) Assessment of athletic health care facility surfaces for MRSA in the secondary school setting. *J Environ Health* **72**: 8-11; quiz 66.

Moran, C.P., Jr., Lang, N., LeGrice, S.F., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J., and Losick, R. (1982) Nucleotide sequences that signal the initiation of transcription and translation in Bacillus subtilis. *Mol Gen Genet* **186**: 339-346.

Moran, G.J., Krishnadasan, A., Gorwitz, R.J., Fosheim, G.E., McDougal, L.K., Carey, R.B., and Talan, D.A. (2006) Methicillin-resistant S. aureus infections among patients in the emergency department. *N Engl J Med* **355**: 666-674.

Morikawa, K., Inose, Y., Okamura, H., Maruyama, A., Hayashi, H., Takeyasu, K., and Ohta, T. (2003) A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes to Cells* **8**: 699-712.

Morrison-Rodriguez, S.M., Pacha, L.A., Patrick, J.E., and Jordan, N.N. (2010) Community-associated methicillin-resistant Staphylococcus aureus infections at an Army training installation. *Epidemiol Infect* **138**: 721-729.

Morse, D.E., Mosteller, R.D., and Yanofsky, C. (1969) Dynamics of synthesis, translation, and degradation of trp operon messenger RNA in E. coli. *Cold Spring Harb Symp Quant Biol* **34**: 725-740.

Mortara, L.A., and Bayer, A.S. (1993) Staphylococcus aureus bacteremia and endocarditis. New diagnostic and therapeutic concepts. *Infect Dis Clin North Am* **7**: 53-68.

Muffler, A., Fischer, D., and Hengge-Aronis, R. (1996) The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for rpoS translation in Escherichia coli. *Genes Dev* **10**: 1143-1151.

Mulligan, M.E., Brosius, J., and McClure, W.R. (1985) Characterization in vitro of the effect of spacer length on the activity of Escherichia coli RNA polymerase at the TAC promoter. *J Biol Chem* **260**: 3529-3538.

Munch-Petersen, A., and Mygind, B. (1976) Nucleoside transport systems in Escherichia coli K12: specificity and regulation. *J Cell Physiol* **89**: 551-559.

Munch-Petersen, A., Mygind, B., Nicolaisen, A., and Pihl, N.J. (1979) Nucleoside transport in cells and membrane vesicles from Escherichia coli K12. *J Biol Chem* **254**: 3730-3737.

Murakami, K.S., Masuda, S., Campbell, E.A., Muzzin, O., and Darst, S.A. (2002) Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. *Science* **296**: 1285-1290.

Murakami, K.S., and Darst, S.A. (2003) Bacterial RNA polymerases: the wholo story. *Curr Opin Struct Biol* **13**: 31-39.

Murray, T., Popham, D.L., and Setlow, P. (1998) Bacillus subtilis cells lacking penicillin-binding protein 1 require increased levels of divalent cations for growth. *J Bacteriol* **180**: 4555-4563.

Murray, T.R., Downey, K.M., and Yunis, A.A. (1983) Chloramphenicol-mediated DNA damage and its possible role in the inhibitory effects of chloramphenicol on DNA synthesis. *J Lab Clin Med* **102**: 926-932.

Musher, D.M., Lamm, N., Darouiche, R.O., Young, E.J., Hamill, R.J., and Landon, G.C. (1994) The current spectrum of Staphylococcus aureus infection in a tertiary care hospital. *Medicine (Baltimore)* **73**: 186-208.

Mylotte, J.M., McDermott, C., and Spooner, J.A. (1987) Prospective study of 114 consecutive episodes of Staphylococcus aureus bacteremia. *Rev Infect Dis* **9**: 891-907.

Naimi, T.S., LeDell, K.H., Como-Sabetti, K., Borchardt, S.M., Boxrud, D.J., Etienne, J., Johnson, S.K., Vandenesch, F., Fridkin, S., O'Boyle, C., Danila, R.N., and Lynfield, R. (2003) Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. *Jama-Journal of the American Medical Association* **290**: 2976-2984.

Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M., Gill, S., Francois, P., and Cheung, A.L. (2011) Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. *J Bacteriol* **193**: 2332-2335.

Nakamura, S. (1990) [Curing effects of chlorination, ozone and UV treatments on plasmid DNAs]. *Nihon Koshu Eisei Zasshi* **37**: 745-751.

Nakano, M.M., Nakano, S., and Zuber, P. (2002) Spx (YjbD), a negative effector of competence in Bacillus subtilis, enhances ClpC-MecA-ComK interaction. *Mol Microbiol* **44**: 1341-1349.

Nakunst, D., Larisch, C., Huser, A.T., Tauch, A., Puhler, A., and Kalinowski, J. (2007) The extracytoplasmic function-type sigma factor SigM of Corynebacterium glutamicum ATCC 13032 is involved in transcription of disulfide stress-related genes. *J Bacteriol* **189**: 4696-4707.

Nde, C.W., Toghrol, F., Jang, H.J., and Bentley, W.E. (2011) Toxicogenomic response of Mycobacterium bovis BCG to peracetic acid and a comparative analysis of the M. bovis BCG response to three oxidative disinfectants. *Appl Microbiol Biotechnol* **90**: 277-304.

Newton, G.L., Arnold, K., Price, M.S., Sherrill, C., Delcardayre, S.B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R.C., and Davis, C. (1996) Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. *J Bacteriol* **178**: 1990-1995.

Nicholas, R.O., Li, T., McDevitt, D., Marra, A., Sucoloski, S., Demarsh, P.L., and Gentry, D.R. (1999) Isolation and characterization of a sigB deletion mutant of Staphylococcus aureus. *Infect Immun* **67**: 3667-3669.

Nitta, T., Nagamitsu, H., Murata, M., Izu, H., and Yamada, M. (2000) Function of the sigma(E) regulon in dead-cell lysis in stationary-phase Escherichia coli. *J Bacteriol* **182**: 5231-5237.

Nizan-Koren, R., Manulis, S., Mor, H., Iraki, N.M., and Barash, I. (2003) The regulatory cascade that activates the Hrp regulon in Erwinia herbicola pv. gypsophilae. *Mol Plant Microbe Interact* **16**: 249-260.

Noble, W.C., Valkenburg, H.A., and Wolters, C.H. (1967) Carriage of Staphylococcus aureus in random samples of a normal population. *J Hyg (Lond)* **65**: 567-573.

Noble, W.C., Virani, Z., and Cree, R.G. (1992) Co-transfer of vancomycin and other resistance genes from Enterococcus faecalis NCTC 12201 to Staphylococcus aureus. *FEMS Microbiol Lett* **72**: 195-198.

Novick, R.P., Projan, S.J., Kornblum, J., Ross, H.F., Ji, G., Kreiswirth, B., Vandenesch, F., and Moghazeh, S. (1995) The agr P2 operon: an autocatalytic sensory transduction system in Staphylococcus aureus. *Mol Gen Genet* **248**: 446-458.

Novick, R.P., and Jiang, D. (2003a) The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. *Microbiology* **149**: 2709-2717.

Novick, R.P., and Jiang, D.R. (2003b) The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. *Microbiology-Sgm* **149**: 2709-2717.

O'Callaghan, D., Maskell, D., Liew, F.Y., Easmon, C.S., and Dougan, G. (1988) Characterization of aromatic- and purine-dependent Salmonella typhimurium: attention, persistence, and ability to induce protective immunity in BALB/c mice. *Infect Immun* **56**: 419-423.

O'Croinin, T., and Dorman, C.J. (2007) Expression of the Fis protein is sustained in late-exponential- and stationary-phase cultures of Salmonella enterica serovar Typhimurium grown in the absence of aeration. *Mol Microbiol* **66**: 237-251.

O'Neill, A.J. (2010) Staphylococcus aureus SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Lett Appl Microbiol* **51**: 358-361.

O'Riordan, K., and Lee, J.C. (2004) Staphylococcus aureus capsular polysaccharides. *Clin Microbiol Rev* **17**: 218-234.

Ogston, A. (1882) Micrococcus poisoning. *J Anat* **17**: 24-58.

Okabayashi, K., and Mizuno, D. (1974) Surface-bound nuclease of Stapylococcus aureus: purification and properties of the enzymes. *J Bacteriol* **117**: 222-226.

Oller, A.R., Province, L., and Curless, B. (2010) Staphylococcus aureus recovery from environmental and human locations in 2 collegiate athletic teams. *J Athl Train* **45**: 222-229.

Padan, E., and Schuldiner, S. (1993) Na+/H+ antiporters, molecular devices that couple the Na+ and H+ circulation in cells. *J Bioenerg Biomembr* **25**: 647-669.

Paget, M.S., Kang, J.G., Roe, J.H., and Buttner, M.J. (1998) sigmaR, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in Streptomyces coelicolor A3(2). *EMBO J* **17**: 5776-5782.

Paget, M.S., Chamberlin, L., Atrih, A., Foster, S.J., and Buttner, M.J. (1999) Evidence that the extracytoplasmic function sigma factor sigmaE is required for normal cell wall structure in Streptomyces coelicolor A3(2). *J Bacteriol* **181**: 204-211.

Paget, M.S., Bae, J.B., Hahn, M.Y., Li, W., Kleanthous, C., Roe, J.H., and Buttner, M.J. (2001a) Mutational analysis of RsrA, a zinc-binding anti-sigma factor with a thiol-disulphide redox switch. *Mol Microbiol* **39**: 1036-1047.

Paget, M.S., Molle, V., Cohen, G., Aharonowitz, Y., and Buttner, M.J. (2001b) Defining the disulphide stress response in Streptomyces coelicolor A3(2): identification of the sigmaR regulon. *Mol Microbiol* **42**: 1007-1020.

Paget, M.S.B., and Helmann, J.D. (2003) Protein family review - The sigma(70) family of sigma factors. *Genome Biology* **4**.

Palma, M., and Cheung, A.L. (2001) sigma(B) activity in Staphylococcus aureus is controlled by RsbU and an additional factor(s) during bacterial growth. *Infect Immun* **69**: 7858-7865.

Pan, Q., Losick, R., and Rudner, D.Z. (2003) A second PDZ-containing serine protease contributes to activation of the sporulation transcription factor sigmaK in Bacillus subtilis. *J Bacteriol* **185**: 6051-6056.

Pane-Farre, J., Jonas, B., Forstner, K., Engelmann, S., and Hecker, M. (2006) The sigma(B) regulon in Staphylococcus aureus and its regulation. *International Journal of Medical Microbiology* **296**: 237-258.

Park, J.H., and Roe, J.H. (2008) Mycothiol regulates and is regulated by a thiol-specific antisigma factor RsrA and sigma(R) in Streptomyces coelicolor. *Mol Microbiol* **68**: 861-870.

Patti, J.M., Jonsson, H., Guss, B., Switalski, L.M., Wiberg, K., Lindberg, M., and Hook, M. (1992) Molecular characterization and expression of a gene encoding a Staphylococcus aureus collagen adhesin. *J Biol Chem* **267**: 4766-4772.

Patti, J.M., Bremell, T., Krajewska-Pietrasik, D., Abdelnour, A., Tarkowski, A., Ryden, C., and Hook, M. (1994) The Staphylococcus aureus collagen adhesin is a virulence determinant in experimental septic arthritis. *Infect Immun* **62**: 152-161.

Pero, J., Nelson, J., and Fox, T.D. (1975) Highly asymmetric transcription by RNA polymerase containing phage-SP01-induced polypeptides and a new host protein. *Proc Natl Acad Sci U S A* **72**: 1589-1593.

Peschel, A., Otto, M., Jack, R.W., Kalbacher, H., Jung, G., and Gotz, F. (1999) Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **274**: 8405-8410.

Popovich, K.J., Weinstein, R.A., and Hota, B. (2008) Are community-associated methicillin-resistant Staphylococcus aureus (MRSA) strains replacing traditional nosocomial MRSA strains? *Clinical Infectious Diseases* **46**: 787-794.

Prunier, A.L., and Leclercq, R. (2005) Role of mutS and mutL genes in hypermutability and recombination in Staphylococcus aureus. *J Bacteriol* **187**: 3455-3464.

Raivio, T.L., and Silhavy, T.J. (2001) Periplasmic stress and ECF sigma factors. *Annu Rev Microbiol* **55**: 591-624.

Rajagopal, L., Vo, A., Silvestroni, A., and Rubens, C.E. (2005) Regulation of purine biosynthesis by a eukaryotic-type kinase in Streptococcus agalactiae. *Mol Microbiol* **56**: 1329-1346.

Rasigade, J.P., Laurent, F., Hubert, P., Vandenesch, F., and Etienne, J. (2010) Lethal necrotizing pneumonia caused by an ST398 Staphylococcus aureus strain. *Emerg Infect Dis* **16**: 1330.

Ray, P.H., Lillich, T.T., and White, D.C. (1972) Consequences of glycerol deprivation on the synthesis of membrane components in a glycerol auxotroph of Staphylococcus aureus. *J Bacteriol* **112**: 413-420.

Ray, P.H., and White, D.C. (1972) Effect of glycerol deprivation on the phospholipid metabolism of a glycerol auxotroph of Staphylococcus aureus. *J Bacteriol* **109**: 668-677.

Rayssiguier, C., Thaler, D.S., and Radman, M. (1989) The barrier to recombination between Escherichia coli and Salmonella typhimurium is disrupted in mismatch-repair mutants. *Nature* **342**: 396-401.

Repine, J.E., Fox, R.B., and Berger, E.M. (1981) Hydrogen peroxide kills Staphylococcus aureus by reacting with staphylococcal iron to form hydroxyl radical. *J Biol Chem* **256**: 7094-7096.

Rezuchova, B., and Kormanec, J. (2001) A two-plasmid system for identification of promoters recognized by RNA polymerase containing extracytoplasmic stress response sigma(E) in Escherichia coli. *J Microbiol Methods* **45**: 103-111.

Rice, K., Peralta, R., Bast, D., de Azavedo, J., and McGavin, M.J. (2001) Description of staphylococcus serine protease (ssp) operon in Staphylococcus aureus and nonpolar inactivation of sspA-encoded serine protease. *Infect Immun* **69**: 159-169.

Ridley, M., Lynn, R., Barrie, D., and Stead, K.C. (1970) Antibiotic-resistant Staphylococcus aureus and hospital antibiotic policies. *Lancet* **1**: 230-233.

Rivera, F.E., Miller, H.K., Kolar, S.L., Stevens, S.M., Jr., and Shaw, L.N. (2012) The impact of CodY on virulence determinant production in community-associated methicillin-resistant Staphylococcus aureus. *Proteomics* **12**: 263-268.

Rodrigue, S., Provvedi, R., Jacques, P.E., Gaudreau, L., and Manganelli, R. (2006) The sigma factors of Mycobacterium tuberculosis. *FEMS Microbiol Rev* **30**: 926-941.

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D., Alston, M., Stringer, M.F., Betts, R.P., Baranyi, J., Peck, M.W., and Hinton, J.C. (2012) Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. *J Bacteriol* **194**: 686-701.

Ross, C.L., Thomason, K.S., and Koehler, T.M. (2009) An extracytoplasmic function sigma factor controls beta-lactamase gene expression in Bacillus anthracis and other Bacillus cereus group species. *J Bacteriol* **191**: 6683-6693.

Rouviere, P.E., De Las Penas, A., Mecsas, J., Lu, C.Z., Rudd, K.E., and Gross, C.A. (1995) rpoE, the gene encoding the second heat-shock sigma factor, sigma E, in Escherichia coli. *EMBO J* **14**: 1032-1042.

Roux, C.M., Booth, N.J., Bellaire, B.H., Gee, J.M., Roop, R.M., 2nd, Kovach, M.E., Tsolis, R.M., Elzer, P.H., and Ennis, D.G. (2006) RecA and RadA proteins of Brucella abortus do not perform overlapping protective DNA repair functions following oxidative burst. *J Bacteriol* **188**: 5187-5195.

Sabelnikov, A.G., Greenberg, B., and Lacks, S.A. (1995) An extended -10 promoter alone directs transcription of the DpnII operon of Streptococcus pneumoniae. *J Mol Biol* **250**: 144-155.

Saecker, R.M., Tsodikov, O.V., McQuade, K.L., Schlax, P.E., Jr., Capp, M.W., and Record, M.T., Jr. (2002) Kinetic studies and structural models of the association of E. coli sigma(70) RNA polymerase with the lambdaP(R) promoter: large scale conformational changes in forming the kinetically significant intermediates. *J Mol Biol* **319**: 649-671.

Saier, M.H., Jr. (2000) Families of transmembrane transporters selective for amino acids and their derivatives. *Microbiology* **146 (Pt 8)**: 1775-1795.

Sakanyan, V., Charlier, D., Legrain, C., Kochikyan, A., Mett, I., Pierard, A., and Glansdorff, N. (1993) Primary structure, partial purification and regulation of key enzymes of the acetyl cycle of arginine biosynthesis in Bacillus stearothermophilus: dual function of ornithine acetyltransferase. *J Gen Microbiol* **139**: 393-402.

Salgo, M.G., Stone, K., Squadrito, G.L., Battista, J.R., and Pryor, W.A. (1995) Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem Biophys Res Commun* **210**: 1025-1030.

Samant, S., Lee, H., Ghassemi, M., Chen, J., Cook, J.L., Mankin, A.S., and Neyfakh, A.A. (2008) Nucleotide biosynthesis is critical for growth of bacteria in human blood. *PLoS Pathog* **4**: e37.

Sambanthamoorthy, K., Smeltzer, M.S., and Elasri, M.O. (2006) Identification and characterization of msa (SA1233), a gene involved in expression of SarA and several virulence factors in Staphylococcus aureus. *Microbiology* **152**: 2559-2572.

Sambrook, J., Fritsche, E., and Maniatis, T. (1989) *Molecular Cloning (a laboratory manual).* New York: Cold Spring harbour Laboratory.

Sanabria, T.J., Alpert, J.S., Goldberg, R., Pape, L.A., and Cheeseman, S.H. (1990) Increasing frequency of staphylococcal infective endocarditis. Experience at a university hospital, 1981 through 1988. *Arch Intern Med* **150**: 1305-1309.

Sancar, A., and Rupp, W.D. (1983) A novel repair enzyme: UVRABC excision nuclease of Escherichia coli cuts a DNA strand on both sides of the damaged region. *Cell* **33**: 249-260.

Sandre, R.M., and Shafran, S.D. (1996) Infective endocarditis: review of 135 cases over 9 years. *Clin Infect Dis* **22**: 276-286.

Sauviac, L., Philippe, H., Phok, K., and Bruand, C. (2007) An extracytoplasmic function sigma factor acts as a general stress response regulator in Sinorhizobium meliloti. *J Bacteriol* **189**: 4204-4216.

Schaberg, D.R. (1994) Resistant Gram-Positive Organisms. *Ann Emerg Med* **24**: 462-464.

Schell, M.A. (1993) Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* **47**: 597-626.

Schenk, S., and Laddaga, R.A. (1992) Improved method for electroporation of Staphylococcus aureus. *FEMS Microbiol Lett* **73**: 133-138.

Schindler, C.A., and Schuhardt, V.T. (1964) Lysostaphin: A New Bacteriolytic Agent for the Staphylococcus. *Proc Natl Acad Sci U S A* **51**: 414-421.

Schmidt, K.A., Manna, A.C., Gill, S., and Cheung, A.L. (2001) SarT, a repressor of alpha-hemolysin in Staphylococcus aureus. *Infect Immun* **69**: 4749-4758.

Schmitt, C.K., Darnell, S.C., Tesh, V.L., Stocker, B.A.D., and Obrien, A.D. (1994) Mutation of Flgm Attenuates Virulence of Salmonella-Typhimurium, and Mutation of Flia Represses the Attenuated Phenotype. *J Bacteriol* **176**: 368-377.

Schneewind, O., Fowler, A., and Faull, K.F. (1995) Structure of the cell wall anchor of surface proteins in Staphylococcus aureus. *Science* **268**: 103-106.

Schneider, T., Senn, M.M., Berger-Bachi, B., Tossi, A., Sahl, H.G., and Wiedemann, I. (2004) In vitro assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of Staphylococcus aureus. *Mol Microbiol* **53**: 675-685.

Schuldiner, S., Agmon, V., Brandsma, J., Cohen, A., Friedman, E., and Padan, E. (1986) Induction of SOS functions by alkaline intracellular pH in Escherichia coli. *J Bacteriol* **168**: 936-939.

Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Boucher, J.C., and Deretic, V. (1996) Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and Pseudomonas aeruginosa conversion to mucoidy in cystic fibrosis. *J Bacteriol* **178**: 4997-5004.

Sen, R., Nagai, H., and Shimamoto, N. (2000) Polymerase arrest at the lambdaP(R) promoter during transcription initiation. *J Biol Chem* **275**: 10899-10904.

Senn, M.M., Giachino, P., Homerova, D., Steinhuber, A., Strassner, J., Kormanec, J., Fluckiger, U., Berger-Bachi, B., and Bischoff, M. (2005) Molecular analysis and organization of the sigmaB operon in Staphylococcus aureus. *J Bacteriol* **187**: 8006-8019.

Sexton, R., Gill, P.R., Jr., Callanan, M.J., O'Sullivan, D.J., Dowling, D.N., and O'Gara, F. (1995) Iron-responsive gene expression in Pseudomonas fluorescens M114: cloning and characterization of a transcription-activating factor, PbrA. *Mol Microbiol* **15**: 297-306.

Seybold, U., Kourbatova, E.V., Johnson, J.G., Halvosa, S.J., Wang, Y.F., King, M.D., Ray, S.M., and Blumberg, H.M. (2006) Emergence of community-associated methicillin-resistant Staphylococcus aureus USA300 genotype as a major cause of health care-associated blood stream infections. *Clinical Infectious Diseases* **42**: 647-656.

Sharp, M.M., Chan, C.L., Lu, C.Z., Marr, M.T., Nechaev, S., Merritt, E.W., Severinov, K., Roberts, J.W., and Gross, C.A. (1999) The interface of sigma with core RNA polymerase is extensive, conserved, and functionally specialized. *Genes Dev* **13**: 3015-3026.

Shaw, L., Golonka, E., Potempa, J., and Foster, S.J. (2004) The role and regulation of the extracellular proteases of Staphylococcus aureus. *Microbiology* **150**: 217-228.

Shaw, L.N., Aish, J., Davenport, J.E., Brown, M.C., Lithgow, J.K., Simmonite, K., Crossley, H., Travis, J., Potempa, J., and Foster, S.J. (2006) Investigations into sigmaB-modulated regulatory pathways governing extracellular virulence determinant production in Staphylococcus aureus. *J Bacteriol* **188**: 6070-6080.

Shaw, L.N., Lindholm, C., Prajsnar, T.K., Miller, H.K., Brown, M.C., Golonka, E., Stewart, G.C., Tarkowski, A., and Potempa, J. (2008) Identification and Characterization of sigma(S), a Novel Component of the Staphylococcus aureus Stress and Virulence Responses. *PLoS One* **3**.

Sheehy, S.H., Atkins, B.A., Bejon, P., Byren, I., Wyllie, D., Athanasou, N.A., Berendt, A.R., and McNally, M.A. (2010) The microbiology of chronic osteomyelitis: prevalence of resistance to common empirical anti-microbial regimens. *J Infect* **60**: 338-343.

Shenoy, M.S., Bhat, G.K., Kishore, A., and Hassan, M.K. (2010) Significance of MRSA strains in community associated skin and soft tissue infections. *Indian J Med Microbiol* **28**: 152-154.

Shuler, M.F., Tatti, K.M., Wade, K.H., and Moran, C.P., Jr. (1995) A single amino acid substitution in sigma E affects its ability to bind core RNA polymerase. *J Bacteriol* **177**: 3687-3694.

Sievert, D.M., Rudrik, J.T., Patel, J.B., McDonald, L.C., Wilkins, M.J., and Hageman, J.C. (2008) Vancomycin-resistant Staphylococcus aureus in the United States, 2002-2006. *Clin Infect Dis* **46**: 668-674.

Sigoillot, F.D., Berkowski, J.A., Sigoillot, S.M., Kotsis, D.H., and Guy, H.I. (2003) Cell cycle-dependent regulation of pyrimidine biosynthesis. *J Biol Chem* **278**: 3403-3409.

Singh, S., Asad, S.F., Ahmad, A., Khan, N.U., and Hadi, S.M. (2001) Oxidative DNA damage by capsaicin and dihydrocapsaicin in the presence of Cu(II). *Cancer Lett* **169**: 139-146.

Sinha, B., Francois, P., Que, Y.A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K.H., Peters, G., and Herrmann, M. (2000) Heterologously expressed Staphylococcus aureus fibronectin-binding proteins are sufficient for invasion of host cells. *Infect Immun* **68**: 6871-6878.

Smith, T.L., Pearson, M.L., Wilcox, K.R., Cruz, C., Lancaster, M.V., Robinson-Dunn, B., Tenover, F.C., Zervos, M.J., Band, J.D., White, E., and Jarvis, W.R. (1999) Emergence of vancomycin resistance in Staphylococcus aureus. Glycopeptide-Intermediate Staphylococcus aureus Working Group. *N Engl J Med* **340**: 493-501.

Smyth, C.J., Mollby, R., and Wadstrom, T. (1975) Phenomenon of hot-cold hemolysis: chelator-induced lysis of sphingomyelinase-treated erythrocytes. *Infect Immun* **12**: 1104-1111.

Sobral, R.G., Ludovice, A.M., Gardete, S., Tabei, K., De Lencastre, H., and Tomasz, A. (2003) Normally functioning murF is essential for the optimal expression of methicillin resistance in Staphylococcus aureus. *Microb Drug Resist* **9**: 231-241.

Sobral, R.G., Ludovice, A.M., de Lencastre, H., and Tomasz, A. (2006) Role of murF in cell wall biosynthesis: isolation and characterization of a murF conditional mutant of Staphylococcus aureus. *J Bacteriol* **188**: 2543-2553.

Spencer, J.P., Wong, J., Jenner, A., Aruoma, O.I., Cross, C.E., and Halliwell, B. (1996) Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem Res Toxicol* **9**: 1152-1158.

Spiegelman, G.B., Hiatt, W.R., and Whiteley, H.R. (1978) Role of the 21,000 molecular weight polypeptide of Bacillus subtilis RNA polymerase in RNA synthesis. *J Biol Chem* **253**: 1756-1765.

Spirig, T., Weiner, E.M., and Clubb, R.T. (2011) Sortase enzymes in Gram-positive bacteria. *Mol Microbiol* **82**: 1044-1059.

Staron, A., Sofia, H.J., Dietrich, S., Ulrich, L.E., Liesegang, H., and Mascher, T. (2009) The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. *Mol Microbiol* **74**: 557-581.

Stiefel, A., Mahren, S., Ochs, M., Schindler, P.T., Enz, S., and Braun, V. (2001) Control of the ferric citrate transport system of Escherichia coli: mutations in region 2.1 of the FecI extracytoplasmic-function sigma factor suppress mutations in the FecR transmembrane regulatory protein. *J Bacteriol* **183**: 162-170.

Studholme, D.J., Wigneshwereraraj, S.R., Gallegos, M.T., and Buck, M. (2000) Functionality of purified sigma(N) (sigma(54)) and a NifA-like protein from the hyperthermophile Aquifex aeolicus. *J Bacteriol* **182**: 1616-1623.

Sugawara, N., Tomita, T., and Kamio, Y. (1997) Assembly of Staphylococcus aureus gamma-hemolysin into a pore-forming ring-shaped complex on the surface of human erythrocytes. *FEBS Lett* **410**: 333-337.

Sullivan, M.A., Yasbin, R.E., and Young, F.E. (1984) New shuttle vectors for Bacillus subtilis and Escherichia coli which allow rapid detection of inserted fragments. *Gene* **29**: 21-26.

Sun, R.G., Converse, P.J., Ko, C., Tyagi, S., Morrison, N.E., and Bishai, W.R. (2004) Mycobacterium tuberculosis ECF sigma factor sigC is required for lethality in mice and for the conditional expression of a defined gene set. *Mol Microbiol* **52**: 25-38.

Tao, L.A., Wu, X.Q., and Sun, B.L. (2010) Alternative Sigma Factor sigma(H) Modulates Prophage Integration and Excision in Staphylococcus aureus. *PLoS Pathog* **6**.

Tatti, K.M., and Moran, C.P., Jr. (1995) sigma E changed to sigma B specificity by amino acid substitutions in its -10 binding region. *J Bacteriol* **177**: 6506-6509.

Tegmark, K., Karlsson, A., and Arvidson, S. (2000) Identification and characterization of SarH1, a new global regulator of virulence gene expression in Staphylococcus aureus. *Mol Microbiol* **37**: 398-409.

Tenover, F.C., and Goering, R.V. (2009) Methicillin-resistant Staphylococcus aureus strain USA300: origin and epidemiology. *Journal of Antimicrobial Chemotherapy* **64**: 441-446.

Thackray, P.D., and Moir, A. (2003) SigM, an extracytoplasmic function sigma factor of Bacillus subtilis, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. *J Bacteriol* **185**: 3491-3498.

Thammavongsa, V., Kern, J.W., Missiakas, D.M., and Schneewind, O. (2009) Staphylococcus aureus synthesizes adenosine to escape host immune responses. *J Exp Med* **206**: 2417-2427.

