

INSIGHTS INTO THE MOLECULAR MECHANISMS OF SENSING AND
RESPONDING TO THE HOST BY *STAPHYLOCOCCUS AUREUS*.

by

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ABSTRACT

Two-component systems (TCSs) are highly conserved