Thompson, R.L., Cabezudo, I., and Wenzel, R.P. (1982) Epidemiology of nosocomial infections caused by methicillin-resistant Staphylococcus aureus. *Ann Intern Med* **97**: 309-317.

Traber, K., and Novick, R. (2006) A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of agr and failure to translate delta- and alpha-haemolysins. *Mol Microbiol* **59**: 1519-1530.

Travers, A.A. (1987) Structure and function of E. coli promoter DNA. *CRC Crit Rev Biochem* **22**: 181-219.

Tristan, A., Bes, M., Meugnier, H., Lina, G., Bozdogan, B., Courvalin, P., Reverdy, M.E., Enright, M.C., Vandenesch, F., and Etienne, J. (2007) Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant Staphylococcus aureus, 2006. *Emerg Infect Dis* **13**: 594-600.

Truong-Bolduc, Q.C., and Hooper, D.C. (2010) Phosphorylation of MgrA and its effect on expression of the NorA and NorB efflux pumps of Staphylococcus aureus. *J Bacteriol* **192**: 2525-2534.

Turgay, K., Hamoen, L.W., Venema, G., and Dubnau, D. (1997) Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of Bacillus subtilis. *Genes Dev* **11**: 119-128.

Turgay, K., Hahn, J., Burghoorn, J., and Dubnau, D. (1998) Competence in Bacillus subtilis is controlled by regulated proteolysis of a transcription factor. *EMBO J* **17**: 6730-6738.

Updegrove, T., Wilf, N., Sun, X., and Wartell, R.M. (2008) Effect of Hfq on RprA-rpoS mRNA pairing: Hfq-RNA binding and the influence of the 5' rpoS mRNA leader region. *Biochemistry* **47**: 11184-11195.

van Sinderen, D., Luttinger, A., Kong, L., Dubnau, D., Venema, G., and Hamoen, L. (1995) comK encodes the competence transcription factor, the key regulatory protein for competence development in Bacillus subtilis. *Mol Microbiol* **15**: 455-462.

Vogel, J., and Wagner, E.G. (2007) Target identification of small noncoding RNAs in bacteria. *Current Opinion in Microbiology* **10**: 262-270.

Voureka, A. (1952) Induced variations in a penicillin-resistant staphylococcus. *J Gen Microbiol* **6**: 352-360.

Wakamatsu, T., Kim, K., Uemura, Y., Nakagawa, N., Kuramitsu, S., and Masui, R. (2011) Role of RecJ-like protein with 5'-3' exonuclease activity in oligo(deoxy)nucleotide degradation. *J Biol Chem* **286**: 2807-2816.

Walsh, C.T. (1989) Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. *J Biol Chem* **264**: 2393-2396.

Walsh, N.P., Alba, B.M., Bose, B., Gross, C.A., and Sauer, R.T. (2003) OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* **113**: 61-71.

Walthers, D., Carroll, R.K., Navarre, W.W., Libby, S.J., Fang, F.C., and Kenney, L.J. (2007) The response regulator SsrB activates expression of diverse Salmonella pathogenicity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS. *Mol Microbiol* **65**: 477-493.

Wang, D., Yu, L., Xiang, H., Fan, J., He, L., Guo, N., Feng, H., and Deng, X. (2008) Global transcriptional profiles of Staphylococcus aureus treated with berberine chloride. *FEMS Microbiol Lett* **279**: 217-225.

Wang, Q.P., and Kaguni, J.M. (1989) A novel sigma factor is involved in expression of the rpoH gene of Escherichia coli. *J Bacteriol* **171**: 4248-4253.

Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H.L., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., Deleo, F.R., and Otto, M. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* **13**: 1510-1514.

Waters, L.S., and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell* **136**: 615-628.

Watson, S.P., Clements, M.O., and Foster, S.J. (1998) Characterization of the starvation-survival response of Staphylococcus aureus. *J Bacteriol* **180**: 1750-1758.

Waxman, D.J., and Strominger, J.L. (1983) Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. *Annual Review of Biochemistry* **52**: 825-869.

Weese, J.S., Archambault, M., Willey, B.M., Hearn, P., Kreiswirth, B.N., Said-Salim, B., McGeer, A., Likhoshvay, Y., Prescott, J.F., and Low, D.E. (2005) Methicillin-resistant Staphylococcus aureus in horses and horse personnel, 2000-2002. *Emerg Infect Dis* **11**: 430-435.

Weese, J.S., Dick, H., Willey, B.M., McGeer, A., Kreiswirth, B.N., Innis, B., and Low, D.E. (2006) Suspected transmission of methicillin-resistant Staphylococcus aureus between domestic pets and humans in veterinary clinics and in the household. *Vet Microbiol* **115**: 148-155.

Weissbach, H., Etienne, F., Hoshi, T., Heinemann, S.H., Lowther, W.T., Matthews, B., St John, G., Nathan, C., and Brot, N. (2002) Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. *Arch Biochem Biophys* **397**: 172-178.

Weissbach, H., Resnick, L., and Brot, N. (2005) Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. *Biochim Biophys Acta* **1703**: 203-212.

White, M.J., He, H., Penoske, R.M., Twining, S.S., and Zahrt, T.C. (2010) PepD participates in the mycobacterial stress response mediated through MprAB and SigE. *J Bacteriol* **192**: 1498-1510.

Wickus, G.G., and Strominger, J.L. (1973) Partial purification and properties of the pyruvate-uridine diphospho-N-acetylglucosamine transferase from Staphylococcus epidermidis. *J Bacteriol* **113**: 287-290.

Wiegert, T., Homuth, G., Versteeg, S., and Schumann, W. (2001) Alkaline shock induces the Bacillus subtilis sigma(W) regulon. *Mol Microbiol* **41**: 59-71.

Wildemauwe, C., Godard, C., Vanhoof, R., Bossuyt, E.V., and Hannecart-Pokorni, E. (1996) Changes in major populations of methicillin-resistant Staphylococcus aureus in Belgium. *J Hosp Infect* **34**: 197-203.

Willetts, N.S. (1967) The elimination of Flac+ from Escherichia coli by mutagenic agents. *Biochem Biophys Res Commun* **27**: 112-117.

Woese, C.R. (1987) Bacterial evolution. *Microbiol Rev* **51**: 221-271.

Woodin, A.M. (1959) Fractionation of a leucocidin from Staphylococcus aureus. *Biochem J* **73**: 225-237.

Woodin, A.M. (1960) Purification of the two components of leucocidin from Staphylococcus aureus. *Biochem J* **75**: 158-165.

Wosten, M.M.S.M. (1998) Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**: 127-150.

Wu, S., de Lencastre, H., and Tomasz, A. (1996) Sigma-B, a putative operon encoding alternate sigma factor of Staphylococcus aureus RNA polymerase: molecular cloning and DNA sequencing. *J Bacteriol* **178**: 6036-6042.

Wyke, A.W. (1984) Isolation of five penicillin-binding proteins from Staphylococcus aureus. *FEMS Micro Lett* **22**.

Yanofsky, C. (1981) Attenuation in the control of expression of bacterial operons. *Nature* **289**: 751-758.

Yeager, C.M., Bottomley, P.J., and Arp, D.J. (2001) Requirement of DNA repair mechanisms for survival of Burkholderia cepacia G4 upon degradation of trichloroethylene. *Appl Environ Microbiol* **67**: 5384-5391.

Yoshimura, M., Asai, K., Sadaie, Y., and Yoshikawa, H. (2004) Interaction of Bacillus subtilis extracytoplasmic function (ECF) sigma factors with the N-terminal regions of their potential anti-sigma factors. *Microbiology-Sgm* **150**: 591-599.

Yu, H., Schurr, M.J., and Deretic, V. (1995) Functional equivalence of Escherichia coli sigma E and Pseudomonas aeruginosa AlgU: E. coli rpoE restores mucoidy and reduces sensitivity to reactive oxygen intermediates in algU mutants of P. aeruginosa. *J Bacteriol* **177**: 3259-3268.

Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R., and Storz, G. (1998) The OxyS regulatory RNA represses rpoS translation and binds the Hfq (HF-I) protein. *EMBO J* **17**: 6061-6068.

Zhang, A., Wassarman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **9**: 11-22.

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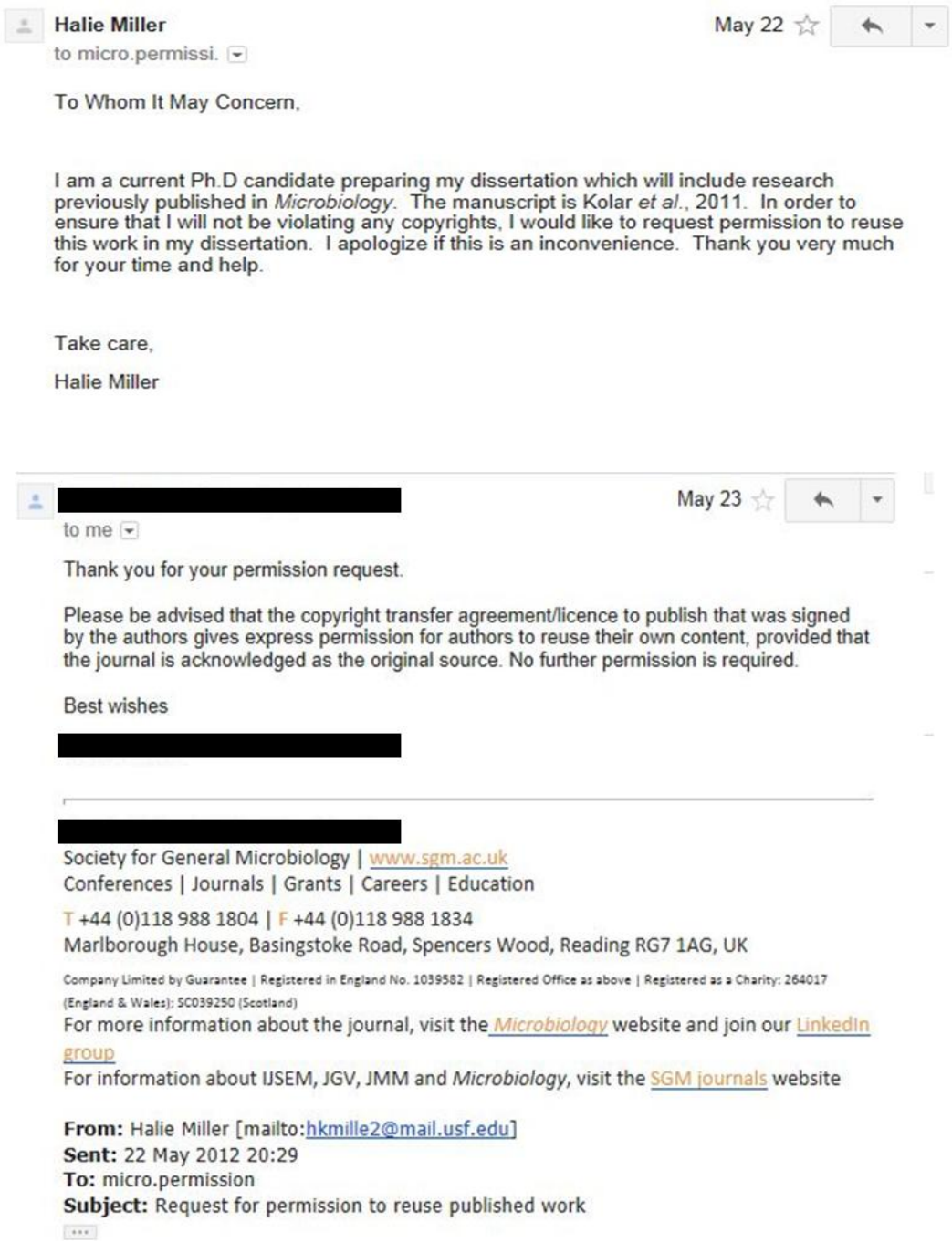

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Identification and Characterization of σ^S, a Novel Component of the *Staphylococcus aureus* Stress and Virulence Responses

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Abstract

S. aureus is a highly successful pathogen that is speculated to be the most common cause of human disease. The progression of disease in *S. aureus* is subject to multi-factorial regulation, in response to the environments encountered during growth. This adaptive nature is thought to be central to pathogenesis, and is the result of multiple regulatory mechanisms employed in gene regulation. In this work we describe the existence of a novel *S. aureus* regulator, an as yet uncharacterized ECF-sigma factor (σ^S), that appears to be an important component of the stress and pathogenic responses of this organism. Using biochemical approaches we have shown that σ^S is able to associates with core-RNAP, and initiate transcription from its own coding region. Using a mutant strain we determined that σ^S is important for *S. aureus* survival during starvation, extended exposure to elevated growth temperatures, and Triton X-100 induced lysis. Coculture studies reveal that a σ^S mutant is significantly outcompeted by its parental strain, which is only exacerbated during prolonged growth (7 days), or in the presence of stressor compounds. Interestingly, transcriptional analysis determined that under standard conditions, *S. aureus* SH1000 does not initiate expression of *sigS*. Assays performed hourly for 72h revealed expression in typically background ranges. Analysis of a potential anti-sigma factor, encoded downstream of *sigS*, revealed it to have no obvious role in the upregulation of *sigS* expression. Using a murine model of septic arthritis, *sigS*-mutant infected animals lost significantly less weight, developed septic arthritis at significantly lower levels, and had increased survival rates. Studies of mounted immune responses reveal that *sigS*-mutant infected animals had significantly lower levels of IL-6, indicating only a weak immunological response. Finally, strains of *S. aureus* lacking *sigS* were far less able to undergo systemic dissemination, as determined by bacterial loads in the kidneys of infected animals. These results establish that σ^S is an important component in *S. aureus* fitness, and in its adaptation to stress. Additionally it appears to have a significant role in its pathogenic nature, and likely represents a key component in the *S. aureus* regulatory network.

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Introduction

Staphylococcus aureus is a major human pathogen that is a leading agent of both nosocomial and community acquired infections. It is both a highly successful and dangerous pathogen that poses a significant threat to public health due to the increased prevalence of antibiotic resistant strains, such as methicillin-resistant *S. aureus* (MRSA) [1–4]. The appearance in recent years of true vancomycin-resistant MRSA [5–9] presents us with a frightening prospect of a return to the days of pre-antibiotic medicine, where the vast majority of staphylococcal bloodstream infections proved fatal. One of the overwhelming reasons that *S. aureus* is such a successful and diverse pathogen is the arsenal of virulence determinants encoded within its genome, which include hemolysins, toxins, adhesins and other exoproteins, such as proteases, staphylokinase and protein A [10,11]. 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 [11]. This responsive and adaptive nature is thought to be central to the disease-causing ability of the organism, and is largely the result of the multiple regulatory mechanisms it employs in gene regulation.

The large and wide reaching regulatory network employed by *S. aureus* encompasses a variety of common bacterial regulatory mechanisms, including two-component regulators, DNA binding proteins, regulatory RNAs, sigma factors and a quorum sensing system. There are thought to be sixteen two-component systems in *S. aureus*, including those that are responsible for the modulation of autolysis (ArlRS, LytRS), virulence (AgrAC, SaeRS) cell wall synthesis/drug resistance (GraRS, VraSR), and the sensing of external iron (HssRS) and oxygen (SrrRS) [12–18]. In addition there is a central, master regulator of virulence, the Agr system,

which is encoded by a four-gene locus that regulates pathogenesis, and the shift from localized to invasive phenotypes [19–21]. Further regulators exist, including the 12 members of the SarA family of DNA binding proteins [22], several of which have been shown to be important in virulence factor synthesis (SarA, Rot, SarT) [23–25]. There are also three metal-dependent DNA binding proteins encoded within the *S. aureus* genome, two of which (Fur and PerR) are required for the survival of *S. aureus* in animal models of infection [26].

S. aureus also has 3 known sigma factors: a housekeeping sigma factor, σ^A, originally described by Deora and Misra [27], and two alternative sigma factors, σ^B and σ^H [28,29]. Of these three, σ^B is by far the most widely studied, the effects of which are apparent in a variety of cellular processes, including oxidative stress resistance, pigmentation, protein secretion, biofilm formation, drug resistance, adaptation to stress and the progression of disease [30–32]. Indeed, strains of *S. aureus* lacking a functional σ^B are pleiotropically altered at the phenotypic level, and demonstrate reduced virulence in *in vivo* models of animal infection [30,33]. σ^A, encoded by the *plaC* gene, was first identified over a decade ago based on its homology with σ^A from *B. subtilis* [27]. It is analogous to other primary sigma factors in that it is essential for growth, and controls much of the day-to-day house-keeping transcription. Documentation of a third sigma factor, σ^H, in *S. aureus* recently appeared in a study by Morikawa *et al.* [28]. Here it was shown that *S. aureus* possesses a homologue of the genetic competence sigma factor, σ^H, from *B. subtilis*.

While the primary sigma factor directs much of the transcription during growth, most organisms possess alternative sigma factors that direct the transcription of specific regulons during unusual physiological conditions. ECF, or extra-cytoplasmic function, sigma factors form a distinct and diverse subfamily within this class of regulators that often share distant or divergent identity with other known σ factors. As a group, they are by far the most numerate of the sigma factor families [34,35], with *Streptomyces coelicolor* possessing more than 50 such elements within its genome. Other organisms, including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Bacillus anthracis* encode 10 or more such factors [34]. They have been identified in a variety of Gram-negative and Gram-positive organisms, and have been shown to have wide-ranging and varied roles in cellular physiology. These include the adaptation to: antimicrobial compounds, salt stress, elevated or reduced growth temperatures, acidic pH, detergents, oxidative stress, disulphide stress, iron starvation, osmotic stress, carbon and nitrogen stress, high pressure and light [36–45]. More importantly however, as the number of ECF-sigma factors identified grows, attention is turning to their often considerable roles in the virulence of pathogenic organisms [46].

Unusually, *S. aureus* seemingly achieves its versatile and adaptive nature with only a limited selection of sigma factors. So far only three have been documented, and only one of these (σ^B) has been shown to have a role in cellular adaptation and virulence. In this work we describe the characterization of a fourth *S. aureus* sigma factor, an apparent ECF-sigma factor, which is seemingly involved in cellular fitness and the adaptation to stress. Additionally it appears to have a significant role in the pathogenic nature of *S. aureus*, and likely represents an additional, key component in the regulatory network of this organism.

Results

Identification of SACOL1827 as a putative ECF-sigma factor

During work in our laboratory on the membrane proteases of *S. aureus*, we generated a mutation in RseP. Multiple publications on RseP proteases in *E. coli*, *B. subtilis* and *Pseudomonas aeruginosa* demonstrate that they commonly serve to cleave the anti-sigma factors of extra-cytoplasmic function (ECF)-sigma factors [47–53]. As it has previously been proposed by Helmann that the genome of *S. aureus* likely contains an ECF-sigma factor [34], we undertook an exploration of the *S. aureus* genome so as to determine whether an as yet unidentified ECF sigma factor was present. Using the protein sequence of the 7 known *B. subtilis* ECF-sigma factors, a novel protein (SACOL1827) bearing homology to the ECF-sigma factors σ^M and σ^{YlaC} was discovered in the *S. aureus* genome (Table 1). BLAST analysis with this protein sequence revealed homology with other ECF-sigma factors from a variety of organisms (Table 1). The gene coding this protein is present in the genome of all of the sequenced strains of *S. aureus*. Equally, it is present in the four other sequenced Staphylococcal genomes: *S. epidermidis* ATCC 12228 and RP62A, as well as *S. haemolyticus* and *S. saprophyticus*. Our initial investigations of SACOL1827, using *in silico* protein analysis, demonstrated the presence of both regions 2 and region 4 of σ^{70}. Further, *in silico* protein folding analysis (using the 3D-JIGSAW, FUGUE and PHYRE databases) generated strong homology scores for both of these regions (between 95–100% certainty for region 2, and 90–95% certainty for region 4). Overall our predictive protein folding and modeling analyses returned a probability value of $p = <0.001$ for σ^S against the founding-member of the ECF-sigma factors, σ^E of *E. coli*.

A common observance of ECF-family proteins is that the genes encoding the sigma factors are contiguous to a coding region specifying an anti-sigma factor. Analysis of the SACOL1827 locus revealed a putatively transcriptionally-linked downstream gene (SACOL1828) that is separated from SACOL1827 by 112 bp. SACOL1828 is a conserved hypothetical protein with no discernable homology to other proteins within the databases, other than its direct homologues in staphylococci. *In silico* analysis determined that these two genes are found clustered in this arrangement in all of the sequenced *S. aureus* genomes (including the RF122 bovine mastitis strain); in *S. epidermidis* ATCC 12228 and RP62A; and in *S. haemolyticus* and *S. saprophyticus*. Commonly the anti-sigma factors of ECF-sigma factors possess membrane associated domains, however analysis of SACOL1828 using a Kyte-Doolittle hydrophobicity plot revealed no such region. Interestingly, some ECF anti-sigma factors possess an H(XXX)C(XX)C motif, as is the case in *Streptomyces coelicolor* and *Mycobacterium tuberculosis* [54–56]. SACOL1828 bears a similar sequence of H(LETN)C(VFH)C, which correlates well with that found in other organisms.

Biochemical characterization of SACOL1827 reveals it to be a sigma factor

Sigma factors bind to core RNAP in a reversible way in order to induce transcription. To test the ability of *S. aureus* SACOL1827 to bind to core-RNAP we generated recombinant protein using standard *E. coli* overexpression techniques, and the 6HIS-tagging vector pET24d (Novagen), as described previously [57]. Pulldown assays were then performed using the purified protein and *E. coli* core-RNAP (Epicentre). Recombinant SACOL1827 was coupled to Ni-NTA agarose beads (via the HIS tag), followed by the addition of core RNAP. Beads were then washed, resuspended in sample buffer and loaded onto a SDS-PAGE gel. As a control, this analysis was repeated in parallel omitting purified SACOL1827. We determined that in the absence of SACOL1827, core-RNAP was unable to bind the Ni-NTA beads, whilst in the presence of SACOL1827 core-RNAP copurified upon elution (Fig. 1A).

Another common feature of ECF-sigma factors is that they have a role in the autoregulation of their own expression. With this in

Table 1. Blast analysis for proteins homologous to SACOL1827 from *Staphylococcus aureus*.

Organism	Assignment	Identities	Positives
		No. of identical residues/total no. of aligned residues	No. of similar residues/total no. of aligned residues
B. subtilis	σ^M ECF σ factor	33/164 (20%)	73/164 (44%)
B. subtilis	σ^{YlaC} ECF σ factor	29/143 (20%)	69/143 (48%)
Idiomarina loihiensis	ECF σ factor	36/128 (28%)	69/128 (53%)
B. thetaiotaomicron	ECF σ factor	36/134 (26%)	72/134 (53%)
Pseudoalteromonas atlantica	ECF σ factor	35/121 (28%)	62/121 (51%)
B. cereus	σ^M ECF σ factor	31/108 (28%)	55/108 (50%)
V. parahaemolyticus	ECF σ factor	37/140 (26%)	70/140 (50%)
Oceanobacillus iheyensis	ECF σ factor	35/121 (28%)	59/121 (48%)
C. botulinum	BotR/A σ^{70} family	33/147 (22%)	78/147 (53%)

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mind we decided to test the ability of SACOL1827 to initiate transcription from its own locus by transcriptional run off analysis. Core-RNAP was preincubated with purified SACOL1827 protein for 15 mins at 4°C, before the addition of an 1168 bp DNA-fragment containing the *sigS* coding region and 945 bp of upstream sequence. After further incubation (at 37°C for 15mins) transcription was initiated by the addition of rNTPs, and allowed to proceed for 30 mins. The mixture was then cleaned via two acid-phenolchloroform extractions (to remove DNA contamination), and an isopropanol precipitation. The purified mRNA transcript was then subject to a 1-step RT-PCR reaction with primers internal to the SACOL1827 coding region (104 bp from the initiation codon to 137 bp from the termination codon). This experiment was repeated with controls, where either purified SACOL1827 protein or core-RNAP was omitted from the reaction mixture. The RT-PCR reactions were then resolved on a 2% agarose gel (Fig. 1B), and revealed that only the SACOL1827-core-RNAP complex lane yielded the expected DNA fragment of 274 bp. The 2 control lanes demonstrated an absence of bands, indicating that the acid-phenolchloroform extractions effectively removed the template DNA. As the SACOL1827-core-RNAP complex is capable of specifically initiating transcription, we termed the SACOL1827 gene *sigS*, and its resultant protein σ^S.

Analysis of a *sigS* mutant reveals a role for σ^S in the *S. aureus* stress response

A common role of ECF-sigma factors is to protect bacterial cells against external stress. In order to investigate if *sigS* has such a purpose in *S. aureus* we created a SH1000 *sigS*::*tet* insertionally inactivated mutant strain. Growth of the mutant was compared to the wild-type and found to be indistinguishable in TSB media under standard conditions (data not shown). However when long term survival experiments were conducted (11 days, aerobic growth, standard conditions) the *sigS* mutant showed a more pronounced decrease in viability than the parental strain (Fig. 2A).

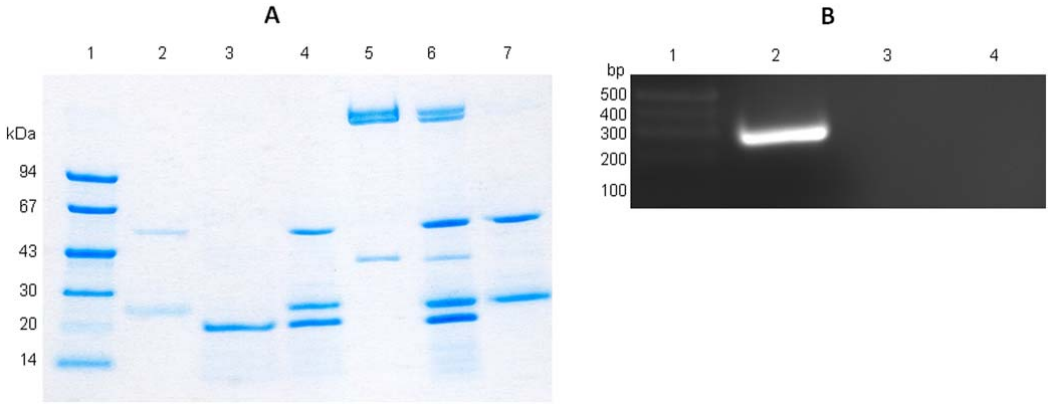

Figure 1. Biochemical characterization of the SACOL1827 protein. (A) Pulldown Assay showing association of SACOL1827 with core-RNAP. Lane order: L1, LMW Markers; L2, Monoclonal Anti-poly Histidine–Agarose antibody (with beads); L3, SACOL1827; L4, SACOL1827 (with beads); L5, core-RNAP; L6, SACOL1827 + core-RNAP (with beads); L7, core-RNAP (with beads); L8, HMW Markers. (B) Transcription run-off assay. Lane order: L1, DNA size markers; L2, transcription run-off conducted with core-RNAP + purified SACOL1827; L3, transcription run-off conducted with core-RNAP only; L4, transcription run-off conducted with purified SACOL1827 only.
doi:10.1371/journal.pone.0003844.g001

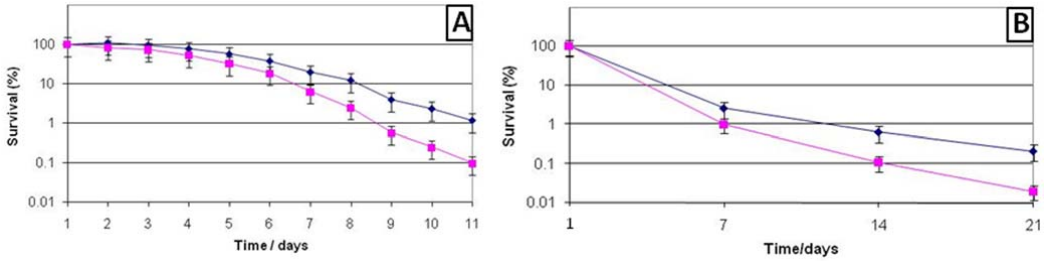

Figure 2. Long term survival of the *sigS* mutant. The SH1000 *sigS* (■) mutant, along with its parental strain (◆), were grown in TSB for 11 (A) or 21 (B) days. CFU/ml were determined at the specified intervals and are expressed as percentage survival.
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The *sigS* mutant strain lost viability at a consistently greater rate than that of the parental strain, an effect that became more pronounced as the experiment was prolonged. In order to assess the long term implications of this, the mutant and parental strain were subjected to starvation survival experiments over a period of 3 weeks (Fig. 2B). As with the 11 day experiment, the mutant strain had a decreased viability during long term starvation when compared to the parental strain.

ECF-sigma factors in a number of organisms have been shown to be important in the response to elevated temperature stress [38,44,58]. Therefore we tested the ability of the *sigS* mutant to grow at elevated temperatures (40°C and 45°C), and to survive heat shock (exponential cultures placed at 55°C for 15 mins before being returned to growth at standard conditions). In each case the *sigS* mutant strain responded to alterations in heat in a manner akin to that of the parental strain (data not shown). However, when we tested the viability of exponentially growing cultures subjected to growth at 55°C, it was found that over a 2 hour period the *sigS* mutant was more sensitive to killing by the elevated temperature (Fig 3A). Following this, further death curves were performed using the *sigS* mutant and its parental strain, in the presence of 0.4 mg ml^{-1} penicillin G, 50 µg ml^{-1} lysostaphin and 0.05% Triton X-100. In the case of lysostaphin and penicillin G no obvious difference was determined between the *sigS* mutant and its parental strain. However, when Triton X-100 was used as a lytic agent, the *sigS* mutant lysed at a quicker initial rate than that of the parental strain (Fig. 3B). This early variations in lysis was not borne out through the entire experimental time course, as the parent and mutant strain reached equivalent levels of survival after approximately 2 h.

Further to these experiments we set out to explore the role of *sigS* in *S. aureus* physiology by subjecting the mutant strain to growth analysis under a variety of different stress conditions. Disk diffusions assays were conducted with SH1000 and the *sigS* mutant in the presence of oxidative stress inducing compounds (30% H_2O_2, 80% cumene hydroperoxide, 500mM diamide, 2M methyl viologen, 1% menadione, 100mM plumbagin, 400 mg ml^{-1} pyrogallol), nitric oxide stress inducing compounds (100mM sodium nitroprusside), detergent stress (10% SDS, 10% Triton X-100), acid (12M HCl) and alkali stress (6M NaOH), alcohol stress (95% ethanol) and the antibiotics bacitracin (2 mg ml^{-1}), vancomycin (2 mg ml^{-1}), penicillin G (5 mg ml^{-1}) and puromycin (20 mg ml^{-1}). In each case no alteration in the zones of growth inhibition were observed (data not shown). The mutant and parental strain were tested further by growing them separately in liquid media containing 1 M and 2.5 M NaCl, 20 mM Glucose, and acidic and alkaline adjusted media (pH 5, with HCl; and pH 9, with NaOH). Again no alterations in growth were detected between the wild-type and mutant strain (data not shown).

Competitive growth analysis reveals the σ^S mutant has a decreased fitness for survival

Competitive growth experiments were undertaken to assess the viability of the SH1000 σ^S mutant when grown in coculture

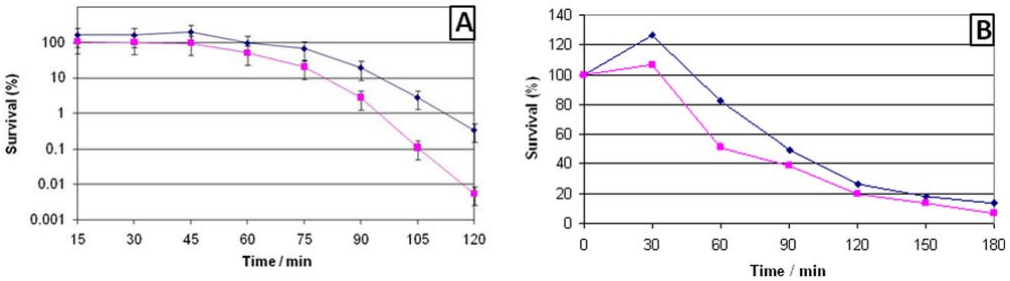

Figure 3. (A) Death curves of the *sigS* mutant and parental strain. (A), The effect of elevated temperature (55°C) on cellular viability. Exponentially growing SH1000 (◆) and the *sigS* mutant (■) were shifted from growth at 37°C to growth at 55°C, and viabilities were determine by CFU/ml at the time intervals specified. The standard deviation of five replicate cultures is shown in the form of error bars. (B) Triton X-100 induced lysis of the *sigS* mutant and its parental strain. SH1000 (◆) and the *sigS* mutant (■) were lysed using 0.05% Triton X-100 and the CFU/ml determined at the time intervals specified.
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experiments with its parental strain SH1000. These experiments are facilitated by the fact that the σ^S mutant is marked with a tetracycline resistance cassette; thus plating dilutions of the coculture on both TSA (Tryptic Soy Agar) and TSA containing tetracycline, allows derivation of exact colony counts for each strain, and thus calculation of the competitive index (CI). What was found was that SH1000 inoculated with the σ^S mutant in a 1:1 ratio resulted in a 1:0.28 ratio after 24 hours growth (Fig. 4). The mutant was even further impaired in its competitive abilities against the parental strain after 7 days of growth, resulting in a growth ratio of 1:0.04. As ECF-sigma factors commonly serve to protect the cell during times of stress we hypothesized that *sigS* mutant would show additional decline in coculture experiments with the parent when grown in the presence of sub-inhibitory concentrations of stress-inducing compounds. Indeed, whilst little variation from non-stressed conditions was observed after 24 hours growth, significant differences were observed after 7 days growth. When the experiments were repeated using the oxidative stress inducing chemicals hydrogen peroxide (1 mM) and diamide (1.5 mM) 7 day ratios were found to be 1:0.02 and 1:0.01, respectively. Additionally when the pH was altered in coculture flasks using HCl (10 mM) or NaOH (10 mM) further declines were seen, yielding 7 day ratios of 1:0.005 and 1:0.0006, respectively. Similarly coculture experiments using the metal ion chelator EDTA (0.1 mM) produced 7 day ratios of 1:0.003. Finally, and most dramatically, experiments using penicillin G (0.01 μg ml^{-1}) and ethanol (5%) yielded no detectable *sigS* mutant colonies after 7 days of growth with the parental strain.

Transcription profiling analysis of *sigS* expression

In order to determine the timing and levels of *sigS* expression in *S. aureus* we created a *lacZ* reporter-fusion strain of SH1000. We cloned a 1405 bp fragment into the suicide vector pAZ106, which bears a promoterless *lacZ* cassette. This 1405 bp fragment runs from 945 basepairs upstream to 354 basepairs downstream of the *sigS* initiation codon. The possibility of additional promoter elements being present in this fragment was excluded by analysis of the *sigS* locus, revealing that SACOL1826 is located 199 bp from the *sigS* initiation codon, and is transcribed in a divergent orientation. This plasmid was first introduced into RN4220 before being transferred to SH1000. Analysis of this strain on TSA containing X-Gal revealed no blue coloration, even after incubation of up to 1 week. We then grew the SH1000 *sigS-lacZ* strain in liquid media for 3 days, removing aliquots at 1 hour intervals in order to assay for specific *sigS* expression. We found that even after 3 full days of growth, we could determine no expression of *lacZ* from the *sigS* reporter strain (Fig 5; maximum miller units were 19 at 52 h). The construct and mutant were

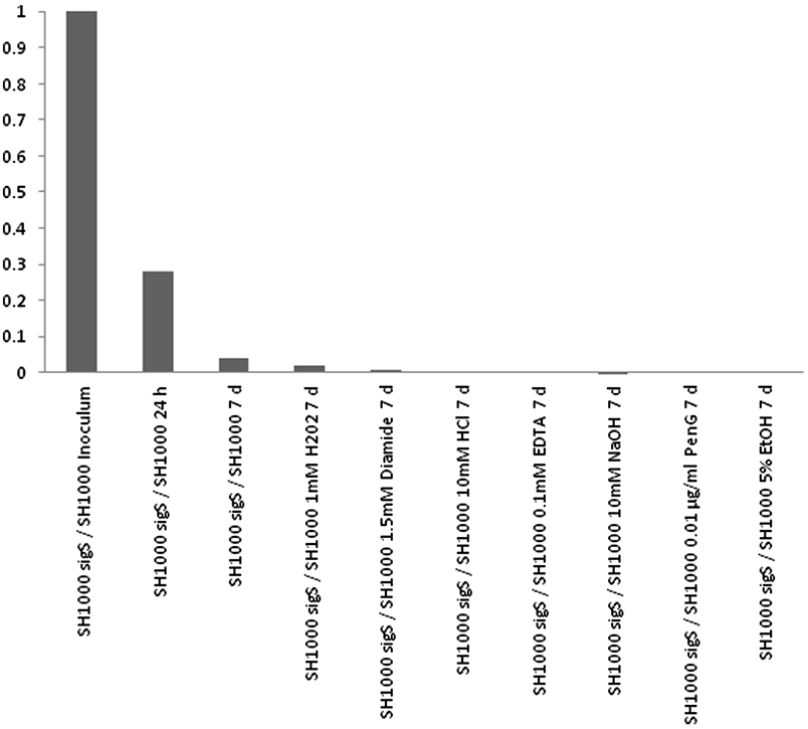

Figure 4. Competitive growth analysis of the *sigS* mutant and its parental strain. SH1000 and its *sigS* mutant derivative were cocultured in TSB or TSB containing subinhibitory concentrations of: hydrogen peroxide (1 mM), diamide (1.5 mM), HCl (10 mM), NaOH (10 mM), EDTA (0.1 mM), penicillin G (0.01 μg ml^{-1}) or ethanol (5%). The competitive index (CI) was determined for each strain after the respective growth periods and represents the relative proportion of the two strains after inoculation at a 1:1 ratio. Data is representative of at least 3 independent cultures.
doi:10.1371/journal.pone.0003844.g004

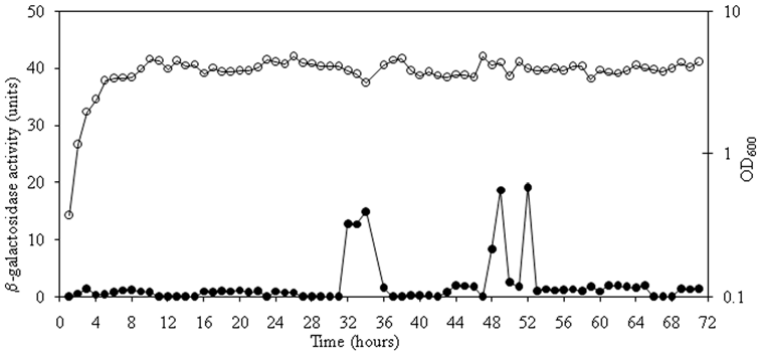

Figure 5. Expression analysis of *sigS* using a *lacZ* reporter-fusion strain. An SH1000 *sigS-lacZ* strain was grown for 72 hours, with samples withdrawn every hour to quantify the relative amount of *sigS* expression (•). The OD600 of the strain was also measured at each time point, and is shown (○).
doi:10.1371/journal.pone.0003844.g005

independently regenerated 2 additional times to ensure that no unwanted genetic rearrangements had occurred with the plasmid, or plasmid bearing strains; yet in each case no *sigS* expression, as determined by β-Galactosidase activity, was detectable.

Studies of ECF-sigma factors in other organisms have demonstrated the induction of ECF-sigma factor expression in response to stress inducing compounds. Specifically, in one such study by Cao et al [59], an elegant disc-diffusion reporter-gene fusion method was employed to define conditions conducive to the expression of σ^W in *B. subtilis*. Thus we employed a similar technique using our *sigS-lacZ* fusion strain. TSA plates were overlayed with TSB top agar (0.7% w/v) seeded with exponentially growing SH1000 *sigS-lacZ* cells, and containing 40 μg ml^{-1} X-GAL. Sterile filter discs were overlayed onto these plates (3 per plate), before being inoculated with 10 μl of the following stress inducing chemicals: 30% H_20_2, 80% cumene hydroperoxide, 500mM diamide, 2M methyl viologen, 1% menadione, 100mM plumbagin, 400 mg ml^{-1} pyrogallol, 100mM sodium nitroprusside, 10% SDS, 10% Triton X-100, 12M HCl, 6M NaOH, 95% ethanol, 2 mg ml^{-1} bacitracin, 2 mg ml^{-1} vancomycin, 5 mg ml^{-1} penicillin G and 20 mg ml^{-1} puromycin. Plates were incubated for 24 h at 37°C and screened for conditions conducive to σ^S expression as determined by a blue halo around the edge of the filter discs. Upon analysis we found that none of the chemicals tested resulted in the induction of σ^S expression, as determined by a lack of blue coloration on any of the test plates (data not shown).

Investigating the effect of SACOL1828 on *sigS* expression

As referred to above, ECF-sigma factors are often encoded upstream of an ORF that specifies an anti-sigma factor. Whilst SACOL1828 would be an unusual anti-sigma factor, as it lacks any obviously membrane associated domains, we decided to assess its role on *sigS* expression. As σ^S seems to have a role in autoinducing its own transcription, it follows that if SACOL1828 were to inhibit the activity of the σ^S protein, then a SACOL1828 mutant would have higher *sigS* expression, as a result of an increase in free σ^S protein. Thus we generated a SACOL1828::*tet* mutant in SH1000, before transducing it with the *sigS-lacZ* reporter-gene fusion. The presence of both mutation and reporter-fusion were confirmed by PCR analysis, and the strain was assayed for β-Galactosidase activity. Much like that seen with the SH1000 *sigS-lacZ* fusion alone, we found that the inactivation of SACOL1828 had no effect on *sigS* expression. Indeed no β-Galactosidase activity was detectable in this strain even after 1 week of growth on TSA containing X-GAL. Because of the close proximity of the integration sites for the *sigS-lacZ* and SACOL1828 mutation we regenerated this strain via an alternative manner. Electrocompetent RN4220 SACOL1828::*tet* cells were prepared, and used as recipients for electroporation with the *sigS-lacZ* construct. Clones were analyzed for the presence of both the mutation and reporter-fusion by PCR analysis, before 2 representative clones were used to generate phage lysate using ϕ11. These lysates was then used to transduce SH1000, with transductants selected for on the basis of the resistances of either the mutation (tetracycline) or the reporter-fusion (erythromycin). Clones were screened by PCR to confirm the efficient constransduction of each marker. Again as with the *sigS-lacZ* reporter-fusion strain, the regeneration of this strain did not result in detectable β-Galactosidase activity.

σ^S is required for the full virulence of *Staphylococcus aureus*

As the number of ECF-sigma factors identified grows, attention is turning to their often considerable roles in bacterial virulence [46]. Therefore we studied the impact of σ^S on the virulence of *S. aureus* infection in a murine model of septic arthritis. Mice were intravenously inoculated with either the parental strain (SH1000) or its *sigS* mutant derivative. In initial experiments using higher doses of bacteria, ranging from 4.5×10^6 to 8×10^6 bacteria per mouse, infection with the *sigS* mutant gave rise to significantly less mortality when compared to animals infected with SH1000 (Fig. 6A). Data from 3 pooled experiments showed that only 3 out of 30 mice infected with the *sigS* mutant died during the 14 day experimental period, compared with 10 out of 30 mice infected with SH1000 ($p<0.05$). In addition, mice infected with the *sigS* mutant lost significantly less weight than mice infected with SH1000. At day 5 post-inoculation, mice infected with the *sigS* mutant had lost on average only 4.4% (−13.3% to +2.2%, IQR) of their body weight, whereas SH1000 infected mice had a median weight loss of 10.4% (−20.2% to −5%, IQR) ($p<0.05$, Fig. 6B). At later time points the weight changes in surviving animals were similar in the two groups, probably due to the markedly higher mortality of mice infected with SH1000. The development of clinical arthritis was significantly less frequent in mice infected with

the *sigS* mutant, than in mice given the same dose of SH1000 (Fig. 6C). At 7 days post-inoculation with the *sigS* mutant only 2 out of 17 mice (12%) had clinically overt arthritis, as compared to 10 out of 17 mice (59%) infected with SH1000 ($p<0.05$). In addition, the severity of clinical arthritis at this time point was significantly reduced in the *sigS* mutant-infected mice, as compared to SH1000-infected mice ($p<0.05$, fig. 6D).

Fourteen days after inoculation all limbs from the mice inoculated with 3×10^6 to 4×10^6 bacteria per mouse were subjected to histopathological evaluation. As shown in figure 7A, infection with the *sigS* mutant induced much less erosion of bone and cartilage as compared to infection with the parental strain ($p<0.05$). In addition, infection with the *sigS* mutant also induced somewhat milder joint inflammation than SH1000 (Fig 7A), although these results were not found to be statistically significant. The systemic immune responses of mice infected with the *sigS* mutant and SH1000 were also compared by analyzing the levels of the proinflammatory cytokine interleukin (IL)-6 in serum 14 days post-inoculation. Mice infected with 3×10^6 bacteria of the *sigS* mutant had a median serum IL-6 concentration of 147 pg/ml (IQR 130–202 pg/ml; n = 10), which was markedly lower than the IL-6 concentration found in mice infected with SH1000, which had a median of 358 pg/ml (IQR 219–729 pg/ml; n = 10) ($p<0.001$, Fig 7B). Finally we investigated the ability of the strains to persist in host tissues, by determining the CFU/ml in kidney tissue homogenates. For this purpose, samples were taken from the kidneys 14 days after inoculation with 3×10^6–4×10^6 staphylococci per mouse. The *sigS* mutant clearly showed a reduced capacity to colonize host tissues, as it could not be detected in the kidneys of 6 out of 17 mice (35.3%). In contrast, growth of SH1000 was seen in the majority of infected animals, with only 2 out of 17 mice having negative kidney cultures (11.8%). The median number of staphylococci in the kidneys was 5×10^4 (IQR 0–3.4×10^7) bacteria after inoculation with the *sigS* mutant, as compared to 3.2×10^7 (IQR 2.5×10^5–1.3×10^8) after inoculation with SH1000. Similar results were obtained after inoculation with higher doses of bacteria (data not shown).

Discussion

S. aureus is a complex and versatile pathogen, which employs many different strategies in order to bring about its pathogenic response. It possess a diverse and wide-reaching network of regulatory elements that serve to fine-tune the coordinated expression of virulence determinants [13,15,20,23,24], so as to specifically bring about infection in a targeted manner. Additionally, there are a number of regulatory elements that contribute to the *S. aureus* virulence process, by controlling cellular physiology, and the adaptation to external conditions. The presumably facilitate both adaptation and proliferation in the harsh environment of the host [17,18,26,31]. Such loci, whilst not always directly controlling virulence determinant production, are no less important to the virulence process, as they facilitate the rapid physiological switching that is a hallmark of *S. aureus*. This kind of

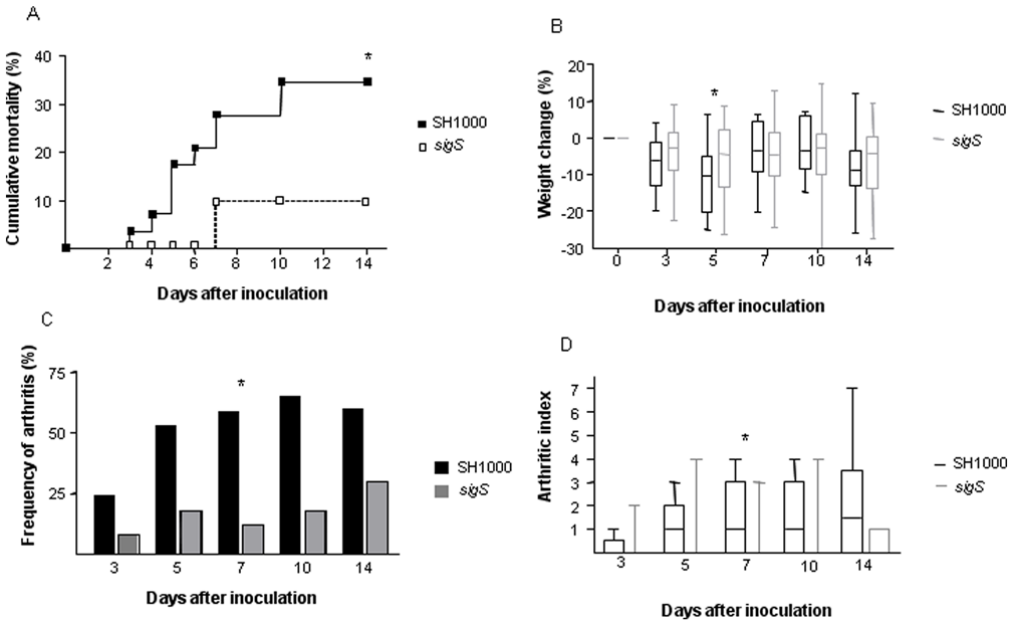

Figure 6. σ^S is required for the full virulence of *S. aureus* in a murine model of septic arthritis. (A), The cumulative mortality of mice (assessed by a log rank test, $p<0.05$). N = 30 per group. (B), Changes of body weight in the same mice as in A (*$p<0.05$ as compared using a Mann-Whitney U test.). (C), Frequency of clinical arthritis in mice inoculated with either wild-type *S. aureus* (SH1000) or its isogenic *sigS* mutant. The data from 2 separate experiments were pooled, n = 25 per group at day 3, n = 18 per group at days 5–10, and n = 10 per group at day 14. Statistical comparisons were performed using a chi-square test with Yates correction (*$p<0.05$). (D), Severity of clinical arthritis in the same mice as in C. Data is presented as medians (horizontal lines); inter-quartile ranges (bars) and ranges (error bars). An arthritic index was calculated by scoring all four limbs of each animal. Statistical comparisons were performed using a Mann-Whitney U test (*$p<0.05$).
doi:10.1371/journal.pone.0003844.g006

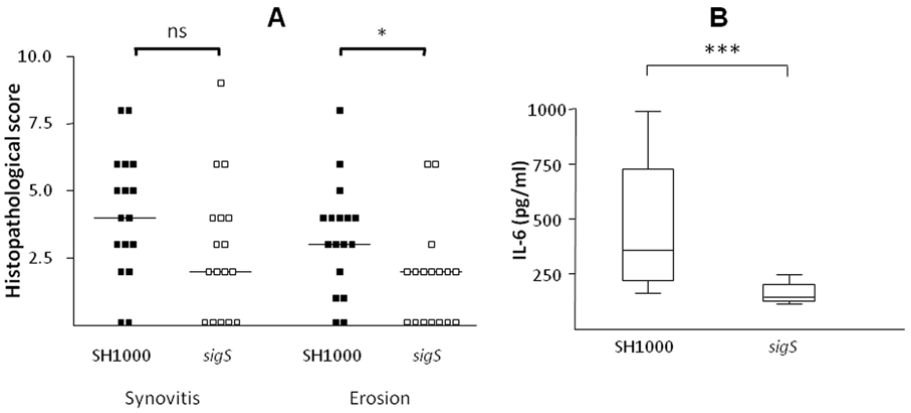

Figure 7. Analysis of the requirement for σ^S in *S. aureus* infection as measured via histopathological evaluation and mounted immune response analysis. (A), Histopathological evaluation of all limbs from mice 14 days post infection. The levels of synovitis and erosion (*$p<0.05$) were measured and mean scores are represented by vertical bars. (B), Serum IL-6 concentrations were determined for infected mice. All samples were run in triplicate. ***$p<0.001$.
doi:10.1371/journal.pone.0003844.g007

responsiveness is commonly induced in other organisms by sigma factors, as they present a rapid and direct way of modulating stimulons in response to change. Rather unusually, *S. aureus* seemingly achieves its versatile and adaptive nature with only a limited selection of sigma factors. So far only three have been documented [27,28,29], and only one of these (σ^B) has been shown to have a role in cellular adaptation and virulence [30,31,33]. The work presented in this current study demonstrates that an additional, and as yet uncharacterized, 4th sigma factor (σ^S) exists in *S. aureus*. σ^S appears to be a member of the ECF-family of sigma factors, and likely represents an important component of the stress and pathogenic responses of this organism.

Using biochemical approaches we have shown that σ^S is able to associates with core-RNAP, and initiate transcription from its own coding region. The autoregulation of ECF-sigma factor expression is a common hallmark of this family of regulators, and has been observed amongst a great many of their number [34]. Additionally, using a *sigS* mutant of *S. aureus*, we have shown that σ^S contributes to the protection against external stress, and plays a role in cellular fitness and survival. This is not unexpected, as the majority of ECF-sigma factors studied have been shown to function in the adaptation to stressful conditions [36–45]. In this study we present that σ^S is important for *S. aureus* cellular survival when faced with prolonged starvation, and extended exposure to elevated growth temperatures. Additionally a *sigS* mutant is seemingly less able to survive, at least initially, the attack on cell wall stability posed by Triton X-100. The observation of these phenotypes for σ^S is not out of keeping with other ECF-sigma factors, as a number are known to contribute to either heat shock responses and/or modulate cell wall stability [34].

On the other hand, using disc diffusion analysis, we were unable to find any increased sensitivity of the *sigS* mutant to a variety of chemical stresses, including those generating oxidative stress (H_20_2, cumene hydroperoxide, diamide, methyl viologen, menadione, plumbagin, pyrogallol), nitric oxide stress (sodium nitroprusside), detergent stress (SDS, Triton X-100), acid and alkali stress (HCl, NaOH), alcohol stress (ethanol) and antibiotic stress (vancomycin, penicillin G, puromycin). Whilst this may appear unusual, given that a number of ECF-sigma factors in other organisms respond to these conditions, it is not entirely inexplicable. ECF-sigma factors are selectively induced in response to the specific stress that they are intended to combat. Thus it is likely the case that in *S. aureus*, σ^S is not the primary arbiter of adaptation to the stresses listed above. This is particularly pertinent to oxidative and antibiotic stress, as *S. aureus* has a variety of mechanisms by which to circumnavigate and survive these threats [60–69]. Therefore it is probably that the efforts exerted in the present study have yet to hit upon the specific condition to which σ^S is required to respond. Indeed it possible, given the data generated by our animal studies, that the specific stress(es) σ^S responds to are not ones that can be simulated *in vitro*, but are uniquely associated with the *in vivo* lifestyle of *S. aureus*.

With that said, it is apparent that *sigS* does present some benefit to the cell during *in vitro* growth. In our coculture studies, where the parent and mutant strain were grown together under a variety of conditions, it was clear that σ^S was a significant aid to the survival and fitness of *S. aureus*. When the SH1000 *sigS* mutant was forced to compete with its parental strain, it displayed significantly reduced abilities for growth and survival. This phenotype was only exacerbated during prolonged growth periods (7 days), or in the presence of external stressor compounds. This would tend to suggest that *sigS* presents a selective advantage to *S. aureus* cells both during standard growth conditions, as well as during times of starvation and/or stress. Therefore it would seem logical that σ^S is a valuable component for maintaining cellular harmony and stability, and as such likely represents an important mechanism by which *S. aureus* protects itself against the harsh environments encountered during growth.

Our transcription profiling studies of *sigS* turned up some interesting information regarding its expression. It appears that during growth under standard conditions, *S. aureus* SH1000 cells do not initiate expression from the *sigS* locus. Our studies, which were sampled every hour for 3 days, consistently revealed expression in the typically background range of 0–1 Miller units. Only in 2 instances during growth did we detect anything higher than these values (32–36 h, and 48–52 h), and even then maximal expression was only 19 Miller units. We have generated a number of *lacZ* reporter-fusion strains in a variety of *S. aureus* backgrounds

[30,70–72] (unpublished data), and have never seen a strain that displays such limited expression under specific analysis. Even upon the analysis of apparently very lowly expressed genes (e.g. SH1000 *ssp-lacZ* fusion), which display little to no blueness on TSA X-gal plates, we routinely observe expression units in the hundreds [30]. With this in mind, and given the length of our transcription experiment, it is likely that even these 2 windows of minor expression may be the result of something other than actual induction of the *sigS* operon (e.g. cellular lysis). Therefore, as asserted above, this would tend to suggest that *sigS* is not expressed in SH1000 during growth under standard conditions.

This is certainly an unusual observation, but as ECF-sigma factors are commonly inducibley expressed in response to stress conditions, it perhaps not surprising. Indeed, analysis of the ECF-sigma factors of *B. subtilis* provides similar examples of transcriptional regulation. For example it has been reported that transcription of the ECF-sigma factor σ^Z from *B. subtilis* is undetectable during growth in rich and minimal media [73]. Further, specific analysis of *B. subtilis* ECF-sigma factor expression, conducted by Asai et al [74], revealed that the expression of σ^V, σ^Y and σ^{YlaC}, in addition to σ^Z, were all equally low, and barely detectable during growth under standard conditions. In a study aimed at defining conditions conducive to σ^W expression in *B. subtilis*, by Cao et al [59], an elegant disc-diffusion reporter-gene fusion method was employed. Cells bearing a *sigW-lacZ* fusion were grown on LB agar containing X-GAL, and overlayed with filter discs containing a variety of antibiotics. Using this approach, chemicals conducive to σ^W expression yielded a halo of blue around the edge of the filter disc. We employed just such an approach with our SH1000 *sigS-lacZ* fusion, using the chemicals previously tested in sensitivity assays with the SH1000 *sigS* mutant. Perhaps unsurprisingly, we found none of the chemicals tested resulted in an increase in σ^S expression. This would tend to add further weight to our assertion that in the present study have yet to hit upon the specific condition to which σ^S is induced in *S. aureus*.

Further transcriptional analysis focused on the role of SACOL1828 on σ^S expression. As referred to above, ECF-sigma factors are often encoded upstream of an ORF that specifies an anti-sigma factor. As σ^S seems to have a role in autoinducing its own transcription, it follows that if SACOL1828 were to inhibit the activity of the σ^S protein, then a mutation in SACOL1828 would have higher *sigS* expression as a result of more free and active σ^S protein. Indeed similar approaches have been used to analyze the putative anti-sigma factors of *B. subtilis* ECF-sigma factors, including σ^{YlaC} and σ^X [75,76]. Our analysis found that inactivating SACOL1828 did not result in an increase in *sigS* expression, as would have been predicted if SACOL1828 were to function as an anti-sigma factor. We suggest, however that this observation may be explained by the apparent lack of *sigS* expression in SH1000. If, as we find, there is little to no *sigS* expression in SH1000 during growth under standard conditions, then it follows that there is little to no σ^S protein present in the cell. Therefore the inactivation of a σ^S anti-sigma factor would not bring about the predicted snowballing of *sigS* expression, resulting from free σ^S protein being able to auto-stimulate its own transcription. Thus it appears that further investigation is required before we can specifically determine whether SACOL1828 plays any role in the regulation of σ^S activity.

The most striking, and indeed important, role we have defined for σ^S is its role in the virulence of *S. aureus*. Using our murine model of septic arthritis infection we have demonstrated that in each of the tests applied, to determine the extent and severity of disease, *S. aureus* cells lacking a functional *sigS* gene were significantly impaired in their ability to establish and maintain infection. Mice infected with *S. aureus* in this model lose weight, undergo extreme destruction of joints, bone and cartilage, and ultimately die. However those mice infected with the *sigS* mutant lost significantly less weight, developed septic arthritis at considerably lower levels, and most tellingly, had considerably increased survival rates. In addition, our studies of mounted immune responses by infected mice reveal that those animals infected with the *sigS* mutant had significantly lower levels of IL-6, indicating only a very weak immune response to the invading pathogens. Finally, a major hallmark of septic arthritis is systemic dissemination, moving from the site of infection into the kidneys. Our analysis reveals that mice infected with the parental strain possessed large numbers of *S. aureus* cells in the kidneys of infected mice. However when the same analysis was conducted with the *sigS* mutant it was apparent that strains of *S. aureus* lacking a functional *sigS* gene were far less able to undergo systemic dissemination. Collectively, the virulence data that we present speaks very strongly to the importance of σ^S in the ability of *S. aureus* to cause disease, a fundamental cornerstone of its innate behavior.

From our investigations presented here we have demonstrated that σ^S is important for the *S. aureus* stress response, aiding in the protection against unfavorable conditions. In addition we have shown that it is vital for the infectious nature of *S. aureus*, as a *sigS* mutant is attenuated in virulence in a murine model of septic arthritis infection. However the specific and mechanistic role of σ^S in *S. aureus* biology remains unknown. It is unlikely; thought not impossible, that σ^S wields its role via direct regulation of virulence determinant expression. A more probable scenario is that σ^S, as with other ECF-sigma factors, is responsible for sensing and responding to discrete external cue(s); and changing *S. aureus* gene expression profiles so as to protect the cell. It is the current and future purpose of our laboratory to explore and develop an understanding of the role of σ^S, which will doubtlessly further our knowledge of this important human pathogen and its disease causing abilities.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The *S. aureus* and *E. coli* strains, along with the plasmids used in this study are listed in Table 2. *E. coli* was grown in Luria-Bertani (LB) medium at 37°C. *S. aureus* was grown in 100 ml TSB (1:2.5 flask/volume ratio) at 37°C with shaking at 250 rpm, unless otherwise indicated. For growth analysis experiments, overnight cultures were inoculated into fresh media to an OD_{600} of 1.0 and allowed to grow for 3 hours. These cultures were then in turn used to inoculate fresh TSB to an OD_{600} of 0.01, and these were used as test cultures. CFU/ml counts were determined by the serial dilution of test-cultures onto TSA, followed by enumeration after overnight growth. All CFU/ml values represent the mean from three independent experiments. When required antibiotics were added at the following concentrations: ampicillin 100 $\mu g\ ml^{-1}$ and tetracycline 12.5 $\mu g\ ml^{-1}$ (*E. coli*); tetracycline 5 $\mu g\ ml^{-1}$, erythromycin 5 $\mu g\ ml^{-1}$and lincomycin 25 $\mu g\ ml^{-1}$ (*S. aureus*). Where appropriate, X-GAL was added to media at a concentration of 40 $\mu g\ ml^{-1}$.

Overexpression and Purification of σ^S

The 470bp *sigS* coding region was PCR generated using primer pair OL-389/OL-390 and cloned into the *E. coli* overexpression vector pET24d (Novagen) to create pLES200. The plasmid was subjected to DNA sequence analysis (UGA core facility) to ensure that the coding region was generated without mistake. This

Table 2. Strains, plasmids and primers used in this study. Where applicable restriction sites are underlined.

Strain, Plasmid or Primer	Genotype or Description	Reference/Source
E. coli		
DH5α	ϕ80 Δ((*lacZ*)*M15* Δ((*argF-lac*)*U169 endA1 recA1 hsdR*17 ($r_K^-m_K^+$) *deoR thi*-1 *supE*44 *gyrA*96 *relA*1	78
Tuner	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm lacY1*(DE3) pLysS (Cam^R)	Novagen
S. aureus		
RN4220	Restriction deficient transformation recipient	Lab Stocks
SH1000	Functional *rsbU* derivative of 8325-4 $rsbU^+$	30
LES55	SH1000 *sigS*::*tet* $sigS^-$	This Study
LES56	SH1000 SACOL1828::*tet* $SACOL1828^-$	This Study
LES57	SH1000 pAZ106::*sigS-lacZ* $sigS^+$	This Study
LES58	RN4220 SACOL1828::*tet* $SACOL1828^-$	This Study
LES59	RN4220 SACOL1828::*tet* pAZ106::*sigS-lacZ* $sigS^+$ $SACOL1828^-$	This Study
Plasmids		
pAZ106	Promoterless *lacZ erm* insertion vector	77
pET24d	6His-tag overexpression vector	Novagen
pLES200	pET24d containing a 470bp *sigS* fragment	This Study
pLES201	pAZ106 containing a 2.3kb *sigS* fragment	This Study
pLES202	pAZ106 containing a 2.2kb SACOL1828 fragment	This Study
pLES203	pLES201 containing a tetracycline cassette within *sigS*	This Study
pLES204	pLES202 containing a tetracycline cassette within SACOL1828	This Study
pLES205	pAZ106 containing a 1.4kb *sigS* fragment	This Study
Primers		
OL-281	ACTGGATCCCAGTTGCAGATGCATCTCTCC	
OL-282	AGCTAGGCATGCCAAGTCTATCTGGCGTAC	
OL-285	ACTGGATCCGACCATCACGATACATCA	
OL-286	CTTCACTGACAACTATGCCG	
OL-287	GCGATTACATTCTAGAAGTTCC	
OL-288	GGAACTTCTAGAATGTAATCGC	
OL-293	ATGGAATTCGTTTGAGCCATAGTCTTTCTC	
OL-297	ATGGAATTCCTAATTAAAATTATGTTGGCATTTA	
OL-387	GATGAGTATTATCAACTACTCTTG	
OL-389	ATGACCATGGTGAAATTTAATGACGTATAC	
OL-390	ATGACTCGAGATTAAAATTATGTTGGATTTTACGC	
OL-429	TATCAACTACTCTAGATAAAAATGTGGC	
OL-430	GCCACATTTTTATCTAGAGTAGTTGATA	
OL-522	ATGTCTAGAGAGTAATGCTAACATAGC	
OL-523	ATGTCTAGACCCAAAGTTGATCCCTTAACG	

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plasmid was purified from *E. coli* DH5α and transferred to the *E. coli* expression host Tuner (Novagen). Cells were grown at 37°C (in LB supplemented with 34 mg/l chloramphenicol and 30 mg/l kanamycin) before the induction of protein expression with 100 mM IPTG at an OD_{600} of 0.5. The culture temperature was then reduced to 30°C and growth was permitted for a further 4–5 h with vigorous agitation. Cells were harvested by centrifugation (10 min, 4,500 g), resuspended (Buffer A: 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 50 mM imidazole) and disrupted by sonication. Soluble protein fractions, collected by centrifugation (30 min, 14,000g, 4°C), were applied to a Chelating Sepharose (Amersham) Ni^{2+} affinity column (1.5cm×1.6cm). To ensure saturated binding of the recombinant σ^S to the matrix, samples were circulated through the column for 2.5 h using the Akta Explorer system (Amersham), and then washed extensively with Buffer A until the OD_{280} of the eluate dropped to a baseline reading. Recombinant σ^S was eluted from the column in a stepwise manner with buffer A containing imidazole at 140, 320 and 500 mM concentrations. Fractions eluted at 320 mM were pooled and lyophilized in order to concentrate the purified recombinant protein. This was then resuspended in water, desalted (HiTrap desalting column, Amersham) by buffer exchange (20 mM Tris-HCl pH 8.0, 20 mM NaCl) and re-lyophilized. Protein purity was assayed by SDS-PAGE, yielding a

single band with a molecular mass of 19 kDa. The presence of the 6His-Tag in recombinant σ^S was confirmed by Western Blot with anti-HisTag antibodies (Roche).

σ^S-Core RNAP Association Experiments

200 µl of anti-His-tag antibodies conjugated to agarose beads (Sigma) were washed thoroughly (20 mM Tris-HCl pH 8.0, 10 mM NaCl) and incubated with 250 µl of 0.2 mg/ml recombinant σ^S at room temperature for 3 h. The resin was washed thoroughly with 20 mM Tris-HCl pH 8.0, 10 mM NaCl and TBS-Tween, before adding 20 µl of core-RNAP at 1U μl^{-1} (Epicentre). Samples were then incubated for 2(h at room temperature followed by extensive washing. After adding SDS-PAGE sample buffer, samples were boiled and centrifuged (10(min, 16 000(g), and the supernatant subjected to SDS-PAGE.

Transcription Run-Off Experiments

0.25 µg of core-RNAP (Epicentre) was preincubated with 1 µg of σ^S in transcription buffer [30mM Tris-HCl (pH 8.0), 10mM MgCl2, 100mM KCl, 1mM DTT], at 4°C for 15mins. After this time, 1 µg of a 1168 bp DNA-fragment (PCR generated using primer OL-281/OL-297), containing the *sigS* coding region and 945 bp of upstream sequence, was added to the σ^S-core-RNAP complex, and further incubated at 37°C for 15mins. Transcription was initiated by the addition of 2.5 µM rNTPs, and transcription was allowed to proceed for 30 mins at 37°C. After this time the mixture was cleaned via 2 acid-phenolchloroform extractions (to remove DNA contamination), followed by isopropanol precipitation. The purified mRNA transcript was then subjected to a 1-step RT-PCR reaction using primer pair OL-387/OL-293 (104 bp from the initiation codon to 137bp from the termination codon, with a target fragment size of 274bp) and the Superscript III enzyme (Invitrogen). This experiment was repeated, omitting either purified σ^S or core-RNAP as controls. RT-PCR reactions were resolved on a 2% agarose gel and visualized using a BioDocIt Device (UVP).

Construction of the *sigS* and SACOL1828 mutant strains

A plasmid for the mutagenesis of *sigS* was constructed by PCR amplification. Two approximately 1kb fragments were PCR generated surrounding the *sigS* coding region (1 located upstream, primer pairs OL-281/OL-430; and 1 located downstream, primer pairs OL-282/OL-429). Primers OL-429 and OL-430 are identical, but divergent to each other, and each contain base pair mismatching, converting the wild type sequence of TCAAGC (~100bp from the *sigS* Met) to TCTAGA, an XbaI restriction recognition site. These fragments were purified and used together as the template for a further round of PCR with primer pair OL-281/OL-282. The resultant 2.3 kb DNA fragment was digested with BamHI and SphI, and cloned into the suicide vector pAZ106 [77] to generate pLES201, using standard cloning techniques [78].

A plasmid for the mutagenesis of SACOL1828 was constructed in a similar manner with the following exceptions. The two approximately 1kb fragments were generated using primer pairs OL-285/OL-288, and OL-286/OL-287. Primers OL-287 and OL-288 are identical, but divergent to each other, and contain mismatching that converts the wild type sequence of TCTTAA (~100bp from the SACOL1828 Met) to a TCTAGA XbaI site. These fragments were used as the template for further PCR using primer pair OL-285/OL-286. This 2.2 kb DNA fragment was digested with BamHI and SphI and cloned into pAZ106 to generate pLES202 .

The novel XbaI sites in pLES201 and pLES202 were then used as a target sites for the insertion of a tetracycline resistance cassette, generated from pDG1515 [79] using primer pair OL-522/OL-523. The XbaI digested cassette was cloned into pLES201 and pLES202, yielding pLES203 (*sigS*) and pLES204 (SACOL1828). These were then used to transform electrocompetent *S. aureus* RN4220, according to the method of Schenk and Ladagga [80], with clones selected for on the basis of tetracycline and erythromycin resistance. Integrants were confirmed by PCR analysis (data not shown) and used as donors for the transduction of *S. aureus* strain SH1000 using phage ϕ11. Transductants were selected for their resistance to tetracycline (indicating the presence of the cassette) and sensitivity to erythromycin (indicating loss of the plasmid and associated functional copy of *sigS* or SACOL1828), before being confirmed by PCR analysis. This created strains LES55 (*sigS*) and LES56 (SACOL1828).

Construction of a *sigS-lacZ* reporter-fusion strain

The putative promoter region of *sigS* was amplified as a 1.4 kb PCR fragment using primer pair OL-281/OL-293 (Table 2). The purified DNA fragment was digested with BamH1 and EcoRI and cloned into similarly digested pAZ106. *S. aureus* RN4220 was transformed with the resulting plasmid, pLES205, and integrants were confirmed by PCR analysis. A representative clone was then used to transduce *S. aureus* SH1000 using ϕ11, with clones again confirmed by PCR analysis. This created strain LES57 (*sigS-lacZ*).

β-Galactosidase assays

Levels of β-Galactosidase activity were measured as described previously [71]. Fluorescence was measured using a Bio-Tek Synergy II plate reader, with a 0.1 sec count time, and calibrated with standard concentrations of MU (4-methyl umbelliferone). One unit of β-Galactosidase activity was defined as the amount of enzyme that catalyzed the production of 1 pmol MU min^{-1} OD_{600} $unit^{-1}$. Assays were performed on duplicate samples and the values averaged. The results presented here were representative of three independent experiments that showed less than 10% variability.

Disc-Diffusion Assays

Disk diffusion sensitivity assays were performed as follows: 5 ml of TSB top agar (0.7%, wt/vol) was seeded with 5 µl of exponentially growing strains of *S. aureus*, and used to overlay TSA plates. Sterile filter disks were placed in the centre of the overlayed plates, and 10 µl of the test chemicals was applied at the following concentrations: 30% H_20_2, 80% cumene hydroperoxide, 500mM diamide, 2M methyl viologen, 1% menadione, 100mM plumbagin, 400 mg ml^{-1} pyrogallol, 100mM sodium nitroprusside, 10% SDS, 10% Triton X-100, 12M HCl, 6M NaOH, 95% ethanol, 2 mg ml^{-1} bacitracin, 2 mg ml^{-1} vancomycin, 5 mg ml^{-1} penicillin G and 20 mg ml^{-1} puromycin. This technique was also adapted for transcription profiling using the SH1000 *sigS-lacZ* strain. In this situation 5 µl of exponentially growing *sigS-lacZ* cells was seeded into 5 ml of TSB top agar (0.7%, wt/vol) containing X-GAL (40 µg ml^{-1}). This was then used to overlay TSA plates before sterile filter discs (3 per plate) were placed on top of the agar overlay. Filter discs were then seeded with 10 µl of the same stress inducing chemicals listed above.

Cell Wall Lysis Experiments

Lysis kinetics using lysostaphin and Triton X-100 were performed as described previously [81]. Penicillin G lysis was performed as described by Fujimoto & Bayles [82].

Coculture experiments

SH1000 and the SH1000 *sigS* mutant were grown in competitive culture experiments as described previously by

Doherty et al [83]. Briefly, both strains were grown separately for 18h in TSB under standard conditions. Cells were harvested by centrifugation, washed with PBS and used to inoculate fresh TSB with an inoculation ratio of 1:1. These ratios were confirmed by retrospective viable counts of the starting inoculum in triplicate. Cultures were incubated at 37°C for the times specified and viable counts were again determined in triplicate. These experiments are facilitated by the tetracycline resistance cassette used to mark the *sigS* mutant. Therefore plating dilutions of the coculture on both TSA, and TSA containing tetracycline, allows derivation of exact colony counts for each strain, and thus calculation of the competitive index (CI).

Experimental models of *S. aureus* sepsis and arthritis

Female NRMI mice, 6 to 8 weeks old, were purchased from B & K Universal AB (Sollentuna, Sweden) and kept in the animal facility of the Department of Rheumatology and Inflammation Research, Göteborg University. *S. aureus* strain SH1000 and its isogenic *sigS* mutant were cultured on horse-blood agar plates at 37°C for 24 hours, harvested, washed in PBS and resuspended in PBS supplemented with 10% dimethyl sulfoxide and 5% bovine serum albumin. Aliquots of bacterial suspensions with a known CFU/ml, as determined by viable counts, were stored at −20°C. Before inoculation, bacterial cultures were thawed, washed once with PBS and diluted in PBS to the desired concentration. In five independent experiments mice were inoculated intravenously with 200 µl of bacterial suspension in declining bacterial doses (8×10^6, 6×10^6, 4.5×10^6, 4×10^6, and 3×10^6 CFU/mouse). Viable counts of the inoculum were performed in each experiment to confirm the accuracy of each dose. Mice were individually monitored for up to 14 days by an observer (CL) blinded to the identity of the experimental groups for general appearance, weight change, mortality, and the development of arthritis, before being sacrificed. Clinical arthritis, defined by visible erythema and/or swelling of at least one joint, was scored from 0 to 3 for each limb (1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema). An arthritic index was generated by adding the scores for each limb of a given animal. Histopathological evaluations of the limbs were performed after routine paraformaldehyde fixation, decalcification, paraffin embedding, and hematoxylin and eosin staining. Tissue sections were evaluated for synovitis and joint destruction by an observer (CL) blinded to the identity of the groups. Synovitis and cartilage/bone destruction were scored separately as 0, none; 1, mild; 2, moderate; and 3, for severe synovial hypertrophy and joint damage. The sum of all of the limbs was used to calculate a histopathology score.

Bacterial persistence in host tissues was evaluated by aseptically removing the kidneys, homogenizing them and performing viable counts after serial dilution in PBS. The CFU/ml were determined after 24 hours of cultivation on horse blood agar plates. Serum IL-6 concentrations were determined as previously described, using a bioassay in which the murine hybridoma cell line B9 is dependent on IL-6 for growth, [84]. All samples were run in triplicate, and the statistical evaluations of weight change and severity of clinical and histopathological arthritis between groups was performed using a Mann-Whitney U test. A chi-square test was used for comparison of frequency of clinical arthritis between groups, whilst the comparison of mortality was done by a log rank test. A p-value <0.05 (after Bonferroni correction for multiple comparisons) was deemed to indicate statistically significant differences.

Acknowledgments

Dedication: This paper is dedicated to the memory of Dr. Andrej Tarkowski who sadly passed away on Sunday 1st June, 2008.

Author Contributions

Conceived and designed the experiments: LNS JP. Performed the experiments: LNS CL TKP HKM MCB EG. Analyzed the data: LNS CL EG AT JP. Contributed reagents/materials/analysis tools: LNS GCS AT JP. Wrote the paper: LNS CL.

References

1. Daum RS (2007) Clinical practice. Skin and soft-tissue infections caused by methicillin-resistant *Staphylococcus aureus*. N Engl J Med 357: 380–390.
2. de Lencastre H, Oliveira D, Tomasz A (2007) Antibiotic resistant *Staphylococcus aureus*: a paradigm of adaptive power. Curr Opin Microbiol 10: 428–435.
3. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, et al. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. Jama 298: 1763–1771.
4. Mwangi MM, Wu SW, Zhou Y, Sieradzki K, de Lencastre H, et al. (2007) Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. Proc Natl Acad Sci U S A 104: 9451–9456.
5. (2002) *Staphylococcus aureus* resistant to vancomycin–United States, 2002. MMWR Morb Mortal Wkly Rep 51: 565–567.
6. (2002) Vancomycin-resistant *Staphylococcus aureus*–Pennsylvania, 2002. MMWR Morb Mortal Wkly Rep 51: 902.
7. Tiwari HK, Sen MR (2006) Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. BMC Infect Dis 6: 156.
8. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, et al. (2003) Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science 302: 1569–1571.
9. Weigel LM, Donlan RM, Shin DH, Jensen B, Clark NC, et al. (2007) High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. Antimicrob Agents Chemother 51: 231–238.
10. Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339: 520–532.
11. Novick RP (2006) Staphylococcal Pathogenesis and Pathogenicity Factors: Genetics and regulation. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, eds. Gram-positive pathogens. Washington, D.C.: ASM Press. pp 496–516.
12. Meehl M, Herbert S, Gotz F, Cheung A (2007) Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 51: 2679–2689.
13. Giraudo AT, Raspanti CG, Calzolari A, Nagel R (1994) Characterization of a Tn551-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. Can J Microbiol 40: 677–681.
14. Brunskill EW, Bayles KW (1996) Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*. J Bacteriol 178: 611–618.
15. Fournier B, Klier A, Rapoport G (2001) The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol Microbiol 41: 247–261.
16. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, et al. (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol Microbiol 49: 807–821.
17. Torres VJ, Stauff DL, Pishchany G, Bezbradica JS, Gordy LE, et al. (2007) A *Staphylococcus aureus* regulatory system that responds to host heme and modulates virulence. Cell Host Microbe 1: 109–119.
18. Yarwood JM, McCormick JK, Schlievert PM (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. J Bacteriol 183: 1113–1123.
19. Janzon L, Lofdahl S, Arvidson S (1989) Identification and nucleotide sequence of the delta-lysin gene, hld, adjacent to the accessory gene regulator (agr) of *Staphylococcus aureus*. Mol Gen Genet 219: 480–485.
20. Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, et al. (1995) The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Gen Genet 248: 446–458.
21. Peng HL, Novick RP, Kreiswirth B, Kornblum J, Schlievert P (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in *Staphylococcus aureus*. J Bacteriol 170: 4365–4372.
22. Cheung AL, Zhang G (2002) Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. Front Biosci 7: d1825–1842.
23. Cheung AL, Koomey JM, Butler CA, Projan SJ, Fischetti VA (1992) Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (sar) distinct from agr. Proc Natl Acad Sci U S A 89: 6462–6466.

24. McNamara PJ, Milligan-Monroe KC, Khalili S, Proctor RA (2000) Identification, cloning, and initial characterization of rot, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J Bacteriol 182: 3197–3203.
25. Schmidt KA, Manna AC, Gill S, Cheung AL (2001) SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. Infect Immun 69: 4749–4758.
26. Horsburgh MJ, Ingham E, Foster SJ (2001) In *Staphylococcus aureus*, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. J Bacteriol 183: 468–475.
27. Deora R, Misra TK (1995) Purification and characterization of DNA dependent RNA polymerase from *Staphylococcus aureus*. Biochem Biophys Res Commun 208: 610–616.
28. Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, et al. (2003) A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells 8: 699–712.
29. Wu S, de Lencastre H, Tomasz A (1996) Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J Bacteriol 178: 6036–6042.
30. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, et al. (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. J Bacteriol 184: 5457–5467.
31. Kullik I, Giachino P, Fuchs T (1998) Deletion of the alternative sigma factor sigmaB in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J Bacteriol 180: 4814–4820.
32. Rachid S, Ohlsen K, Wallner U, Hacker J, Hecker M, et al. (2000) Alternative transcription factor sigma(B) is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. J Bacteriol 182: 6824–6826.
33. Jonsson IM, Arvidson S, Foster S, Tarkowski A (2004) Sigma factor B and RsbU are required for virulence in *Staphylococcus aureus*-induced arthritis and sepsis. Infect Immun 72: 6106–6111.
34. Helmann JD (2002) The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol 46: 47–110.
35. Paget MS, Helmann JD (2003) The sigma70 family of sigma factors. Genome Biol 4: 203.
36. Chi E, Bartlett DH (1995) An rpoE-like locus controls outer membrane protein synthesis and growth at cold temperatures and high pressures in the deep-sea bacterium *Photobacterium* sp. strain SS9. Mol Microbiol 17: 713–726.
37. Dartigalongue C, Missiakas D, Raina S (2001) Characterization of the *Escherichia coli* sigma E regulon. J Biol Chem 276: 20866–20875.
38. Fernandes ND, Wu QL, Kong D, Puyang X, Garg S, et al. (1999) A mycobacterial extracytoplasmic sigma factor involved in survival following heat shock and oxidative stress. J Bacteriol 181: 4266–4274.
39. Horsburgh MJ, Moir A (1999) Sigma M, an ECF RNA polymerase sigma factor of *Bacillus subtilis* 168, is essential for growth and survival in high concentrations of salt. Mol Microbiol 32: 41–50.
40. Keith LM, Bender CL (1999) AlgT (sigma22) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. J Bacteriol 181: 7176–7184.
41. Turner MS, Helmann JD (2000) Mutations in multidrug efflux homologs, sugar isomerases, and antimicrobial biosynthesis genes differentially elevate activity of the sigma(X) and sigma(W) factors in *Bacillus subtilis*. J Bacteriol 182: 5202–5210.
42. Visca P, Leoni L, Wilson MJ, Lamont IL (2002) Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. Mol Microbiol 45: 1177–1190.
43. Wei ZM, Beer SV (1995) hrpL activates Erwinia amylovora hrp gene transcription and is a member of the ECF subfamily of sigma factors. J Bacteriol 177: 6201–6210.
44. Wu QL, Kong D, Lam K, Husson RN (1997) A mycobacterial extracytoplasmic function sigma factor involved in survival following stress. J Bacteriol 179: 2922–2929.
45. Xiao Y, Heu S, Yi J, Lu Y, Hutcheson SW (1994) Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 hrp and hrmA genes. J Bacteriol 176: 1025–1036.
46. Bashyam MD, Hasnain SE (2004) The extracytoplasmic function sigma factors: role in bacterial pathogenesis. Infect Genet Evol 4: 301–308.
47. Cezairliyan BO, Sauer RT (2007) Inhibition of regulated proteolysis by RseB. Proc Natl Acad Sci U S A 104: 3771–3776.
48. Grigorova IL, Chaba R, Zhong HJ, Alba BM, Rhodius V, et al. (2004) Fine-tuning of the *Escherichia coli* sigmaE envelope stress response relies on multiple mechanisms to inhibit signal-independent proteolysis of the transmembrane anti-sigma factor, RseA. Genes Dev 18: 2686–2697.
49. Heinrich J, Wiegert T (2006) YpdC determines site-1 degradation in regulated intramembrane proteolysis of the RsiW anti-sigma factor of *Bacillus subtilis*. Mol Microbiol 62: 566–579.
50. Koide K, Maegawa S, Ito K, Akiyama Y (2007) Environment of the active site region of RseP, an *Escherichia coli* regulated intramembrane proteolysis protease, assessed by site-directed cysteine alkylation. J Biol Chem 282: 4553–4560.
51. Makinoshima H, Glickman MS (2006) Site-2 proteases in prokaryotes: regulated intramembrane proteolysis expands to microbial pathogenesis. Microbes Infect 8: 1882–1888.
52. Qiu D, Eisinger VM, Rowen DW, Yu HD (2007) Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 104: 8107–8112.
53. Schobel S, Zellmeier S, Schumann W, Wiegert T (2004) The *Bacillus subtilis* sigmaW anti-sigma factor RsiW is degraded by intramembrane proteolysis through YluC. Mol Microbiol 52: 1091–1105.
54. Bae JB, Park JH, Hahn MY, Kim MS, Roe JH (2004) Redox-dependent changes in RsrA, an anti-sigma factor in Streptomyces coelicolor: zinc release and disulfide bond formation. J Mol Biol 335: 425–435.
55. Paget MS, Bae JB, Hahn MY, Li W, Kleanthous C, et al. (2001) Mutational analysis of RsrA, a zinc-binding anti-sigma factor with a thiol-disulphide redox switch. Mol Microbiol 39: 1036–1047.
56. Song T, Dove SL, Lee KH, Husson RN (2003) RshA, an anti-sigma factor that regulates the activity of the mycobacterial stress response sigma factor SigH. Mol Microbiol 50: 949–959.
57. Moon JL, Shaw LN, Mayo JA, Potempa J, Travis J (2006) Isolation and properties of extracellular proteinases of *Penicillium marneffei*. Biol Chem 387: 985–993.
58. Resto-Ruiz SI, Sweger D, Widen RH, Valkov N, Anderson BE (2000) Transcriptional activation of the htrA (High-temperature requirement A) gene from *Bartonella henselae*. Infect Immun 68: 5970–5978.
59. Cao M, Wang T, Ye R, Helmann JD (2002) Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* sigma(W) and sigma(M) regulons. Molecular microbiology 45(5): 1267–1276.
60. Clauditz A, Resch A, Wieland KP, Peschel A, Gotz F (2006) Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. Infection and immunity 74(8): 4950–4953.
61. Cosgrove K, Coutts G, Jonsson IM, Tarkowski A, Kokai-Kun JF, et al. (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. Journal of bacteriology 189(3): 1025–1035.
62. Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infection and immunity 69(6): 3744–3754.
63. Horsburgh MJ, Wharton SJ, Cox AG, Ingham E, Peacock S, et al. (2002) MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. Molecular microbiology 44(5): 1269–1286.
64. Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, et al. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. The Journal of experimental medicine 202(2): 209–215.
65. Frees D, Qazi SN, Hill PJ, Ingmer H (2003) Alternative roles of ClpX and ClpP in *Staphylococcus aureus* stress tolerance and virulence. Molecular microbiology 48(6): 1565–1578.
66. Singh VK, Utaida S, Jackson LS, Jayaswal RK, Wilkinson BJ, et al. (2007) Role for dnaK locus in tolerance of multiple stresses in *Staphylococcus aureus*. Microbiology (Reading, England) 153(Pt 9): 3162–3173.
67. Gardete S, Wu SW, Gill S, Tomasz A (2006) Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. Antimicrobial agents and chemotherapy 50(10): 3424–3434.
68. McAleese F, Wu SW, Sieradzki K, Dunman P, Murphy E, et al. (2006) Overexpression of genes of the cell wall stimulon in clinical isolates of *Staphylococcus aureus* exhibiting vancomycin-intermediate- *S. aureus*-type resistance to vancomycin. Journal of bacteriology 188(3): 1120–1133.
69. Utaida S, Dunman PM, Macapagal D, Murphy E, Projan SJ, et al. (2003) Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. Microbiology (Reading, England) 149(Pt 10): 2719–2732.
70. Shaw L, Golonka E, Potempa J, Foster SJ (2004) The role and regulation of the extracellular proteases of *Staphylococcus aureus*. Microbiology (Reading, England) 150(Pt 1): 217–228.
71. Shaw LN, Aish J, Davenport JE, Brown MC, Lithgow JK, et al. (2006) Investigations into sigmaB-modulated regulatory pathways governing extracellular virulence determinant production in *Staphylococcus aureus*. Journal of bacteriology 188(17): 6070–6080.
72. Shaw LN, Jonsson IM, Singh VK, Tarkowski A, Stewart GC (2007) Inactivation of traP has no effect on the agr quorum-sensing system or virulence of *Staphylococcus aureus*. Infection and immunity 75(9): 4519–4527.
73. Horsburgh MJ, Thackray PD, Moir A (2001) Transcriptional responses during outgrowth of *Bacillus subtilis* endospores. Microbiology (Reading, England) 147(Pt 11): 2933–2941.
74. Asai K, Yamaguchi H, Kang CM, Yoshida K, Fujita Y, et al. (2003) DNA microarray analysis of *Bacillus subtilis* sigma factors of extracytoplasmic function family. FEMS microbiology letters 220(1): 155–160.
75. Matsumoto T, Nakanishi K, Asai K, Sadaie Y (2005) Transcriptional analysis of the ylaABCD operon of *Bacillus subtilis* encoding a sigma factor of extracytoplasmic function family. Genes Genet Syst 80: 385–393.
76. Huang X, Decatur A, Sorokin A, Helmann JD (1997) The *Bacillus subtilis* sigma(X) protein is an extracytoplasmic function sigma factor contributing to survival at high temperature. Journal of bacteriology 179(9): 2915–2921.

77. Kemp EH, Sammons RL, Moir A, Sun D, Setlow P (1991) Analysis of transcriptional control of the gerD spore germination gene of *Bacillus subtilis* 168. J Bacteriol 173: 4646–4652.
78. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual. New York: Cold Spring Harbor Laboratory.
79. Guerout-Fleury AM, Shazand K, Frandsen N, Stragier P (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167: 335–336.
80. Schenk S, Laddaga RA (1992) Improved method for electroporation of *Staphylococcus aureus*. FEMS Microbiol Lett 73: 133–138.
81. Shaw LN, Golonka E, Szmyd G, Foster SJ, Travis J, et al. (2005) Cytoplasmic control of premature activation of a secreted protease zymogen: deletion of staphostatin B (SspC) in *Staphylococcus aureus* 8325-4 yields a profound pleiotropic phenotype. J Bacteriol 187: 1751–1762.
82. Fujimoto DF, Bayles KW (1998) Opposing roles of the *Staphylococcus aureus* virulence regulators, Agr and Sar, in Triton X-100- and penicillin-induced autolysis. J Bacteriol 180: 3724–3726.
83. Doherty N, Holden MT, Qazi SN, Williams P, Winzer K (2006) Functional analysis of luxS in *Staphylococcus aureus* reveals a role in metabolism but not quorum sensing. J Bacteriol 188: 2885–2897.
84. Helle M, Boeije L, Aarden LA (1988) Functional discrimination between interleukin 6 and interleukin 1. Eur J Immunol 18: 1535–1540.

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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	*ϕ*80 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

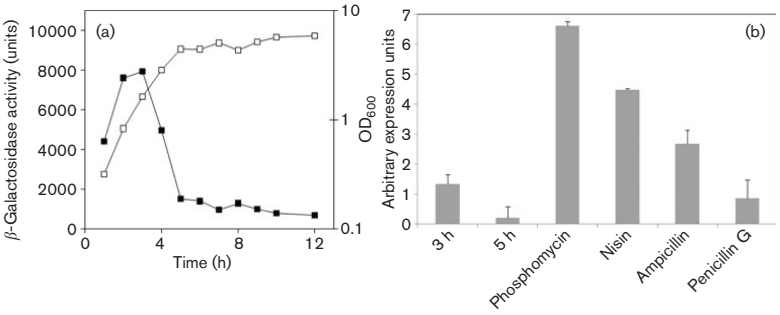

Fig. 1. Transcriptional profiling of *nsaRS*. (a) A reporter *lacZ* fusion was created for *nsaRS* in the SH1000 background, and analysed using 4-MU. Cultures were grown in TSB and OD_{600} (□) and β-galactosidase activity (■) was measured every hour throughout growth. Data shown are from at least three 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 μg ml^{-1} for bacitracin, whilst MW2 has an MIC of 64 μg ml^{-1}. The fact that Matsuo and colleagues reported an MIC of 16 μg 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

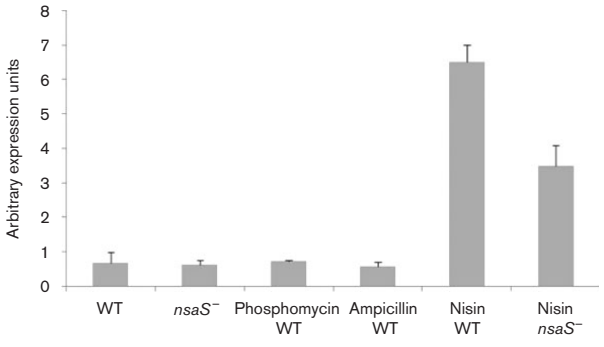

Fig. 2. Role of NsaRS in regulating the transporter *nsaAB*. Transcription of *nsaAB* was initially determined under standard conditions in SH1000 (WT) and its *nsaS* mutant grown for 3 h. These analyses were repeated using TSB containing subinhibitory concentrations of phosphomycin, ampicillin and nisin 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

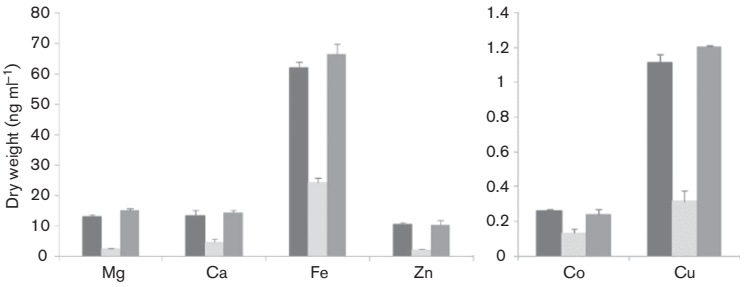

Fig. 3. The transport of divalent metal ions under low-abundance conditions requires a functional NsaRS. The SH1000 wild-type (black), its *nsaS* mutant (light grey) and the complemented strain (dark grey) were grown for 4 h in metal-ion-limiting media (CLR) containing 400 μM magnesium sulfate and 0.2 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

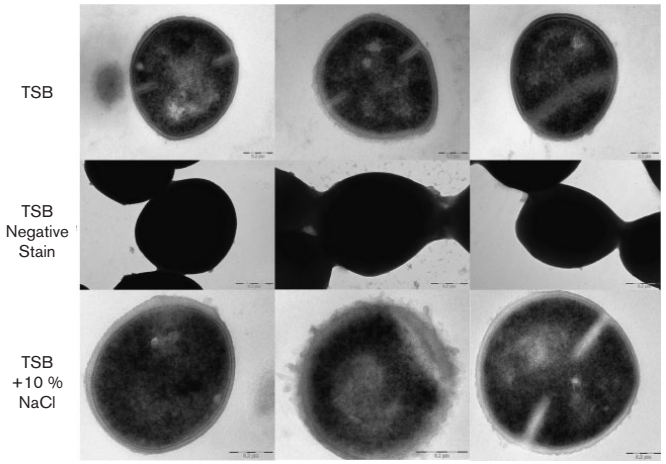

Fig. 4. NsaRS mediates remodelling of cell envelope architecture. SH1000, its *nsaS* mutant and the complemented strain were grown in TSB for thin section (3 h cultures) and negative stain (6 h cultures) analyses. Additionally, thin section images were captured for strains grown in TSB containing 10 % 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

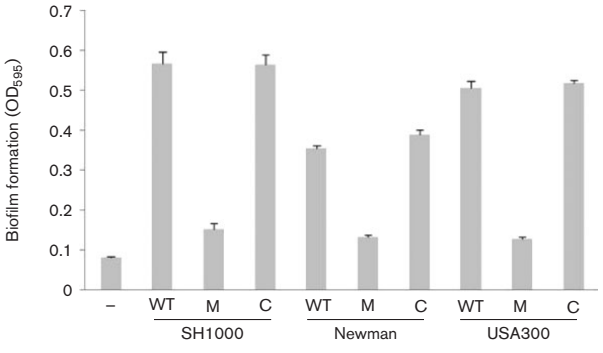

Fig. 5. The NsaRS TCS contributes to biofilm formation in *S. aureus*. *nsaS* mutants in the SH1000, Newman and USA300 background were assayed for biofilm formation. WT, Wild-type; M, *nsaS* mutant; C, *nsaS* mutant strain complemented *in trans* within *nsaRS*. The negative control (–) was the non-biofilm-forming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

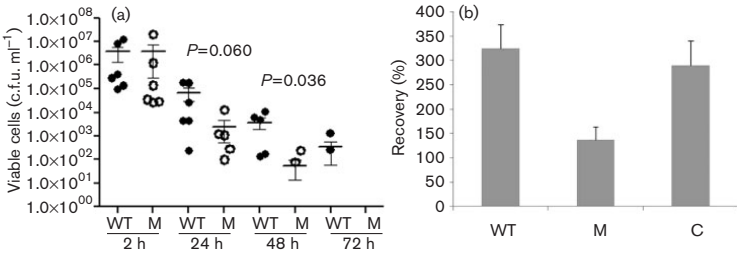

Fig. 6. NsaRS plays an important role during the interaction of *S. aureus* with components of the human innate immune system. (a) Phagocytosis assays were carried out for the Newman strain (●) and *nsaS* mutant (○) at an m.o.i. of 1 : 50 (hMdMs). After challenge, cultures were maintained for the desired 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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REFERENCES

Beenken, K. E., Blevins, J. S. & Smeltzer, M. S. (2003). Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. *Infect Immun* **71**, 4206–4211.

Beenken, K. E., Dunman, P. M., McAleese, F., Macapagal, D., Murphy, E., Projan, S. J., Blevins, J. S. & Smeltzer, M. S. (2004). Global gene expression in *Staphylococcus aureus* biofilms. *J Bacteriol* **186**, 4665–4684.

Beenken, K. E., Mrak, L. N., Griffin, L. M., Zielinska, A. K., Shaw, L. N., Rice, K. C., Horswill, A. R., Bayles, K. W. & Smeltzer, M. S. (2010). Epistatic relationships between *sarA* and *agr* in *Staphylococcus aureus* biofilm formation. *PLoS ONE* **5**, e10790.

Blake, K. L., Randall, C. P. & O'Neill, A. J. (2011). In vitro studies indicate a high resistance potential for the lantibiotic nisin in *Staphylococcus aureus* and define a genetic basis for nisin resistance. *Antimicrob Agents Chemother* **55**, 2362–2368.

Boles, B. R. & Horswill, A. R. (2008). Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog* **4**, e1000052.

Boles, B. R., Thoendel, M., Roth, A. J. & Horswill, A. R. (2010). Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. *PLoS ONE* **5**, e10146.

Brunskill, E. W. & Bayles, K. W. (1996). Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*. *J Bacteriol* **178**, 611–618.

Cao, M., Wang, T., Ye, R. & Helmann, J. D. (2002). Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. *Mol Microbiol* **45**, 1267–1276.

Cheung, A. L., Koomey, J. M., Butler, C. A., Projan, S. J. & Fischetti, V. A. (1992). Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. *Proc Natl Acad Sci U S A* **89**, 6462–6466.

Cheung, G. Y., Rigby, K., Wang, R., Queck, S. Y., Braughton, K. R., Whitney, A. R., Teintze, M., DeLeo, F. R. & Otto, M. (2010). *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLoS Pathog* **6**, e1001133.

Corrigan, R. M., Rigby, D., Handley, P. & Foster, T. J. (2007). The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* **153**, 2435–2446.

de Kruijff, B., van Dam, V. & Breukink, E. (2008). Lipid II: a central component in bacterial cell wall synthesis and a target for antibiotics. *Prostaglandins Leukot Essent Fatty Acids* **79**, 117–121.

Delaune, A., Poupel, O., Mallet, A., Coic, Y. M., Msadek, T. & Dubrac, S. (2011). Peptidoglycan crosslinking relaxation plays an important role in *Staphylococcus aureus* WalKR-dependent cell viability. *PLoS ONE* **6**, e17054.

Delgado, A., Zaman, S., Muthaiyan, A., Nagarajan, V., Elasri, M. O., Wilkinson, B. J. & Gustafson, J. E. (2008). The fusidic acid stimulon of *Staphylococcus aureus*. *J Antimicrob Chemother* **62**, 1207–1214.

Fournier, B., Klier, A. & Rapoport, G. (2001). The two-component system ArlS–ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. *Mol Microbiol* **41**, 247–261.

Fuchs, S., Pané-Farré, J., Kohler, C., Hecker, M. & Engelmann, S. (2007). Anaerobic gene expression in *Staphylococcus aureus*. *J Bacteriol* **189**, 4275–4289.

Gardete, S., Wu, S. W., Gill, S. & Tomasz, A. (2006). Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **50**, 3424–3434.

Giraudo, A. T., Raspanti, C. G., Calzolari, A. & Nagel, R. (1994). Characterization of a Tn*551*-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. *Can J Microbiol* **40**, 677–681.

Hasper, H. E., Kramer, N. E., Smith, J. L., Hillman, J. D., Zachariah, C., Kuipers, O. P., de Kruijff, B. & Breukink, E. (2006). An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* **313**, 1636–1637.

Herbert, S., Bera, A., Nerz, C., Kraus, D., Peschel, A., Goerke, C., Meehl, M., Cheung, A. & Götz, F. (2007). Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PLoS Pathog* **3**, e102.

Highlander, S. K., Hultén, K. G., Qin, X., Jiang, H., Yerrapragada, S., Mason, E. O., Jr, Shang, Y., Williams, T. M., Fortunov, R. M. & other authors (2007). Subtle genetic changes enhance virulence of methicillin resistant and sensitive *Staphylococcus aureus*. *BMC Microbiol* **7**, 99.

Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E. & Foster, S. J. (2001a). PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect Immun* **69**, 3744–3754.

Horsburgh, M. J., Ingham, E. & Foster, S. J. (2001b). In *Staphylococcus aureus*, *fur* is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J Bacteriol* **183**, 468–475.

Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K. & Foster, S. J. (2002). σB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**, 5457–5467.

Hyde, A. J., Parisot, J., McNichol, A. & Bonev, B. B. (2006). Nisin-induced changes in *Bacillus* morphology suggest a paradigm of antibiotic action. *Proc Natl Acad Sci U S A* **103**, 19896–19901.

Joseph, P., Fichant, G. &, Quentin, Y. & Denizot, F. (2002). Regulatory relationship of two-component and ABC transport systems and clustering of their genes in the *Bacillus*/*Clostridium* group, suggest a functional link between them. *J Mol Microbiol Biotechnol* **4**, 503–513.

Kemp, E. H., Sammons, R. L., Moir, A., Sun, D. & Setlow, P. (1991). Analysis of transcriptional control of the gerD spore germination gene of Bacillus subtilis 168. *J Bacteriol* **173**, 4646–4652.

Koprivnjak, T., Mlakar, V., Swanson, L., Fournier, B., Peschel, A. & Weiss, J. P. (2006). Cation-induced transcriptional regulation of the *dlt* operon of *Staphylococcus aureus*. *J Bacteriol* **188**, 3622–3630.

Koziel, J., Maciag-Gudowska, A., Mikolajczyk, T., Bzowska, M., Sturdevant, D. E., Whitney, A. R., Shaw, L. N., DeLeo, F. R. & Potempa, J. (2009). Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. *PLoS ONE* **4**, e5210.

Kraus, D., Herbert, S., Kristian, S. A., Khosravi, A., Nizet, V., Götz, F. & Peschel, A. (2008). The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defenses. *BMC Microbiol* **8**, 85.

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., Golda, A., Maciag-Gudowska, A., Brix, K. & other authors (2008). A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS ONE* **3**, e1409.

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H. & Hiramatsu, K. (2003). Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol Microbiol* **49**, 807–821.

Levy, O. (2000). Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. *Blood* **96**, 2664–2672.

Li, M., Cha, D. J., Lai, Y., Villaruz, A. E., Sturdevant, D. E. & Otto, M. (2007). The antimicrobial peptide-sensing system *aps* of *Staphylococcus aureus*. *Mol Microbiol* **66**, 1136–1147.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**, 402–408.

Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N Engl J Med* **339**, 520–532.

Mascher, T. (2006). Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. *FEMS Microbiol Lett* **264**, 133–144.

Matsuo, M., Kato, F., Oogai, Y., Kawai, T., Sugai, M. & Komatsuzawa, H. (2010). Distinct two-component systems in methicillin-resistant *Staphylococcus aureus* can change the susceptibility to antimicrobial agents. *J Antimicrob Chemother* **65**, 1536–1537.

McNamara, P. J., Milligan-Monroe, K. C., Khalili, S. & Proctor, R. A. (2000). Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. *J Bacteriol* **182**, 3197–3203.

Meehl, M., Herbert, S., Götz, F. & Cheung, A. (2007). Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **51**, 2679–2689.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., Lopez, J. A., Foster, T. J., Penadés, J. R. & Lasa, I. (2009). Protein A-mediated multicellular behavior in *Staphylococcus aureus*. *J Bacteriol* **191**, 832–843.

Muthaiyan, A., Silverman, J. A., Jayaswal, R. K. & Wilkinson, B. J. (2008). Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. *Antimicrob Agents Chemother* **52**, 980–990.

Nilsson, R. P., Beijer, L. & Rutberg, B. (1994). The *glpT* and *glpQ* genes of the glycerol regulon in *Bacillus subtilis*. *Microbiology* **140**, 723–730.

Novick, R. P. (2006). Staphylococcal pathogenesis and pathogenicity factors: genetics and regulation. In *Gram-positive Pathogens*, pp. 496–516. Edited by V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy & J. I. Rood. Washington, DC: American Society for Microbiology.

Novick, R. P., Projan, S. J., Kornblum, J., Ross, H. F., Ji, G., Kreiswirth, B., Vandenesch, F., Moghazeh, S. & Novick, R. P. (1995). The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. *Mol Gen Genet* **248**, 446–458.

Pagels, M., Fuchs, S., Pané-Farré, J., Kohler, C., Menschner, L., Hecker, M., McNamarra, P. J., Bauer, M. C., von Wachenfeldt, C. & other authors (2010). Redox sensing by a Rex-family repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus aureus*. *Mol Microbiol* **76**, 1142–1161.

Petek, M., Baebler, S., Kuzman, D., Rotter, A., Podlesek, Z., Gruden, K., Ravnikar, M. & Urleb, U. (2010). Revealing fosfomycin primary effect on *Staphylococcus aureus* transcriptome: modulation of cell envelope biosynthesis and phosphoenolpyruvate induced starvation. *BMC Microbiol* **10**, 159.

Pietiäinen, M., François, P., Hyyryläinen, H. L., Tangomo, M., Sass, V., Sahl, H. G., Schrenzel, J. & Kontinen, V. P. (2009). Transcriptome analysis of the responses of *Staphylococcus aureus* to antimicrobial peptides and characterization of the roles of *vraDE* and *vraSR* in antimicrobial resistance. *BMC Genomics* **10**, 429.

Ray, P. H., Lillich, T. T. & White, D. C. (1972). Consequences of glycerol deprivation on the synthesis of membrane components in a glycerol auxotroph of *Staphylococcus aureus*. *J Bacteriol* **112**, 413–420.

Riordan, J. T., Tietjen, J. A., Walsh, C. W., Gustafson, J. E. & Whittam, T. S. (2010). Inactivation of alternative sigma factor 54 (RpoN) leads to increased acid resistance, and alters locus of enterocyte effacement (LEE) expression in *Escherichia coli* O157 : H7. *Microbiology* **156**, 719–730.

Schlag, S., Fuchs, S., Nerz, C., Gaupp, R., Engelmann, S., Liebeke, M., Lalk, M., Hecker, M. & Götz, F. (2008). Characterization of the oxygen-responsive NreABC regulon of *Staphylococcus aureus*. *J Bacteriol* **190**, 7847–7858.

Schneider, T. & Sahl, H. G. (2010). Lipid II and other bactoprenol-bound cell wall precursors as drug targets. *Curr Opin Investig Drugs* **11**, 157–164.

Shaw, L., Golonka, E., Potempa, J. & Foster, S. J. (2004). The role and regulation of the extracellular proteases of *Staphylococcus aureus*. *Microbiology* **150**, 217–228.

Shaw, L. N., Golonka, E., Szmyd, G., Foster, S. J., Travis, J. & Potempa, J. (2005). Cytoplasmic control of premature activation of a secreted protease zymogen: deletion of staphostatin B (SspC) in *Staphylococcus aureus* 8325-4 yields a profound pleiotropic phenotype. *J Bacteriol* **187**, 1751–1762.

Shaw, L. N., Aish, J., Davenport, J. E., Brown, M. C., Lithgow, J. K., Simmonite, K., Crossley, H., Travis, J., Potempa, J. & Foster, S. J. (2006). Investigations into σ^B-modulated regulatory pathways governing extracellular virulence determinant production in *Staphylococcus aureus*. *J Bacteriol* **188**, 6070–6080.

Shaw, L. N., Jonsson, I. M., Singh, V. K., Tarkowski, A. & Stewart, G. C. (2007). Inactivation of *traP* has no effect on the *agr* quorum-sensing system or virulence of *Staphylococcus aureus*. *Infect Immun* **75**, 4519–4527.

Shaw, L. N., Lindholm, C., Prajsnar, T. K., Miller, H. K., Brown, M. C., Golonka, E., Stewart, G. C., Tarkowski, A. & Potempa, J. (2008). Identification and characterization of σ, a novel component of the *Staphylococcus aureus* stress and virulence responses. *PLoS ONE* **3**, e3844.

Staroń, A., Finkeisen, D. E. & Mascher, T. (2011). Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*. *Antimicrob Agents Chemother* **55**, 515–525.

Sullivan, M. A., Yasbin, R. E. & Young, F. E. (1984). New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. *Gene* **29**, 21–26.

Sun, J., Zheng, L., Landwehr, C., Yang, J. & Ji, Y. (2005). Identification of a novel essential two-component signal transduction system, YhcSR, in *Staphylococcus aureus*. *J Bacteriol* **187**, 7876–7880.

Torres, V. J., Stauff, D. L., Pishchany, G., Bezbradica, J. S., Gordy, L. E., Iturregui, J., Anderson, K. L., Dunman, P. M., Joyce, S. & Skaar, E. P. (2007). A *Staphylococcus aureus* regulatory system that responds to host heme and modulates virulence. *Cell Host Microbe* **1**, 109–119.

Tsang, L. H., Cassat, J. E., Shaw, L. N., Beenken, K. E. & Smeltzer, M. S. (2008). Factors contributing to the biofilm-deficient phenotype of *Staphylococcus aureus sarA* mutants. *PLoS ONE* **3**, e3361.

Yarwood, J. M., McCormick, J. K. & Schlievert, P. M. (2001). Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. *J Bacteriol* **183**, 1113–1123.

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DATASET BRIEF

The impact of CodY on virulence determinant production in community-associated methicillin-resistant *Staphylococcus aureus*

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Staphylococcus aureus is a leading human pathogen of both hospital and community-associated diseases worldwide. This organism causes a wealth of infections within the human host as a result of the vast arsenal of toxins encoded within its genome. Previous transcriptomic studies have shown that toxin production in *S. aureus* can be strongly impacted by the negative regulator CodY. CodY acts by directly, and indirectly (via Agr), repressing toxin production during times of plentiful nutrition. In this study, we use iTRAQ-based proteomics for the first time to study virulence determinant production in *S. aureus*, so as to correlate transcriptional observations with actual changes in protein synthesis. Using a *codY* mutant in the epidemic CA-MRSA clone USA300 we demonstrate that deletion of this transcription factor results in a major upregulation of toxin synthesis in both post-exponential and stationary growth. Specifically, we observe hyper-production of secreted proteases, leukocidins and hemolysins in both growth phases in the USA300 *codY* mutant. Our findings demonstrate the power of mass spectrometry-based quantitative proteomics for studying toxin production in *S. aureus*, and the importance of CodY to this central process in disease causation and infection.

Keywords:
Microbiology / Regulation / *Staphylococcus aureus* / Toxins / Virulence determinants

The transcription factor CodY is well conserved within the low G+C Gram-positive bacteria, where it has been shown to be an important regulator of metabolism and virulence [1]. It functions by sensing intracellular levels of branched-chain amino acids (BCAAs) and GTP during growth, and responds by repressing genes involved in starvation behaviors in nutrient-rich conditions, such as amino acid transport, sporulation, and competence [2–5]. As BCAA and GTP levels decline, CodY loses its affinity for DNA binding, bringing about de-repression of target genes [1]. This results in a physiological transition from growth and division to amino acid metabolism and stress tolerance. In this study, we focus on the impact of CodY on toxin production in the major human pathogen *Staphylococcus aureus*. Previous reports have shown that, in addition to its role in nutritional regulation, CodY impacts toxin gene expression in *S. aureus* by repressing transcription of another regulator, *agr*. Agr is a quorum-sensing, two-component system that is maximally expressed during post-exponential growth, where it represses surface proteins and induces transcription of toxins [6]. As such, CodY functions to couple toxin gene regulation in *S. aureus* to the nutritional status of the cell. Thus far, a variety of studies have documented the effects of CodY on *S. aureus* toxin gene transcription [7–9], but, to date, a study of how these changes affect toxin synthesis is lacking.

Accordingly, we have employed iTRAQ to assess the impact of CodY on toxin synthesis in the human clinical isolate, CA-MRSA USA300. A *codY* mutant was generated in

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strain USA300-HOU-MRSA [10] using techniques described previously [11]. Post-exponential phase (5 h) or stationary phase (15 h) cultures of wild-type and mutant strains (Supporting Information Fig. 1) were prepared in TSB as described previously [10, 11]. Secretomes were harvested by centrifugation, sterilized by filtration, and concentrated using Millipore Centricon Plus-70 filter units with a 3 kDa cutoff. Proteins were precipitated overnight at 4°C using 10% trichloroacetic acid. Precipitates were collected by centrifugation and pellets washed thrice with 100% ice-cold ethanol, before being air-dried. Samples were resuspended in iTRAQ dissolution buffer at 100 μg/mL, in a volume of 20 μL. One μL of denaturant and 2 μL of reducing reagent were added to each sample, and incubated at 60°C for 1 h. Next, 1 μL of cysteine blocking reagent was added and incubated for 10 min at room temperature. Trypsin was added in a ratio of 1:30, and samples digested for 12–16 h at 37°C. Labeling with iTRAQ reagents was completed according to manufacturer's instructions. Briefly, peptide samples were individually labeled with iTRAQ reagents (USA300-116, USA300-*codY*-117) for 1 h at room temperature. After incubation, samples were combined and dried using a SpeedVac centrifuge, and resuspended in 1 mL of 0.1% formic acid in water. A C-18 Vydac column was used to desalt samples, with peptides eluted using 0.1% formic acid in ACN. After peptide elution, iTRAQ-labeled samples were dried using a SpeedVac centrifuge, resuspended in 0.1% formic acid in water, and analyzed using a hybrid linear ion trap-Orbitrap instrument mass spectrometer (LTQ Orbitrap XL, Thermo) operated with Xcalibur (v2.0.7) data acquisition software. Five μL of protein digest was loaded onto a 75 μm id × 2 cm ProteoPep II C18 trap (New Objective, Woburn, MA, USA) and desalted online before injection onto a 75 μm id × 10 cm Proteopep II C18 analytical column, where a linear gradient was carried out to 40% acetonitrile in 90 min at 250 nL/min, using a NanoUltra 2D-HPLC system (Eksigent, Dublin, CA, USA). Data-dependent acquisition consisted of MS/MS analysis in the LTQ linear ion trap of the top three most intense precursor ions from initial high-resolution (30 000 at m/z 400) survey scans, followed by high-energy collision dissociation (HCD) and Orbitrap fragment ion detection (7500 at m/z 400) of the same three ions.

MS/MS spectra generated from data-dependent acquisition via the LTQ Orbitrap XL were extracted by extractMSN.exe (BioWorks v.3.3, Thermo), and searched against a concatenated subset database containing both normal and randomized sequences of *S. aureus* USA300_FPR3757 obtained from Uniprot.org (downloaded 07-27-2010, 5214 entries) using the MASCOT search algorithm (v2.2.2). MASCOT was searched with a fragment ion mass tolerance of 0.60 Da, and a parent ion tolerance of 10 ppm, assuming full trypsin specificity with 1 possible missed cleavage. Oxidation of methionine, as well as iTRAQ labeling of peptide N-terminus, lysine, and tyrosine, were included as variable modifications in MASCOT. Fixed modifications included MMTS modification of cysteine. MASCOT results were compiled in ProteoIQ (v2.1.11, Nusep) for final identification and relative quantitation. The MASCOT database search score cutoff was adjusted within ProteoIQ to achieve a 1% false discovery rate at the protein level. Relative quantitation of iTRAQ reporter ions was performed by ProteoIQ followed by normalization to the dataset median (assuming a global median ratio of 1) using Microsoft Excel. The ratios are reported as 117 reporter ion intensity (USA300-*codY*)/116 reporter ion intensity (USA300), and considered significant if the ratio was at least ± 1 standard deviation away from the median. Quantitative proteomic analysis using iTRAQ was performed using three biological replicates. All MASCOT identification data are deposited in the PRIDE proteomics identification database (http://www.ebi.ac.uk/pride) under accession numbers 17700–17705 [12]. The data were converted using the PRIDE Converter [13].

Tables 1 and 2 show iTRAQ-based relative quantitation for proteins that are known or putative secreted virulence factors of *S. aureus*; however, all proteins and corresponding iTRAQ ratios are reported in Supporting Information Tables 1 and 2. It should be noted that the complete data sets in Supplementary Information contain a number of intracellular proteins within the secretomes of both strains. It has been our experience, and that of others [14], that subproteome contamination is common, and can be the result of a number of factors, including natural cell lysis during growth. Additionally, there is growing evidence to suggest that intracellular proteins are intentionally exported, and have novel functions outside the cell [14]. For the data presented herein, we analyzed only those proteins that were significantly altered between the two strains based on our filtering criteria, and are either known secreted proteins, or contained signal peptides, as determined by the SignalP algorithm.

Post-exponential secretomes of the *codY* mutant compared with the wild-type (Table 1) demonstrate a dramatic increase in several *agr*-regulated proteases, such as aureolysin (14.1-fold), Spl proteases (SplE = 7.3-fold, SplB = 6.6-fold), and Staphopain B (4.4-fold). Most pronounced, however, was V8 protease, which was produced at levels 16.8-fold higher in the mutant. Furthermore, synthesis of multiple leukocidins, including LukD (3.5-fold) and LukE (5.1-fold), along with the Panton-Valentine leukocidins, LukF-PV (4.9-fold) and LukS-PV (6.9-fold), were increased in the mutant during post-exponential growth. Additionally, subunits of the γ-hemolysin were found in greater abundance in the *codY*-null strain (HlgB = 3.0-fold, HlgC = 6.7-fold). Interestingly, during post-exponential growth in the mutant, we observed decreased levels of two phenol soluble modulins: δ-hemolysin and Psmβ1 (−4.8-fold and −5.0-fold, respectively). It is possible that this unexpected downregulation may result from overproduction of proteases in the *codY* mutant. Indeed, it has previously been suggested that the V8 protease may modulate self-

Table 1. Quantitative proteomic profiling of virulence determinant production in CA-MRSA USA300 and its *codY* mutant during post-exponential growth

Accession number	Gene	Protein name	CodY ratio[a)]	Standard deviation[b)]
Upregulated				
SAUSA300_0951	*sspA*	V8 protease	4.07 (16.8)*	2.28
SAUSA300_2572	*aur*	Zinc metalloproteinase aureolysin	3.82 (14.1)*	2.71
SAUSA300_1754	*splE*	Serine protease	2.86 (7.3)	0.46
SAUSA300_1382	*lukS-PV*	Panton-Valentine leukocidin	2.79 (6.9)	1.73
SAUSA300_1759		Putative uncharacterized protein	2.76 (6.8)	2.06
SAUSA300_2366	*hlgC*	Gamma-hemolysin component C	2.75 (6.7)	2.1
SAUSA300_1757	*splB*	Serine protease	2.72 (6.6)	2.29
SAUSA300_1769	*lukE*	Leukocidin	2.34 (5.1)	2.18
SAUSA300_1381	*lukF-PV*	Panton-Valentine leukocidin	2.29 (4.9)	2
SAUSA300_0950	*sspB*	Cysteine protease	2.15 (4.4)	2.13
SAUSA300_0409		Putative uncharacterized protein	1.86 (3.6)	0.58
SAUSA300_1768	*lukD*	Leukocidin	1.79 (3.5)	1.89
SAUSA300_2367	*hlgB*	Gamma-hemolysin component B	1.61 (3.0)	1.91
Downregulated				
SAUSA300_1067	*psmβ1*	Antibacterial protein	−2.23 (−4.8)	1.02
SAUSA300_1988	*hld*	Delta-hemolysin	−2.31 (−5.0)	1.48

∗ Indicates ratio value that is ≥2 standard deviations away from median.
a) Ratio values are Log_2 transformed; number in parenthesis represents fold change.
b) Standard deviation (SD) values are calculated based on ratio values obtained from all peptides across all replicates ($n = 3$); SD values are Log_2 transformed.

Table 2. Quantitative proteomic profiling of virulence determinant production in CA-MRSA USA300 and its *codY* mutant during stationary growth

Accession number	Gene	Protein name	CodY ratio[a)]	Standard deviation[b)]
Upregulated				
SAUSA300_1758	*splA*	Serine protease	4.06 (16.6)*	0.96
SAUSA300_2572	*aur*	Zinc metalloproteinase aureolysin	3.78 (13.7)*	2
SAUSA300_1754	*splE*	Serine protease	3.70 (13.0)*	2.03
SAUSA300_2366	*hlgC*	Gamma-hemolysin component C	3.54 (11.6)*	2.24
SAUSA300_1382	*lukS-PV*	Panton-Valentine leukocidin	3.50 (11.3)*	1.94
SAUSA300_0274		Putative uncharacterized protein	3.38 (10.4)*	1.92
SAUSA300_1753	*splF*	Serine protease	3.12 (8.7)*	1.46
SAUSA300_1755	*splD*	Serine protease	3.12 (8.7)*	1.8
SAUSA300_1757	*splB*	Serine protease	3.08 (8.5)*	2.01
SAUSA300_1381	*lukF-PV*	Panton-Valentine leukocidin	3.04 (8.2)*	1.61
SAUSA300_0815	*ear*	Ear protein	3.04 (8.2)*	2.21
SAUSA300_0951	*sspA*	V8 protease	2.86 (7.3)*	2.51
SAUSA300_1759		Putative uncharacterized protein	2.76 (6.8)*	2.28
SAUSA300_2365	*hlgA*	Gamma-hemolysin component A	2.38 (5.2)*	2.42
SAUSA300_0950	*sspB*	Cysteine protease	2.34 (5.0)	2.11
SAUSA300_1058	*hla*	Alpha-hemolysin	2.24 (4.7)	1.47
SAUSA300_0883		Putative surface protein	2.10 (4.3)	1.93
SAUSA300_1890	*scpA*	Staphopain A	1.76 (3.4)	1.75
SAUSA300_2164		Hypothetical protein	1.58 (3.0)	1.25
SAUSA300_1768	*lukD*	Leukocidin	1.44 (2.7)	2.13
SAUSA300_0955	*atl*	Bifunctional autolysin	1.38 (2.6)	2.36
SAUSA300_1988	*hld*	Delta-hemolysin	1.28 (2.4)	1.89

∗ Indicates ratio value that is ≥2 standard deviations away from median.
a) Ratio values are Log_2 transformed; number in parenthesis represents fold change.
b) Standard deviation (SD) values are calculated based on ratio values obtained from all peptides across all replicates ($n = 3$); SD values are Log_2 transformed.

derived toxin stability [15]; whilst more recently our group has shown that aureolysin degrades both Hla and the PSMs [16]. This observation might also explain why we did not observe significant alteration in α-hemolysin protein levels during post-exponential growth, despite our validation assays detecting elevated *hla* (and *hld*) transcription in the *codY* mutant (Supporting Information Fig. 2).

When exploring the effects of *codY* mutation on stationary-phase toxin synthesis (Table 2), we observed additional alterations when compared with the wild-type. Specifically, the hyperactive *agr* phenotype of USA300 [17] is more pronounced in the absence of CodY during stationary phase, with significant overproduction of *agr*-regulated proteins. As with our post-exponential findings, the major class of proteins with increased synthesis was again secreted proteases. Of the ten known enzymes, nine are increased in stationary-phase cultures of the mutant, with only SplC not shown to be significantly altered. Specifically, Spl enzymes increased from 8.5-fold (SplB) to 16.6-fold (SplA). In addition, we observed upregulation of both cysteine proteases (SspB = 5.0-fold, ScpA = 3.4-fold), aureolysin (13.7-fold), and V8 protease (7.3-fold). These findings are again consistent with our transcriptional validation work, which reveals strong upregulation from major protease loci (*aur*, *scp* and *ssp*), throughout growth in the *codY* mutant (Supporting Information Fig. 2). Leading on from this, given that secreted proteases negatively impact *S. aureus* biofilm formation [10, 16], one would predict that a USA300 *codY* mutant would be impaired in this behavior. As such, we analyzed biofilm formation in the *codY* mutant, and found an approximately 3-fold reduction when compared with the parental and complemented strains (Fig. 1A).

We also observed accumulation of cytolytic toxins in stationary phase cultures of the mutant, with both PVL components increased (LukS-PV = 11.3-fold, LukF-PV = 8.2-fold), as was LukD (2.7-fold). There was also a global increase in hemolysin production in the mutant, with α-hemolysin (4.7-fold), γ-hemolysin (HlgA = 5.2-fold, HlgC = 11.6-fold) and δ-hemolysin (2.4-fold) all displaying greater protein levels. This former finding is in strong agreement with our validation assays, which reveal a 5.8-fold increase in α-hemolysin activity in the *codY* mutant during this growth phase (Fig. 1B). This latter finding is of interest as it is in contrast to the post-exponential data, where we observed decreased δ-hemolysin. While protease levels remain high during stationary phase, it is possible that they suffer a decrease in enzymatic activity as cultures age, explaining the increase in δ-hemolysin accumulation. Furthermore, δ-hemolysin is transcribed at very high levels in the run up to stationary phase (Supporting Information Fig. 2); therefore, despite proteolytic degradation, its abundance is still detectably increased.

Our findings with regards to the high levels and activity of proteolytic enzymes raise an important technical point. Owing to the high abundance of these enzymes in cultures

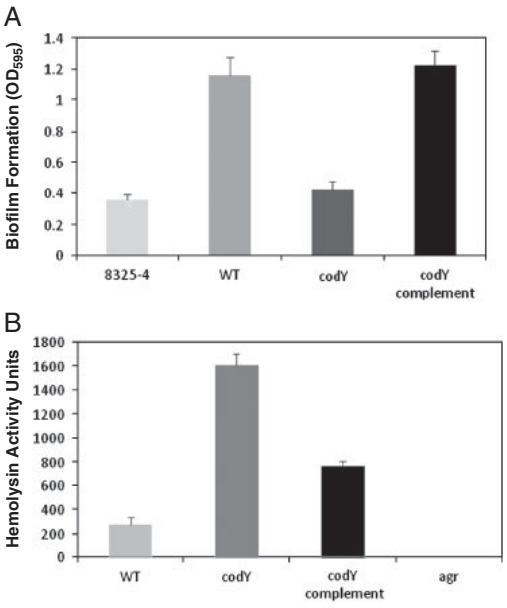

Figure 1. Deletion of *codY* in *S. aureus* USA300 results in decreased biofilm formation and increased hemolytic activity. The USA300 *codY* mutant, and its parental and complemented strains, were assayed for biofilm formation (A) and hemolytic activity (B), as described previously [10, 13]. 8325-4 (A) and a USA300 *agr* mutant (B) were used as the negative controls. Hemolysis data are derived from 15 h cultures. Data presented are from three biological replicates. Error bars are shown as ±SEM.

at both time points, the standard deviation values for ratios determined may be elevated due to biological variability associated with proteolytic degradation. This result would be expected, giving variability at the peptide level within biological replicates, and producing alterations in sequence coverage obtained by MS analysis. Additionally, iTRAQ-labeled peptides derived from the relatively complex secretomes used in this study were not fractionated prior to LC-MS/MS which may lead to iTRAQ ratio compression for certain peptides and therefore, underestimation and/or increased variability of relative changes determined at the protein level [18]. In spite of this, we suggest that such proteomic analyses are of significant value, and perhaps provide more insight than those focused on transcriptional changes. Specifically, changes in gene expression may not directly lead to alterations in protein abundance; resulting from the post-translational events described herein, and in other works [15, 16]. Even though these findings are from in vitro studies, one would predict that such events also occur in vivo, and are thus physiologically relevant and important.

We also observed an increase in the production of a number of uncharacterized secreted proteins in the *codY* mutant. Specifically, SAUSA300_0409 and SAUSA300_0274 were upregulated in post-exponential and stationary phases,

respectively. While no obvious domains or homology could be detected for these proteins, the same is not true for SAUSA300_1759, which was upregulated in the mutant during both growth phases at the same level (6.8-fold). SAUSA300_1759 has homology to the Ear protein, which was also found to be upregulated in the *codY* mutant (8.2-fold). The Ear protein currently has unknown function, but has been labeled a putative toxin in strain MW2, and is found on the ψSa3 pathogenicity island [19, 20]. Other putative toxins were also found to have altered levels, with two Eap/Map domain proteins SAUSA300_0883 (4.3-fold) and SAUSA300_2164 (3.0-fold) both showing increases in the mutant. While these two proteins have yet to be studied, they are part of a family of proteins in *S. aureus* that contain such domains. Eap/Map proteins are both surface associated and extracellular virulence determinants, with a variety of roles in pathogenesis, including adherence and immune-subversion [21, 22].

The present study is the first of its kind to use iTRAQ proteomic techniques to map toxin production in the major human pathogen *S. aureus*. It is apparent from our studies that this approach can be an effective method for relative protein quantitation in this bacterium, as a way to corroborate transcriptional analyses. We show that a variety of known and novel toxins encoded within the genome of CA-MRSA USA300 are hyper-produced as a result of *codY* inactivation. As such, this information provides additional insight into the role of this important regulator, and an understanding of its contribution to disease causation.

All MASCOT identification data are available in the PRIDE database (http://www.ebi.ac.uk/pride) under accession numbers 17700–17705.

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The authors have declared no conflict of interest.

References

[1] Stenz, L., Francois, P., Whiteson, K., Wolz, C. et al., The CodY pleiotropic repressor controls virulence in Gram-positive pathogens. *FEMS Immunol. Med. Microbiol.* 2011, *62*, 123–139.

[2] Ferson, A. E., Wray, L. V., Jr., Fisher, S. H., Expression of the *Bacillus subtilis gabP* gene is regulated independently in response to nitrogen and amino acid availability. *Mol. Microbiol.* 1996, *22*, 693–701.

[3] Fisher, S. H., Rohrer, K., Ferson, A. E., Role of CodY in regulation of the *Bacillus subtilis hut* operon. *J. Bacteriol.* 1996, *178*, 3779–3784.

[4] Fujita, M., Losick, R., Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev.* 2005, *19*, 2236–2244.

[5] Mirel, D. B., Estacio, W. F., Mathieu, M., Olmsted, E. et al., Envi-
ronmental regulation of *Bacillus subtilis* sigma(D)-dependent gene expression. *J. Bacteriol.* 2000, *182*, 3055–3062.

[6] Novick, R. P., Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 2003, *48*, 1429–1449.

[7] Majerczyk, C. D., Sadykov, M. R., Luong, T. T., Lee, C. et al., *Staphylococcus aureus* CodY negatively regulates virulence gene expression. *J. Bacteriol.* 2008, *190*, 2257–2265.

[8] Pohl, K., Francois, P., Stenz, L., Schlink, F. et al., CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. *J. Bacteriol.* 2009, *191*, 2953–2963.

[9] Somerville, G. A., Proctor, R. A., At the crossroads of bacterial metabolism and virulence factor synthesis in staphylococci. *Microbiol. Mol. Biol. Rev.* 2009, *73*, 233–248.

[10] Kolar, S. L., Nagarajan, V., Oszmiana, A., Rivera, F. E. et al., NsaRS is a cell-envelope-stress sensing two-component system of *Staphylococcus aureus*. *Microbiology* 2011, *157*, 2206–2219.

[11] Shaw, L. N., Lindholm, C., Prajsnar, T. K., Miller, H. K. et al., Identification and characterization of σ^S, a novel component of the *Staphylococcus aureus* stress and virulence responses. *PLoS One* 2008, *3*, e3844.

[12] Martens, L., Hermjakob, H., Jones, P., Adamski, M. et al., PRIDE: the proteomics identifications database. *Proteomics* 2005, *5*, 3537–3545.

[13] Barsnes, H., Vizcaino, J. A., Reisinger, F., Eidhammer, I., Martens, L., Submitting proteomics data to PRIDE using PRIDE Converter. *Methods Mol. Biol.* 2011, *694*, 237–253.

[14] Dreisbach, A., Hempel, K., Buist, G., Hecker, M. et al., Profiling the surfacome of *Staphylococcus aureus*. *Proteomics* 2010, *10*, 3082–3096.

[15] Lindsay, J. A., Foster, S. J., Interactive regulatory pathways control virulence determinant production and stability in response to environmental conditions in *Staphylococcus aureus*. *Mol. Gen. Genet.* 1999, *262*, 323–331.

[16] Zielinska, A. K., Beenken, K. E., Joo, H. S., Mrak, L. N. et al., Defining the strain-dependent impact of the staphylococcal accessory regulator (*sarA*) on the alpha toxin phenotype of *Staphylococcus aureus*. *J. Bacteriol.* 2011, *193*, 2948–2958.

[17] Montgomery, C. P., Boyle-Vavra, S., Adem, P. V., Lee, J. C. et al., Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. *J. Infect. Dis.* 2008, *198*, 561–570.

[18] Ow, S. Y., Salim, M., Noirel, J., Evans, C. et al., iTRAQ underestimation in simple and complex mixtures: "the good, the bad and the ugly". *J. Proteome Res.* 2009, *8*, 5347–5355.

[19] Shukla, S. K., Karow, M. E., Brady, J. M., Stemper, M. E. et al., Virulence genes and genotypic associations in nasal

carriage, community-associated methicillin-susceptible and methicillin-resistant USA400 *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* 2010, *48*, 3582–3592.

[20] Baba, T., Bae, T., Schneewind, O., Takeuchi, F., Hiramatsu, K., Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. *J. Bacteriol.* 2008, *190*, 300–310.

[21] Jonsson, K., McDevitt, D., McGavin, M. H., Patti, J. M., Hook, M., *Staphylococcus aureus* expresses a major histocompatibility complex class II analog. *J. Biol. Chem.* 1995, *270*, 21457–21460.

[22] Hussain, M., Haggar, A., Heilmann, C., Peters, G. et al., Insertional inactivation of Eap in *Staphylococcus aureus* strain Newman confers reduced staphylococcal binding to fibroblasts. *Infect. Immun.* 2002, *70*, 2933–2940.

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The ECF Sigma Factor, σ^S, Protects Against Both Cytoplasmic and Extracytoplasmic Stresses in *Staphylococcus aureus*

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Abstract

Previously we identified a novel component of the *S. aureus* regulatory network, an extracytoplasmic-function σ-factor, σ^S, involved in stress response and disease causation. Here we present additional characterization of σ^S, demonstrating a role for it in protection against DNA damage, cell wall disruption and interaction with components of the innate immune system. Promoter mapping reveals the existence of three unique *sigS* start sites, one of which appears to be subject to auto-regulation. Transcriptional profiling revealed that *sigS* expression remains low in a number of *S. aureus* wild-types, but is upregulated in the highly mutated strain RN4220. Further analysis demonstrates *sigS* expression is inducible upon exposure to a variety of chemical stressors that elicit DNA damage, including methyl methanesulfonate and ciprofloxacin, as well as those that disrupt cell wall stability, such as ampicillin and oxacillin. Significantly, expression of *sigS* is highly induced during growth in serum and upon phagocytosis by RAW 264.7 murine macrophage-like cells. Phenotypically, σ^S mutants display sensitivity to a broad range of DNA damaging agents and cell wall targeting antibiotics. Further to this, the survivability of σ^S mutants is strongly impacted during challenge by components of the innate immune system. Collectively, our data suggests that σ^S likely serves dual functions within the *S. aureus* cell, protecting against both cytoplasmic and extracytoplasmic stresses. This further argues for its important, and perhaps novel, role in the *S. aureus* stress and virulence responses.

Introduction

Staphylococcus aureus is an exceedingly virulent and successful pathogen, capable of causing a wide range of infections, from relatively benign skin lesions to life threatening septicemia. With an overwhelming ability to adapt to its environment, *S. aureus* has become the most common cause of both hospital and community acquired infections, and is believed to be the leading cause of death by a single infectious agent in the United States (20, 34). The threat posed by this organism to human health is further heightened by the rapid and continued emergence of multi-drug resistant isolates (1, 20, 34, 43).

Many components govern the adaptive nature of *S. aureus*, including complex regulatory networks, which allow it to respond to constantly changing environments via rapid shifts in gene expression. There are a number of different elements that mediate this fine-tuning, including DNA-binding proteins, two-component systems, regulatory RNAs and alternative σ factors (10, 11, 18, 21, 22, 32, 44, 50, 51). This latter class acts by binding to core-RNA polymerase and redirecting promoter recognition to coordinate gene expression, bringing about expedient and wide-reaching alterations within the cell.

From a classification perspective, σ factors are divided into five discrete subfamilies, with the essential housekeeping factors (σ^A or σ^{70}), which are responsible for the majority of transcription, comprising group 1. The remaining families (2-5) contain alternative σ factors, which are important for niche-specific transcriptional regulation in response to environmental change (24, 27, 40, 41). These elements provide the ability to readily adapt to an ever-changing environment by discrete alterations in transcription profiles. As such, bacteria typically encode a number of alternative σ factors within their genome that fulfill a wide range of functions. Of the alternative families, group 4, comprising the ECF (extracytoplasmic function) σ factors, are by far the most numerate of all such elements (27); for example, *Streptomyces*

ceolicolor contains approximately 65 σ factors, around 50 of which are of the ECF subtype (27). Interestingly, *S. aureus* relies on only 4 σ factors to oversee the execution of its gene expression. In addition to a primary σ factor, σ^A, (16, 17), *S. aureus*, as with the majority of firmicutes, possesses a σ^B alternative σ factor, which controls the general stress response (18, 28, 37, 54, 63). A third σ factor, σ^H, has recently been reported, demonstrating homology to σ^H from *Bacillus subtilis*, and has been shown to regulate competence genes and the integration and excision of prophages (46, 69). Finally, a recent discovery in our laboratory demonstrated the existence of a fourth σ factor, σ^S, belonging to the ECF-family (64). Unlike many other organisms, which commonly possess multiple ECF σ factors, σ^S is the only such element discovered in this organism thus far (27, 64).

Previous work by our group revealed a role for σ^S in the stress and virulence response of *S. aureus* (64). Specifically, we showed that σ^S is important in extended survival during starvation and by lysis with Triton X-100. Competitive growth analysis revealed a decreased ability of a *sigS* mutant to compete against its parental strain both under standard conditions, and in the presence of stress. Interestingly, transcriptional analysis of *sigS* in the laboratory strain SH1000 revealed only baseline expression during growth in rich media over a 72 h period. Finally, using a murine model of septic arthritis, we demonstrated a role for σ^S in systemic infections, as mice infected with a *sigS* mutant displayed significantly decreased weight-loss, mortality, severity of infection, systemic dissemination and mounted immune response by the host.

In this study we have further explored the role and regulation of σ^S, in an effort to understand the conditions under which *S. aureus* utilizes this transcriptional regulator. We show that not only is *sigS* transcription seemingly subject to genetic control in *S. aureus* cells, but it is highly inducible in response to a variety of stresses, including those that elicit DNA-damage and cell wall perturbations. Additionally, we reveal that *sigS* is strongly upregulated upon exposure to

serum, and following phagocytosis by macrophage-like cells. Finally, we present a role for σ^S in the response to DNA damage and cell wall stress, as well as a role in protection against components of the innate immune system.

Materials & Methods

Bacterial strains, plasmids and growth conditions. *S. aureus* and *E. coli* strains and plasmids are listed in Table 1. *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. Synchronized cultures were obtained as described previously (64). When required, antibiotics were added at the following concentrations: ampicillin 100 mg l^{-1} (*E. coli*), tetracycline 5 mg l^{-1} (*S. aureus*), erythromycin 5 mg l^{-1} (*S. aureus*), lincomycin 25 mg l^{-1} (*S. aureus*), and chloramphenicol 5 mg l^{-1} (*S. aureus*). Where specified, chemically defined minimal media (CDM) and metal ion limiting media (CL), were prepared as described previously (29, 75). Porcine serum agar was created by adding filter sterilized porcine serum (Sigma) to preautoclaved and cooled 2% agar in dH_20.

Construction of the *sigS* mutant and *sigS-lacZ* fusion strains. All strains used in this study, other than those described below, were created via φ11 mediated transduction from strains previously described (Table 1).

Construction of *sigS* complement strains. The complement construct generated contains approximately 1 kb of upstream and 710 bp of downstream DNA, relative to the *sigS* coding region. This was PCR amplified using primer pair OL281 and OL1715 (Table 1), and cloned into the Gram-positive shuttle vector pMK4, creating pHKM1. *S. aureus* RN4220 was transformed with this construct, with clones confirmed by PCR analysis, using a combination of gene and vector specific primers (OL-281/OL-1036). A representative clone was selected to transduce the RN4220 and USA300 *sigS* mutants. Clones were again confirmed by PCR analysis, creating strains HKM06 and HKM08.

β-Galactosidase assays. Levels of β-galactosidase activity were measured as described previously (35). The results presented herein are representative of three independent replicates that showed less than 10 % variability.

Real-time PCR. Quantitative real-time PCR analysis was conducted as described previously (35) using primers listed in Table 1 specific for *sigS* (OL1275/OL1276). Control primers were for the 16s rRNA gene, as described previously (36). Values were calculated from three independent replicates, and the data analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Primer extension analysis. Primer extension analysis was carried out as described previously (62) using the AMV reverse transcriptase primer extension system (Promega) according to the manufacturer's guidelines. RNA for primer extension reactions was extracted using an RNeasy kit (Qiagen) as described previously (35). For primer extension analysis 32 µg RNA was used with primer OL1528.

Plate based assay to determine alterations in transcription resulting from external stress. These assays were performed using *sigS-lacZ* fusion strains as described previously (64), with the following stress 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% isopropanol, 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 ml^{-1} pyrogallol, 1 M sodium nitroprusside, 1 M ethyl methane sulfonate, 1 M methyl methane sulfonate, 5 mg ml^{-1} penicillin G, 5 mg ml^{-1} ciprofloxacin, 5 mg ml^{-1} nalidixic acid, 5 mg ml^{-1} cefotaxime, 5 mg ml^{-1} vancomycin, 2 mg ml^{-1} phosphomycin, 5 mg ml^{-1} spectinomycin, 100 mg ml^{-1} ampicillin, 100 mg ml^{-1} oxacillin, 5 mg ml^{-1} gramicidin, 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, 4.21 M peracetic acid, 0.1 M EDTA, 1 M DTT. Plates were incubated for 24 h at 37°C and screened for blue halos, indicating expression.

Transcriptional analysis during growth in porcine serum. Synchronous cultures of the *sigS-lacZ* fusion strains were standardized to an OD_{600} of 0.5, pelleted and washed twice in PBS before being resuspended in 1 ml of filter sterilized porcine serum (Sigma). The suspension was then incubated at 37°C in a rotator device for a period of 1, 5 or 24 h. At the appropriate time point 1 ml samples were pelleted and stored in -20°C for future analysis. Concomitantly, the CFU per ml for each sample was determined via serial dilution and plating on TSA. Harvested bacterial cells were assayed for β-galactosidase production as described previously (35), with the following alterations. Arbitrary expression units were calculated as a measure of substrate cleavage (4-MUG) by β-galactosidase into 4MU, which was evaluated by measuring the fluorescence of each sample at 355 / 460 nm, 0.1 sec, divided by the CFU ml^{-1}. Samples collected from the initial inocula were analyzed for β-galactosidase activity, and used as a measure of baseline expression to identify changes in transcription, as described previously (68, 74). The data presented was generated from 3 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Macrophage cell culture and *S. aureus* intracellular transcriptional analysis. Assays were carried out using the RAW 264.7 murine leukaemic monocyte macrophage cell line (ATCC TIB-71) as described previously (74). Cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin solution (Sigma) until infection, at which time antibiotics were used as described below. RAW 264.7 cells were seeded into 6-well plates and allowed to grow to a density of 2.5 x 10^6 cells per well. These

198 were then infected with *S. aureus* strains resuspended in cell culture medium at 2.5 x 10^8 CFU
199 per well to give an MOI of 100. To synchronize infections and facilitate contact between bacteria
200 and RAW 264.7 cells, plates were centrifuged at 1000 rpm for 10 min. Cells were subsequently
201 incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 to allow phagocytosis.
202 After this time, wells were washed twice with PBS and any remaining non-phagocytosed
203 bacteria were killed by the addition of media containing 30 µg ml^{-1} gentamicin, for 1 h. This was
204 then replaced with fresh DMEM containing 5 µg ml^{-1} gentamicin, and incubated for 24 h.
205 Following this, RAW 264.7 cells were washed twice with PBS, and lysed using 500 µl PBS
206 containing 0.5% Triton X-100. Samples were withdrawn to determine bacterial numbers and the
207 remaining bacteria were pelleted by centrifugation. Harvested bacterial cells were assayed for
208 β-galactosidase production as described previously (35), with the modifications described above
209 for pig serum studies. The data presented was generated from 6 independent replicates and
210 analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.
211
212 **DNA damage sensitivity assays.** Exponentially growing cultures were washed and
213 resuspended in PBS before the addition of DNA damaging agents: 150 mM H_2O_2, 20 mM MMS
214 or 2 mg ml^{-1} EtBr. These were placed at 37°C with shaking, and aliquots removed at the time
215 intervals specified. Samples were then serially diluted, and CFU ml^{-1} determined alongside
216 control samples that were removed prior to exposure. Percent survival was calculated by
217 comparing initial CFU ml^{-1} to final CFU ml^{-1} from three independent assays, and the data was
218 analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.
219 Data is presented as fold change of percent survival relative to that of the wild-type strain.
220
221 **Ultraviolet radiation survival assay.** This assay was performed as previously described (9).
222 Briefly, strains were synchronized to an OD_{600} of 0.05 and allowed to grow for 4 h. Cultures
223 were then serially diluted, and 10^{-2} through 10^{-6} dilutions plated on TSA. Dilutions 10^{-2} and 10^{-3}

were subjected to UV irradiation at 4,000 μJ/cm^2 using a CL-1000 Ultraviolet Crosslinker (UVP). Dilutions 10^{-4} through 10^{-6} served as unexposed controls. All plates were incubated in the dark at 37°C overnight. Survival rates were calculated from three independent experiments, and the data analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

MIC determinations of cell wall targeting antibiotics. These were performed using a variety of cell-wall targeting antibiotics, and a microbroth dilution assay, described previously (5).

Whole Blood Survival Assay. The USA300 wild-type and its isogenic *sigS* mutant were subjected to analysis using a whole human blood model of survival as described previously (35). Pooled and deidentified whole human blood was purchased from Bioreclamation. Survival rates were calculated from three independent replicates, and the data analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Macrophage cell culture and *S. aureus* intracellular survival assay. Infections were carried out as described above for transcription studies, with the following alterations. RAW 264.7 cells were infected with *S. aureus* strains resuspended in cell culture medium at 2.5 x 10^6 CFU per well to give an MOI of 1. Samples were withdrawn 24 hours post phagocytosis and CFU ml^{-1} determined via serial dilution and plating on TSA. The data presented was generated from 3 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Results

σ^S is differentially expressed in *S. aureus* wild-type strains. An unusual finding from our previous study on σ^S was that no expression of the *sigS* gene was detected in SH1000 under standard conditions (64). To assess whether this is a conserved phenomenon, transcriptional analysis using a *sigS-lacZ* fusion was performed in a variety of laboratory strains, including RN4220, 8325-4, SH1000 and Newman, as well as the clinical isolate, USA300. Expression of *sigS* again exhibited only baseline activity over a 24 h period in complex liquid media in every strain, apart from RN4220 (Figure 1A). Surprisingly, in this latter strain we observed an approximate 5-fold increase in *sigS* expression when compared to other *S. aureus* isolates. To ensure our findings were not an artifact of the fusion construct, we performed quantitative real-time PCR on these wild-type strains during a window of maximal *sigS* expression (3 h). Using this approach we again observed robust expression of *sigS* in RN4220, with minimal transcription detected in the other isolates (Figure 1B). Curiously, whilst low expression was observed for the other backgrounds, transcriptional activity in Newman was almost entirely negligible. To determine if nucleotide alterations in the promoter region contributed to this variable expression, we sequenced a 945 bp region immediately 5' of the annotated *sigS* start codon for each of these strains. Interestingly, all sequences were identical to each other, and, where available, matched publicly available genome data for the requisite strains. As such, the differential expression of *sigS* is seemingly not mediated by SNPs within the promoter or regulatory regions of the *sigS* gene.

Mapping the *sigS* transcriptional start site. We previously demonstrated that σ^S acts to upregulate itself from its own promoter region; therefore, in order to elucidate this promoter and any others, we set out to map the *sigS* promoters using RN4220 as a model. To this end, we performed primer extension analysis on RNA extracted from strain RN4220 from exponentially growing cultures (3 h) using a primer located 12 nucleotides downstream of the *sigS* initiation

codon. We identified 3 unique transcription start sites (Figure 2), the longest of which bears an adenine +1 residue, located 150 bp upstream of the translation start site. This is 7 nt away from a putative σ^A promoter, denoted as P1, with a sequence of aTtACA, followed by a 17bp spacer, and then TATtta. Promoter P2 is located 126 nt upstream of the translational start site, beginning with a thymine residue, and appears to have no notable σ^A or σ^B promoter sequences; however, a possible σ^S consensus was identified as CAAAGT 12 bp upstream of TATCA, the putative -10 site. The third transcription start site, positioned 107 nt upstream of the coding region, contains an adenine +1 residue and is 7 nucleotides away from a putative σ^A promoter, denoted as P3, with a sequence of aTcACA, followed by a 11bp spacer, and then acTtAT.

***sigS* deletion results in a growth defect in strain RN4220.** We previously reported that the *sigS* mutant in the SH1000 background displayed no notable growth defect under standard conditions (Shaw 2008). Given the data presented above regarding differential expression of *sigS* in *S. aureus* wild-type strains, we next performed growth analysis of the *sigS* mutant in the RN4220, 8325-4, SH1000 and USA300 LAC backgrounds. Interestingly, whilst mutation of σ^S led to no notable growth defects in these latter 3 strains, we observed a significant growth defect in strain RN4220 (Figure 3). Specifically, *sigS* deletion in this strain resulted in a significant defect upon exit from stationary phase, which continued through exponential growth. At hour 2 we noted a 4.4-fold decrease in optical density of the σ^S mutant compared to the wild-type, which peaks at hour 3 with a fold change of 5.3. This trend continues through hour 4 with a fold decrease of 3. By hours 5 growth of the σ^S mutant is comparable to that of the parent strain. This observation is particularly interesting, as *sigS* expression peaks at hour 3 in RN4220, which corresponds to the time point at which we observe the highest fold decrease in growth for the mutant strain.

299

300 ***sigS* expression is inducible in response to external stimuli.** Given the differential nature of
301 *sigS* expression amongst *S. aureus* strains, we next set out to explore whether transcription
302 could be induced from this locus via external stress, as with other ECF-sigma factors (27, 55,
303 67). This was performed using a disk diffusion assay previously described by us (35, 64), and
304 builds on a pilot screen conducted with SH1000 (64). As such, *sigS-lacZ* fusion strains in 8325-
305 4, Newman, SH1000 and USA300 were grown in the presence of a plethora of stress conditions
306 (see materials and methods). Whilst we were unable to detect upregulation of *sigS* in most
307 strains, we did observe significant inducibility in 8325-4. Specifically, we noted *sigS* expression
308 in the presence of a variety of chemicals (Table 2), including a number of agents that induce cell
309 wall stress, as well as compounds know to elicit DNA damage. We also noted *sigS* upregulation
310 in amino acid limiting media, metal ion limiting media, and during growth on pig serum. These
311 minimal media studies were of particular interest, as they correlate with our previous studies
312 demonstrating a starvation survival defect for *sigS* mutant strains (64). Again to rule out artifacts
313 of the screen, we sought to verify these findings during continuous growth in liquid media. This
314 was performed with the 8325-4 *sigS-lacZ* fusion strain grown in TSB containing sub-lethal
315 concentrations of select chemicals (MMS, H_2O_2 and NaOH) from Table 2, and revealed
316 increased expression of *sigS* in each instance (Figure 4A-C). Specifically, in the presence of
317 MMS, expression peaked at 5 h, with a 48.7-fold increase compared to standard conditions.
318 Maximal expression with both NaOH and H_2O_2 occurred at 10 h, with fold increases of 10 and
319 4.4 respectively, compared to unsupplemented media. Additional qRT-PCR analysis was
320 performed (Figure 4D) to verify this data, and again confirmed that the greatest fold increase in
321 *sigS* expression was induced by exposure to MMS, resulting in a 102.6- fold increase in
322 transcription. Transcription of *sigS* upon exposure to NaOH and H_2O_2 was 63.3- and 57.2-fold
323 higher, respectively.

324

To explore if this upregulation was conserved for other *S. aureus* strains, but perhaps below the limit of detection for our plate based assay, we performed experiments with MMS, NaOH and H_2O_2 using SH1000 and USA300 *sigS-lacZ* fusion strains. Interestingly, despite a lack of blue coloration in the plate based assay, we again detected upregulation of *sigS* during growth in liquid media with sub-lethal concentrations of MMS and H_2O_2 (Figure 5A-B). Specifically, expression with MMS in both SH1000 and USA300 was highest at 2 h, with fold increases of 3.6 and 8.1, respectively, compared to standard growth conditions. In the presence of H_2O_2, *sigS* expression in SH1000 increased by 2.6-fold (2 h), and by 2.3-fold in USA300, (7 h). Conversely, we observed no increase in expression for NaOH when grown in these conditions, suggesting that greater, and more lethal, concentrations of this agent may be required to induce expression. We again confirmed this data by qRT-PCR in SH1000 and USA300 grown in the presence of MMS and H_2O_2 (Figure 5C). We determined that SH1000 displays a 16.9-fold increase in *sigS* expression when cultured with MMS, and a 13.5-fold increase when cultured with H_2O_2. Expression of *sigS* in USA300 grown with MMS or H_2O_2 displayed 4.6- and 2.8-fold increases in transcription, respectively. Collectively, these findings demonstrate a significant inducibility of *sigS* in response to external stimuli that is conserved across *S. aureus* strains.

***sigS* is strongly upregulated during challenge by components of the innate immune system.** Despite limited expression under standard laboratory growth conditions, we have previously demonstrated a role for σ^S in the virulence of *S. aureus* (64). Working on a hypothesis that *sigS* transcription would be increased during infection, we performed expression profiling of *sigS-lacZ* fusion strains upon *ex vivo* challenge by components of the innate immune system. Indeed, our plate based analysis already suggests that *sigS* expression is increased by exposure to pig serum (Table 2). In order to confirm these findings, and quantify this increase across different strains, we performed transcriptional analysis using *sigS-lacZ* fusions strains grown in TSB, and then subcultured into pig serum. We determined that after just 1h of growth

in serum, *sigS* expression increased by 3.4-fold in strain 8325-4, compared to TSB (Figure 6A). Expression continued to rise over time, with fold increases of 21.2 and 20.5 observed at hours 5 and 24, respectively. Additionally we observed a similar effect in strains SH1000 and USA300. Specifically, over the course of growth, we noted fold increases of 13.8, 36.2 and 44.0 in SH1000 for hours 1, 5 and 24, respectively. Finally, in USA300, *sigS* expression increased 6.8-, 15.6- and 26.3-fold at hours 1, 5 and 24, respectively. We continued this line of investigation by assessing *sigS* expression upon phagocytosis by RAW 264.7 macrophage-like cells. Accordingly, macrophages were infected with strains 8325-4, SH1000 and USA300 bearing a *sigS-lacZ* fusion for a period of 24 h, before β-galactosidase activity was measured. Expression of *sigS* was significantly increased after phagocytosis in all strains tested (Figure 6B), with the highest levels observed in 8325-4. In this strain we found a 286.6-fold increase in *sigS* expression compared to background levels. Expression in SH1000 and USA300 increased by 7.5- and 7.4-fold, respectively, compared to background levels. As such, these findings support our hypothesis, and suggest that σ^S is required during the interaction of *S. aureus* with its host.

σ^S mutants are sensitive to DNA damage stress and cell-wall targeting antibiotics. Thus far we have demonstrated that chemicals known to induce DNA damage and cell wall stress strongly impact *sigS* transcription. Accordingly, we next sought to perform death-curve kill studies to examine the viability of *sigS* mutants during exposure to lethal concentrations of these agents in strains 8325-4, SH1000 and USA300. We found that when exponentially growing cultures were exposed to 5X the MIC of MMS for 30 min, a consistent decline in mutant cell viability was seen across all strains tested (the data for USA300, which is representative, is presented, (Figure 7A). Specifically, we recovered 3.33-fold less *sigS* mutant cells compared to the wild-type, when exposed to this agent. Complementation of the σ^S mutation reduced the observed growth impairment significantly, although not completely to wild-type levels. This lack of full complementation is likely attributed to plasmid instability in the presence of DNA

damaging conditions, as suggested by others previously (3, 26, 49, 59, 73, 76). Further to this, in order to determine whether the role of σ^S was limited to protection against DNA alkylation (as induced by MMS), we next examined the ability of the USA300 σ^S mutant to survive exposure to agents that induce other types of DNA damage. As such, analysis was carried out during exposure to oxidative stress resulting from the addition of H_2O_2. Following a 5 min exposure to this agent, we observed a 3.9-fold decrease in *sigS* mutant viability compared to the parent strain, which was fully complementable (Figure 7B). We next used the DNA intercalating agent ethidium bromide (EtBr), and found that the *sigS* mutant displayed a 2.1–fold decrease in viability compare to the wild-type strain after 15 min of exposure (Figure 7C). We again saw that complementation was able to abrogate these affects, but not completely to levels of the wild-type strain. This is likely attributed to the ability of EtBr to cure plasmids upon exposure, as observed by others previously (3). Finally, in order to determine if σ^S mediates protection against UV induced lesions and double strand breaks, we compared the survivability of the wild-type strain and its *sigS* mutant. Exponentially growing cultures were serially diluted on TSA and subjected to UV at a dosage of 4,000 μJ cm^{-2}. Exposure at this level resulted in a 2.1-fold decrease in viability for the mutant (Figure 7D). Complementation in this assay is not possible because of plasmid instability, as we observed >83% loss upon exposure (data not shown).

Following this, we next assessed the sensitivity of *sigS* mutants to a variety of cell wall targeting antibiotics (Figure 8). Analysis using bacitracin in the USA300 background revealed a 4.5-fold decrease in MIC for the mutant strain (30 μg ml^{-1}) compared to the parent (135 μg ml^{-1}). We observed a similar degree of sensitivity with ampicillin, resulting in a 4-fold decrease in MIC for the mutant (25 μg ml^{-1}) compared to wild-type USA300 (100 μg ml^{-1}). Finally, analysis performed using penicillin G yielded a striking 60-fold decrease in MIC for the *sigS* mutant (5 μg ml^{-1}) compared to the parent (300 μg ml^{-1}).

σ^S aids in protection of the *S. aureus* cell during interaction with components of the innate immune system. Previously we have shown a role for σ^S in virulence, using a murine model of septic arthritis. We have also demonstrated herein that *sigS* expression increases not only upon exposure to serum, but also during phagocytosis by macrophage-like cells. As such, we sought to determine the importance of σ^s during challenge by components of the innate immune system. This was first performed using whole human blood and the USA300 wild-type, *sigS* mutant, and its complemented strain. As such, exponentially growing cells were inoculated into whole human blood, and incubated for 4 hours. After this time we recovered 2.1-fold less viable cells of the *sigS* mutant, compared to the wild-type strain (Figure 9A). Complementation analysis restored viability to levels similar to that of the wild-type. Following this, we also conducted macrophage survival assays, again in the USA300 background, to assess the ability of the *sigS* mutant to persist upon phagocytosis. At 24 h post-phagocytosis by RAW 264.7 cells, we observed a 4.5-fold decrease in survivability of the *sigS* mutant compared to the parent (Figure 9B). Complementation of this finding increases survivability of the mutant cells, but not completely to levels of the wild-type strain. This is likely explained by significantly instability of the plasmid during phagocytosis, which we routinely observe when performing this assay. Collectively, these findings support our earlier work, which indicates an important role for σ^s in the virulence of *S. aureus*, and confirms our expression analysis, demonstrating *sigS* upregulation during interaction with components of the innate immune system.

Discussion

In this study, we provide new evidence for the role of σ^S, a novel ECF σ factor in *S. aureus*. In our previous works we have shown that σ^S is a functioning sigma factor that controls is own expression (64). Additionally, we have demonstrated a role for it in the stress and virulence responses of this organism. From a gene expression standpoint, we have previously shown that *sigS* expression is minimal during growth under standard laboratory conditions in SH1000 (64). In this study, we reveal that this phenomenon is conserved across a variety of *S. aureus* strains, including laboratory (8325-4, SH1000 and Newman) and clinical (USA300) isolates. In each case, we observed low levels of expression of *sigS* during growth in rich media. These results may not be entirely surprising, as the majority of ECF σ factors are employed to protect the cell during times of stress, and are often only transcribed when required (27, 55, 67).

Interestingly, we did observe robust *sigS* expression in the highly mutated laboratory strain, RN4220. Amongst the many mutations present in this strain are those that render the activity of other global regulators nonfunctional, including *agr* and *sigB* (15, 71). As such, the regulatory circuits in place in this strain are likely to be highly disordered, potentially explaining *sigS* disregulation, and therefore upregulation. Interestingly, these affects do not appear to be mediated directly through either Agr or σ^B, as mutations in these genes alone do not affect *sigS* expression in either SH1000 or USA300 (data not shown). Recent sequencing of the RN4220 genome has revealed a number of SNPs (single-nucleotide polymorphisms) and deletions relative to the parental strain NCTC 8325 (2, 48). Of interest, a number of these are in genes involved in DNA metabolism, replication, recombination and repair. Most notably, RN4220 carries a SNP in UvrC, a component of the UvrABC exonuclease, which in *Escherichia coli* repairs DNA damage induced by a number of mechanisms, including UV light (60). Interestingly, our analysis herein demonstrates σ^S mutants are less able to survive exposure to UV stress. Additionally, SNPs in RN4220 are located in a putative helicase, SAOUHSC_02790

as well as a truncated resolvase, SAOUHSC_02392. Collectively, these observations suggest that RN4220 is perhaps more prone to DNA damage than other wild-type strains, as a result of mutated and non-functional repair pathways. This would then perhaps explain why this strain exhibits stronger *sigS* expression than other wild-types, as we implicate σ^S in influencing the response of *S. aureus* to DNA damage in this study. Indeed we have observed that the increasing levels of *sigS* transcription in different wild-type strains directly correlates with their sensitivity to DNA damaging agents, such as MMS (data not shown).

Promoter mapping of the *sigS* locus reveals three discrete transcriptional start sties. Promoters, P1 and P2, both appear to be under the control of the housekeeping σ factor, σ^A, however both are severely corrupted from consensus sequences and/or spacing. Due to the relative weakness of these promoters it is likely that other regulatory elements must act to activate transcription from these sequences. This again likely explains the low levels of *sigS* expression observed in the majority of *S. aureus* strains, and argues for a genetic regulatory network that controls expression of this regulator. Previously we demonstrated that *sigS*, as with other ECF-sigma factors (27, 45, 65), controls its own expression via auto-regulation. During promoter mapping in the present study we reveal a likely σ^S controlled transcript, P2 (CAAAGT -12 bp-TATCA). Typically ECF sigma factor consensus sequences display a conserved AAC motif in the -35 region (27, 45); however, exceptions exist. Specifically, the ECF sigma factor of *Neisseria gonorrhoeae* does not recognize an AAC motif (25), whilst σ^R of Streptomyces coelicolor recognizes an AAT motif (53). More importantly, σ^X, an ECF sigma factor in several Psuedomonas spp, specifically recognizes an AAG motif, as seen here for σ^S (4), (39). ECF σ factors typically have significant divergence, and decreased homology within their region 2.4 (41, 45), which specifically recognizes -10 promoter elements. Accordingly, such sites are often difficult to ascertain, however the identified putative -10 element is strikingly similar to the TCTGA recognized sequence of σ^E in *E. coli* (13).

In addition to examining expression in wild-type strains, we have also assessed the level to which *sigS* is upregulated in response to external stress. We show that a variety of stressors can induce expression of *sigS*, ranging from those that elicit alkali stress, to others that affect protein synthesis. Interestingly, this is most pronounced in strain 8325-4, which, like RN4220, lacks natural σ^B activity. We are able to demonstrate that the conditions of *sigS* inducibility in 8325-4 hold true for the σ^B functional strains SH1000 and USA300, although not always at the same levels. Of note, when we inactivate *sigB* in SH1000, we do not observe the same robust increases in *sigS* inducibility seen in 8325-4 (data not shown), despite these strains being very closely related. This observation is perhaps explained by the fact that 8325-4 is an *rsbU* mutant, which is an activator of σ^B activity, rather than a true *sigB* null-strain. Given recent findings showing a role for RsbU outside of its influence of σ^B activity (72), it is possible that these differences are mediated by RsbU, rather than σ^B, mechanisms. It has also recently been shown that 8325-4 and SH1000 are more genetically distinct than the 11 bp deletion in *rsbU* (52), perhaps suggesting that SNPs, and other genetic variations between these 2 strains influence *sigS* expression.

With regards to environmental influence on *sigS* expression, those chemicals that induce DNA damage, such as methyl methanesulfonate (MMS), appear to have the most profound effects. These findings correlate well with our phenotypic studies, showing that *sigS* mutants have increased sensitivity to a broad range of DNA damage inducing stresses. These include alkylating and intercalating agents, reactive oxygen species and UV induced damage; each of which leads to the activation of specific and distinct repair pathways. Interestingly, when we analyzed transcription of a number of DNA repair pathway genes (*ogt*, *uvrB* and *mutM*) in both *sigS* mutants and *S. aureus* wild-type strains, we observed no alterations in expression (data not shown). As such, our findings suggest that σ^S is involved in mediating a comprehensive response to DNA damage by an as yet unknown mechanism. These findings are somewhat

501 novel, as the majority of ECF σ factors typically respond to perturbations in the cell wall.
502 However, reports on ECF σ factors from other organisms reveals several examples that function
503 in sensing and responding to cytoplasmic stress. Specifically, both RpoE of *Rhodobacter*
504 *sphaeroides* and Ecf of *Neisseria gonorrhoeae* respond to oxidative stress, which can in turn
505 lead to DNA damage (6, 19, 25).
506
507 Interestingly, a number of agents were identified as inducing *sigS* expression that are not
508 typically thought of as inducing DNA damage, but can also induce this kind of stress. For
509 example, H_20_2 resulted in *sigS* upregulation, and can react with intracellular iron to form hydroxyl
510 radicals, which cause damage to DNA (8, 30, 31, 56). Additionally, SOS and DNA damage
511 repair genes have previously been shown in *Escherichia coli* to be upregulated during alkali
512 stress caused by excess NaOH, which also upregulates *sigS* expression (23, 61). Finally, the
513 protein synthesis inhibiting antibiotic, chloramphenicol, upregulated *sigS*, and has been shown
514 to lead to the degradation of dsDNA and the inhibition of DNA synthesis (47).
515
516 A consideration with these DNA damage agent studies is that they may not be directly
517 upregulating *sigS* expression, but might cause mutations within the *S. aureus* genome, leading
518 to SNPs. In such a scenario this could lead to disregulation of regulatory circuits, leading to
519 *sigS* upregulation in a manner akin to that proposed for RN4220 and 8325-4. To examine this
520 we analyzed 8325-4 *sigS-lacZ* fusion strains exposed to DNA damaging agents for a 24 h
521 period. Upon removal of the stressor, strains were grown on agar plates containing X-gal. We
522 found no detectable blue coloration on such plates (data not shown), indicating DNA damage
523 induced upregulation of *sigS* does not appear to be mediated via heritable SNPs, but results
524 directly from exposure to these agents. As such, the increase in expression of *sigS* is due
525 solely to exposure to agents such as MMS, and suggests that σ^S is utilized by the cell to adapt
526 during times of DNA damage.

527

528 We also observe substantial increases in expression of *sigS* following exposure to a number of
529 cell wall targeting chemicals, suggesting a role for σ^S in protection against this type of stress.
530 This correlates well with other work presented in this study, which demonstrate *sigS* mutants
531 have increased sensitivity to the cell wall targeting antibiotics. These findings also corroborate
532 our previous work, which demonstrates that *sigS* mutants are sensitive to growth in the
533 presence of a number of cell wall disrupting agents, including Triton X-100 and SDS (64). This
534 suggests a role for σ^S in the *S. aureus* cell wall stress response, which is typical of ECF σ
535 factors. For example, RpoE in *Escherichia coli* serves to upregulate genes involved in the heat-
536 shock response, and is triggered by misfolded proteins accumulating in the periplasm and outer
537 membrane (13). Furthermore, σ^W and σ^M of *Bacillus subtilis* both respond to cell wall
538 biosynthesis inhibiting antibiotics, with σ^M proving vital for survival during exposure to
539 phosphomycin (7, 70). This information, alongside the observation that σ^S is the lone ECF σ
540 factor in *S. aureus*, suggests it likely serves dual functions within the cell, protecting against
541 both cytoplasmic and extracytoplasmic stresses.

542

543 We have also demonstrated here that *sigS* transcription is increased considerably when *S.*
544 *aureus* is challenged by complement during growth in pig serum. We also present evidence for
545 *sigS* upregulation during *ex vivo* infection, revealing high levels of expression upon
546 phagocytosis by murine macrophage-like cells. Phenotypically, we show that σ^S is important for
547 survival during growth in whole human blood, and following phagocytosis. Collectively this
548 supports our previous work, which reveals a major requirement for σ^S during virulence (64). As
549 part of the microbicidal mechanism employed by macrophages, reactive oxygen species (ROS)
550 and reactive nitrogen intermediates (RNI) are excreted at very high levels, leading to DNA
551 damage in invading organisms during infection (33, 38, 42, 58, 66). Moreover, it has been
552 observed that pathogenic organisms such as *Burkholderia* spp., *B. abortus* and *Vibrio cholerae*

defective in DNA damage repair mechanisms are attenuated in virulence, underscoring their importance during infection (12, 14, 57, 77). Together this suggests that, upon entry into the host, bacterial pathogens are faced with an array of DNA damaging conditions. Given that these conditions lead to activation of σ^S in *S. aureus*, this likely goes some way to explaining the avirulent phenotype of *sigS* mutants.

In summary, we present extended characterization of the lone, and novel, ECF σ factor, σ^S, in *S. aureus*. We reveal that, under standard conditions, its transcription remains low in a range of wild-type strains, but that it can be upregulated in response to external stimuli. Specifically, chemicals leading to DNA damage and cell wall disruption strongly induce expression of *sigS*. This upregulation is seemingly of importance, as functional characterization reveals *sigS* mutants are sensitive to both of these types of stress. Additionally, we reveal strong upregulation of this gene during growth in pig serum as well as upon phagocytosis by murine macrophage-like cells, which is seemingly protective to the cell. Collectively, our data suggests that σ^S likely serves dual functions within the cell, protecting against both cytoplasmic and extracytoplasmic stresses. This further argues for its important, and perhaps novel, role in the *S. aureus* stress and virulence responses.

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References

1. **Archer, G. L.** 1998. Staphylococcus aureus: a well-armed pathogen. Clin Infect Dis **26:**1179-81.

2. **Berscheid, A., P. Sass, K. Weber-Lassalle, A. L. Cheung, and G. Bierbaum.** 2012. Revisiting the genomes of the Staphylococcus aureus strains NCTC 8325 and RN4220. Int J Med Microbiol.

3. **Bouanchaud, D. H., M. R. Scavizzi, and Y. A. Chabbert.** 1968. Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. J Gen Microbiol **54:**417-25.

4. **Brinkman, F. S., G. Schoofs, R. E. Hancock, and R. De Mot.** 1999. Influence of a putative ECF sigma factor on expression of the major outer membrane protein, OprF, in Pseudomonas aeruginosa and Pseudomonas fluorescens. J Bacteriol **181:**4746-54.

5. **Burda, W. N., K. B. Fields, J. B. Gill, R. Burt, M. Shepherd, X. P. Zhang, and L. N. Shaw.** 2012. Neutral metallated and meso-substituted porphyrins as antimicrobial agents against gram-positive pathogens. Eur J Clin Microbiol Infect Dis **31:**327-35.

6. **Campbell, E. A., R. Greenwell, J. R. Anthony, S. Wang, L. Lim, K. Das, H. J. Sofia, T. J. Donohue, and S. A. Darst.** 2007. A conserved structural module regulates transcriptional responses to diverse stress signals in bacteria. Mol Cell **27:**793-805.

7. **Cao, M., T. Wang, R. Ye, and J. D. Helmann.** 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the Bacillus subtilis sigma(W) and sigma(M) regulons. Mol Microbiol **45:**1267-76.

8. **Chang, W., D. A. Small, F. Toghrol, and W. E. Bentley.** 2006. Global transcriptome analysis of Staphylococcus aureus response to hydrogen peroxide. J Bacteriol **188:**1648-59.

9. **Chen, Z., T. T. Luong, and C. Y. Lee.** 2007. The sbcDC locus mediates repression of type 5 capsule production as part of the SOS response in Staphylococcus aureus. J Bacteriol **189:**7343-50.

10. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr. Proc Natl Acad Sci U S A **89:**6462-6.

11. **Cheung, A. L., C. Wolz, M. R. Yeaman, and A. S. Bayer.** 1995. Insertional Inactivation of a Chromosomal Locus That Modulates Expression of Potential Virulence Determinants in Staphylococcus-Aureus. J Bacteriol **177:**3220-3226.

12. **Cuccui, J., A. Easton, K. K. Chu, G. J. Bancroft, P. C. Oyston, R. W. Titball, and B. W. Wren.** 2007. Development of signature-tagged mutagenesis in Burkholderia pseudomallei to identify genes important in survival and pathogenesis. Infect Immun **75:**1186-95.

13. **Dartigalongue, C., D. Missiakas, and S. Raina.** 2001. Characterization of the Escherichia coli sigma E regulon. J Biol Chem **276:**20866-75.

14. **Davies, B. W., R. W. Bogard, N. M. Dupes, T. A. Gerstenfeld, L. A. Simmons, and J. J. Mekalanos.** 2011. DNA damage and reactive nitrogen species are barriers to Vibrio cholerae colonization of the infant mouse intestine. PLoS Pathog **7:**e1001295.

15. **de Azavedo, J. C., T. J. Foster, P. J. Hartigan, J. P. Arbuthnott, M. O'Reilly, B. N. Kreiswirth, and R. P. Novick.** 1985. Expression of the cloned toxic shock syndrome toxin 1 gene (tst) in vivo with a rabbit uterine model. Infect Immun **50:**304-9.

16. **Deora, R., and T. K. Misra.** 1996. Characterization of the primary sigma factor of Staphylococcus aureus. J Biol Chem **271:**21828-34.

17. **Deora, R., and T. K. Misra.** 1995. Purification and characterization of DNA dependent RNA polymerase from Staphylococcus aureus. Biochem Biophys Res Commun **208:**610-6.

18. **Deora, R., T. Tseng, and T. K. Misra.** 1997. Alternative transcription factor sigmaSB of Staphylococcus aureus: characterization and role in transcription of the global regulatory locus sar. J Bacteriol **179:**6355-9.

19. **Dufour, Y. S., R. Landick, and T. J. Donohue.** 2008. Organization and evolution of the biological response to singlet oxygen stress. J Mol Biol **383:**713-30.

20. **Emori, T. G., and R. P. Gaynes.** 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. Clin Microbiol Rev **6:**428-42.

21. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in Staphylococcus aureus. Mol Microbiol **41:**247-61.

22. **Giraudo, A. T., G. L. Martinez, A. Calzolari, and R. Nagel.** 1994. Characterization of a Tn925-induced mutant of Staphylococcus aureus altered in exoprotein production. J Basic Microbiol **34:**317-22.

23. **Goodson, M.** 1989. Habituation to alkali in Escherichia coli. Lett Appl Microbiol **9:**71-73.

24. **Gruber, T. M., and C. A. Gross.** 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu Rev Microbiol **57:**441-66.

25. **Gunesekere, I. C., C. M. Kahler, C. S. Ryan, L. A. Snyder, N. J. Saunders, J. I. Rood, and J. K. Davies.** 2006. Ecf, an alternative sigma factor from Neisseria gonorrhoeae, controls expression of msrAB, which encodes methionine sulfoxide reductase. J Bacteriol **188:**3463-9.

26. **Hashimoto, H., K. Kono, and S. Mitsuhashi.** 1964. Elimination of Penicillin Resistance of Staphylococcus Aureus by Treatment with Acriflavine. J Bacteriol **88:**261-2.

27. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol **46:**47-110.

644 28. **Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster.** 2002. sigmaB
645 modulates virulence determinant expression and stress resistance: characterization of a
646 functional rsbU strain derived from Staphylococcus aureus 8325-4. J Bacteriol **184:**5457-67.

647 29. **Horsburgh, M. J., M. O. Clements, H. Crossley, E. Ingham, and S. J. Foster.** 2001. PerR controls
648 oxidative stress resistance and iron storage proteins and is required for virulence in
649 Staphylococcus aureus. Infect Immun **69:**3744-54.

650 30. **Imlay, J. A., S. M. Chin, and S. Linn.** 1988. Toxic DNA damage by hydrogen peroxide through the
651 Fenton reaction in vivo and in vitro. Science **240:**640-2.

652 31. **Imlay, J. A., and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302-9.

653 32. **Janzon, L., S. Lofdahl, and S. Arvidson.** 1986. Evidence for a Coordinate Transcriptional Control
654 of Alpha-Toxin and Protein-a Synthesis in Staphylococcus-Aureus. FEMS Microbiol Lett **33:**193-
655 198.

656 33. **Kennedy, L. J., K. Moore, Jr., J. L. Caulfield, S. R. Tannenbaum, and P. C. Dedon.** 1997.
657 Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents. Chem Res
658 Toxicol **10:**386-92.

659 34. **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R.**
660 **Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B.**
661 **Carey, and S. K. Fridkin.** 2007. Invasive methicillin-resistant Staphylococcus aureus infections in
662 the United States. JAMA **298:**1763-71.

663 35. **Kolar, S. L., V. Nagarajan, A. Oszmiana, F. E. Rivera, H. K. Miller, J. E. Davenport, J. T. Riordan,**
664 **J. Potempa, D. S. Barber, J. Koziel, M. O. Elasri, and L. N. Shaw.** 2011. NsaRS is a cell-envelope-
665 stress-sensing two-component system of Staphylococcus aureus. Microbiology **157:**2206-19.

666 36. **Koprivnjak, T., V. Mlakar, L. Swanson, B. Fournier, A. Peschel, and J. P. Weiss.** 2006. Cation-
667 induced transcriptional regulation of the dlt operon of Staphylococcus aureus. J Bacteriol
668 **188:**3622-30.

669 37. **Kullik, I. I., and P. Giachino.** 1997. The alternative sigma factor sigmaB in Staphylococcus aureus:
670 regulation of the sigB operon in response to growth phase and heat shock. Arch Microbiol
671 **167:**151-9.

672 38. **Lancaster, J. R., Jr.** 1997. A tutorial on the diffusibility and reactivity of free nitric oxide. Nitric
673 Oxide **1:**18-30.

674 39. **Lane, W. J., and S. A. Darst.** 2006. The structural basis for promoter -35 element recognition by
675 the group IV sigma factors. PLoS Biol **4:**e269.

676 40. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The sigma 70 family: sequence conservation
677 and evolutionary relationships. J Bacteriol **174:**3843-9.

678 41. **Lonetto, M. A., K. L. Brown, K. E. Rudd, and M. J. Buttner.** 1994. Analysis of the Streptomyces
679 coelicolor sigE gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma
680 factors involved in the regulation of extracytoplasmic functions. Proc Natl Acad Sci U S A
681 **91:**7573-7.

682 42. **MacMicking, J., Q. W. Xie, and C. Nathan.** 1997. Nitric oxide and macrophage function. Annu
683 Rev Immunol **15:**323-50.

684 43. **McDougal, L. K., G. E. Fosheim, A. Nicholson, S. N. Bulens, B. M. Limbago, J. E. Shearer, A. O.**
685 **Summers, and J. B. Patel.** 2010. Emergence of resistance among USA300 methicillin-resistant
686 Staphylococcus aureus isolates causing invasive disease in the United States. Antimicrob Agents
687 Chemother **54:**3804-11.

688 44. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification,
689 cloning, and initial characterization of rot, a locus encoding a regulator of virulence factor
690 expression in Staphylococcus aureus. J Bacteriol **182:**3197-3203.

691 45. **Missiakas, D., and S. Raina.** 1998. The extracytoplasmic function sigma factors: role and
692 regulation. Mol Microbiol **28:**1059-1066.

693 46. **Morikawa, K., Y. Inose, H. Okamura, A. Maruyama, H. Hayashi, K. Takeyasu, and T. Ohta.** 2003.
694 A new staphylococcal sigma factor in the conserved gene cassette: functional significance and
695 implication for the evolutionary processes. Genes to Cells **8:**699-712.

696 47. **Murray, T. R., K. M. Downey, and A. A. Yunis.** 1983. Chloramphenicol-mediated DNA damage
697 and its possible role in the inhibitory effects of chloramphenicol on DNA synthesis. J Lab Clin
698 Med **102:**926-32.

699 48. **Nair, D., G. Memmi, D. Hernandez, J. Bard, M. Beaume, S. Gill, P. Francois, and A. L. Cheung.**
700 2011. Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory
701 strain used in virulence research, identifies mutations that affect not only virulence factors but
702 also the fitness of the strain. J Bacteriol **193:**2332-5.

703 49. **Nakamura, S.** 1990. [Curing effects of chlorination, ozone and UV treatments on plasmid DNAs].
704 Nihon Koshu Eisei Zasshi **37:**745-51.

705 50. **Novick, R. P., and D. R. Jiang.** 2003. The staphylococcal saeRS system coordinates
706 environmental signals with agr quorum sensing. Microbiology-Sgm **149:**2709-2717.

707 51. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S.**
708 **Moghazeh.** 1995. The agr P2 operon: an autocatalytic sensory transduction system in
709 Staphylococcus aureus. Mol Gen Genet **248:**446-58.

710 52. **O'Neill, A. J.** 2010. Staphylococcus aureus SH1000 and 8325-4: comparative genome sequences
711 of key laboratory strains in staphylococcal research. Lett Appl Microbiol **51:**358-61.

53. **Paget, M. S., V. Molle, G. Cohen, Y. Aharonowitz, and M. J. Buttner.** 2001. Defining the disulphide stress response in Streptomyces coelicolor A3(2): identification of the sigmaR regulon. Mol Microbiol **42:**1007-20.

54. **Pane-Farre, J., B. Jonas, K. Forstner, S. Engelmann, and M. Hecker.** 2006. The sigmaB regulon in Staphylococcus aureus and its regulation. Int J Med Microbiol **296:**237-58.

55. **Raivio, T. L., and T. J. Silhavy.** 2001. Periplasmic stress and ECF sigma factors. Annu Rev Microbiol **55:**591-624.

56. **Repine, J. E., R. B. Fox, and E. M. Berger.** 1981. Hydrogen peroxide kills Staphylococcus aureus by reacting with staphylococcal iron to form hydroxyl radical. J Biol Chem **256:**7094-6.

57. **Roux, C. M., N. J. Booth, B. H. Bellaire, J. M. Gee, R. M. Roop, 2nd, M. E. Kovach, R. M. Tsolis, P. H. Elzer, and D. G. Ennis.** 2006. RecA and RadA proteins of Brucella abortus do not perform overlapping protective DNA repair functions following oxidative burst. J Bacteriol **188:**5187-95.

58. **Salgo, M. G., K. Stone, G. L. Squadrito, J. R. Battista, and W. A. Pryor.** 1995. Peroxynitrite causes DNA nicks in plasmid pBR322. Biochem Biophys Res Commun **210:**1025-30.

59. **Salisbury, V., R. W. Hedges, and N. Datta.** 1972. Two modes of "curing" transmissible bacterial plasmids. J Gen Microbiol **70:**443-52.

60. **Sancar, A., and W. D. Rupp.** 1983. A novel repair enzyme: UVRABC excision nuclease of Escherichia coli cuts a DNA strand on both sides of the damaged region. Cell **33:**249-60.

61. **Schuldiner, S., V. Agmon, J. Brandsma, A. Cohen, E. Friedman, and E. Padan.** 1986. Induction of SOS functions by alkaline intracellular pH in Escherichia coli. J Bacteriol **168:**936-9.

62. **Shaw, L., E. Golonka, J. Potempa, and S. J. Foster.** 2004. The role and regulation of the extracellular proteases of Staphylococcus aureus. Microbiology **150:**217-28.

63. **Shaw, L. N., J. Aish, J. E. Davenport, M. C. Brown, J. K. Lithgow, K. Simmonite, H. Crossley, J. Travis, J. Potempa, and S. J. Foster.** 2006. Investigations into sigmaB-modulated regulatory pathways governing extracellular virulence determinant production in Staphylococcus aureus. J Bacteriol **188:**6070-80.

64. **Shaw, L. N., C. Lindholm, T. K. Prajsnar, H. K. Miller, M. C. Brown, E. Golonka, G. C. Stewart, A. Tarkowski, and J. Potempa.** 2008. Identification and Characterization of sigma(S), a Novel Component of the Staphylococcus aureus Stress and Virulence Responses. PLoS One **3**.

65. **Shaw, L. N., C. Lindholm, T. K. Prajsnar, H. K. Miller, M. C. Brown, E. Golonka, G. C. Stewart, A. Tarkowski, and J. Potempa.** 2008. Identification and characterization of sigma, a novel component of the Staphylococcus aureus stress and virulence responses. PLoS One **3:**e3844.

66. **Spencer, J. P., J. Wong, A. Jenner, O. I. Aruoma, C. E. Cross, and B. Halliwell.** 1996. Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin

epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. Chem Res Toxicol **9:**1152-8.

67. **Staron, A., H. J. Sofia, S. Dietrich, L. E. Ulrich, H. Liesegang, and T. Mascher.** 2009. The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. Mol Microbiol **74:**557-81.

68. **T, O. C., and C. J. Dorman.** 2007. Expression of the Fis protein is sustained in late-exponential- and stationary-phase cultures of Salmonella enterica serovar Typhimurium grown in the absence of aeration. Mol Microbiol **66:**237-51.

69. **Tao, L. A., X. Q. Wu, and B. L. Sun.** 2010. Alternative Sigma Factor sigma(H) Modulates Prophage Integration and Excision in Staphylococcus aureus. PLoS Pathog **6**.

70. **Thackray, P. D., and A. Moir.** 2003. SigM, an extracytoplasmic function sigma factor of Bacillus subtilis, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. J Bacteriol **185:**3491-8.

71. **Traber, K., and R. Novick.** 2006. A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of agr and failure to translate delta- and alpha-haemolysins. Mol Microbiol **59:**1519-30.

72. **Truong-Bolduc, Q. C., and D. C. Hooper.** 2010. Phosphorylation of MgrA and its effect on expression of the NorA and NorB efflux pumps of Staphylococcus aureus. J Bacteriol **192:**2525-34.

73. **Voureka, A.** 1952. Induced variations in a penicillin-resistant staphylococcus. J Gen Microbiol **6:**352-60.

74. **Walthers, D., R. K. Carroll, W. W. Navarre, S. J. Libby, F. C. Fang, and L. J. Kenney.** 2007. The response regulator SsrB activates expression of diverse Salmonella pathogenicity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS. Mol Microbiol **65:**477-93.

75. **Watson, S. P., M. O. Clements, and S. J. Foster.** 1998. Characterization of the starvation-survival response of Staphylococcus aureus. J Bacteriol **180:**1750-8.

76. **Willetts, N. S.** 1967. The elimination of Flac+ from Escherichia coli by mutagenic agents. Biochem Biophys Res Commun **27:**112-7.

77. **Yeager, C. M., P. J. Bottomley, and D. J. Arp.** 2001. Requirement of DNA repair mechanisms for survival of Burkholderia cepacia G4 upon degradation of trichloroethylene. Appl Environ Microbiol **67:**5384-91.

780 **Figure Legends**

781

782 **Figure 1. Transcription profiling of *sigS* in a variety of *S. aureus* wild-type strains.**
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823 h, and again at 24 h, to determine β-galactosidase activity. Assays were performed on
824 duplicate samples and the values averaged. The results presented herein were
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826 (**C**) Quantitative real-time PCR analysis was performed with strain USA300 (grey) and
827 SH1000 (black) grown for 2 h under conditions identical to **A-B**, using primers specific

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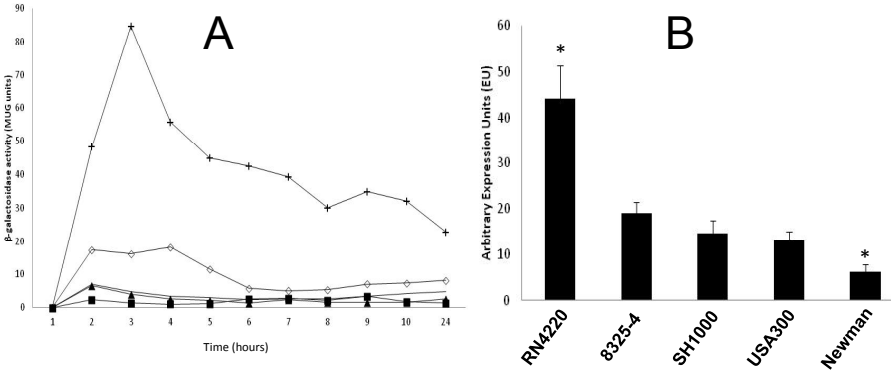

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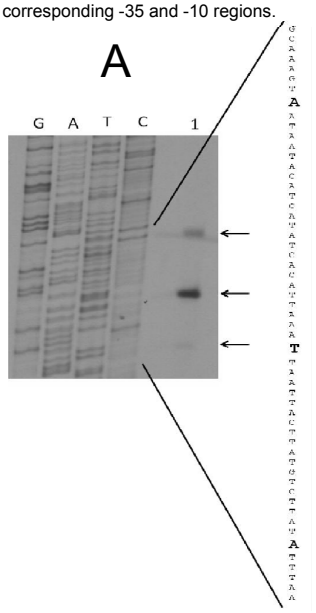

B

$^{-35}_{P1}$

TGTTACCTCCCGATTACAAATTTTGTTCTATCTA

$^{-10}_{P1}$ $^{-35}_{P2}$ $^{+1}_{P1}$ $^{-10}_{P2}$

CTATTTAGCAAAGTAATAATACATCATATCACA

$^{+1}_{P2}$ $^{-35}_{P3}$

TTAAATTAATTACTTATGTCTTATATTTAACAC

$^{-10}_{P3}$ $^{+1}_{P3}$

TTAATTTAGCGATTACGCAAATGAATGATTTTTC

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RBS Start

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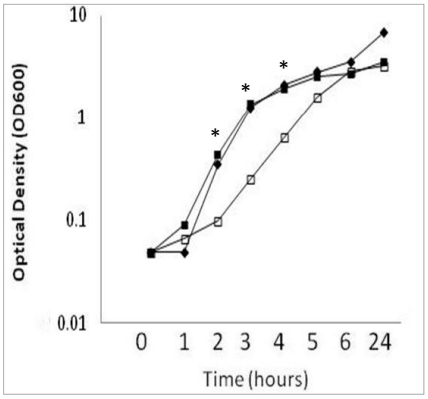

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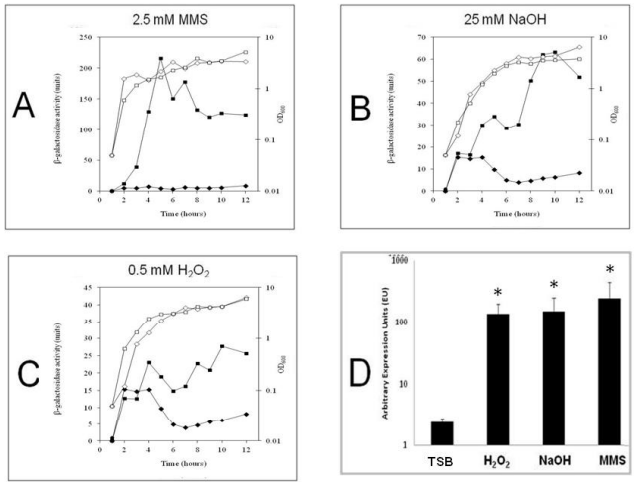

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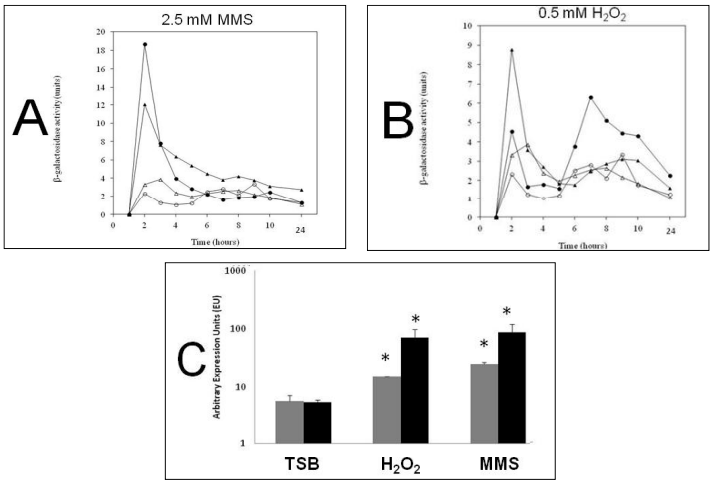

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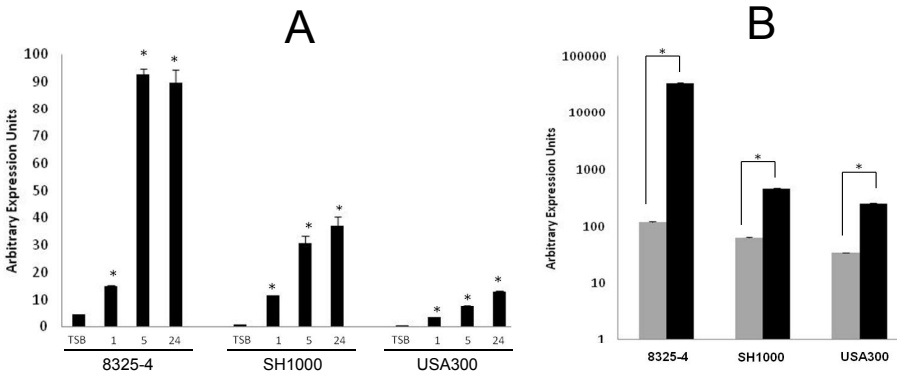

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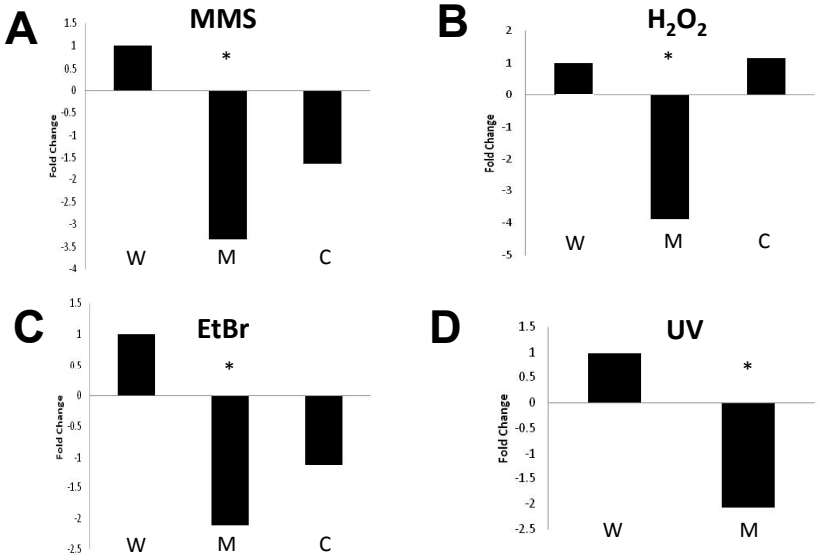

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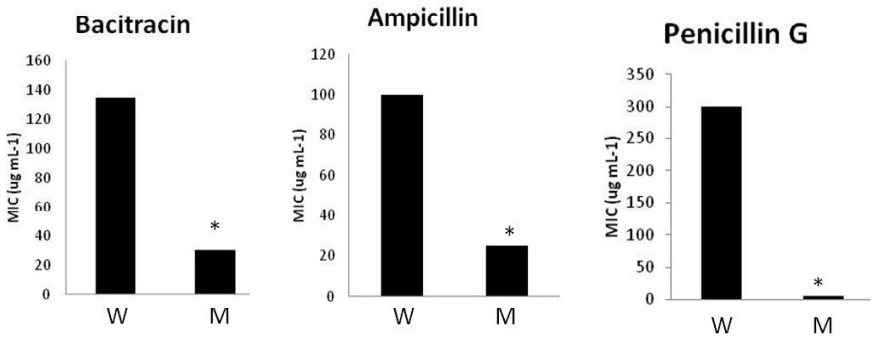

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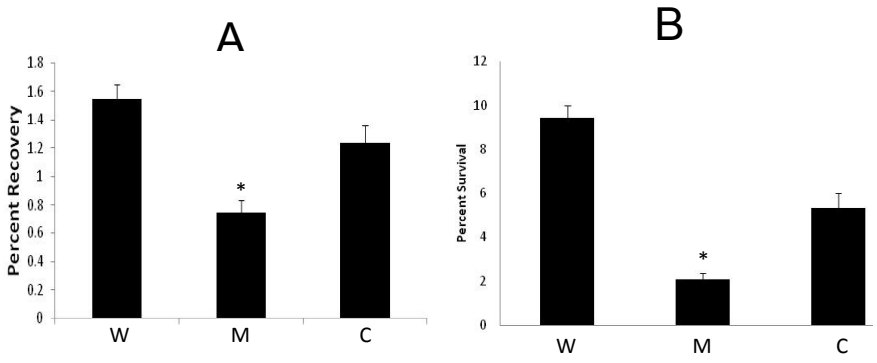

Table 1. Strains, plasmids and primers used in this study.

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	Sambrook *et al.*, 1989
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, human clinical isolate	Lab Stocks
USA300	USA300-LAC MRSA isolate cured of pUSA300-LAC-MRSA	Paul Fey UNMC
LES57	SH1000 pAZ106::*sigS-lacZ sigS* $^+$	Shaw *et al.*, 2008
HKM01	RN4220 pAZ106::*sigS-lacZ sigS* $^+$	This study
HKM02	8325-4 pAZ106::*sigS-lacZ sigS* $^+$	This study
HKM03	Newman pAZ106::*sigS-lacZ sigS* $^+$	This study
HKM04	USA300 pAZ106::*sigS-lacZ sigS* $^+$	This study
HKM05	RN4220 *sigS::tet sigS* $^-$	This study
HKM06	RN4220 *sigS::tet* pMK4::*sigS* $^+$	This study
HKM07	USA300 *sigS::tet sigS* $^-$	This study
HKM08	USA300 *sigS::tet* pMK4::*sigS* $^+$	This study
Plasmids		
pMK4	Shuttle vector	Sullivan *et al.*, 1984
pHKM1	pMK4 containing a 2.1 kb *sigS* fragment	This study
Primers[1]		
OL281	ACT GGA TCC CAG TTG CAG ATG CAT CTC TCC	
OL1715	ATG CTG CAG CAA GTC TAT CTG GCG TAC	
OL1036	CCG CGC ACA TTT CCC CGA AA	
OL1275	ACC TTG AAG GAT ACA AGC AA	
OL1276	GGC ATT TAC GCT TAA CGG AC	
OL1528	GTG GTG TTT GTT GTA TAC GTC	

[1] Restriction sites are underlined.

Table 2. Compounds found to induce expression of a *sigS-lacZ* reporter fusion in strain 8325-4.

Agent / Condition	Stress / Mode of Action	Overall Effect
Sodium Hydroxide (NaOH)	Alkali Stress	DNA damage
Hydrogen Peroxide (H_2O_2)	Oxidative Stress	DNA damage
Methyl methanesulfonate (MMS)	Alkylates DNA	DNA damage
Ethyl methanesulfonate (EMS)	Alkylates DNA	DNA damage
Ciprofloxacin	Inhibits DNA gyrase	DNA damage
Nalidixic Acid	Inhibits DNA gyrase	DNA damage
Chloramphenicol	Inhibits protein synthesis	Miscellaneous
Pig Serum	Components of the humoral immune system	Miscellaneous
Amino acid limiting media	Minimal media	Miscellaneous
Metal limiting media	Minimal media	Miscellaneous
Cefotaxime	Inhibits transpeptidation	Cell wall weakening/disruption
Ampicillin	Inhibits transpeptidation	Cell wall weakening/disruption
Oxacillin	Inhibits transpeptidation	Cell wall weakening/disruption
Sodium Dodecyl Sulfate (SDS)	Disrupts cell walls	Cell wall weakening/disruption
Phosphomycin	Inhibits UDP-N-aceytglucosamine-3-enolpyruvyltransferase	Cell wall weakening/disruption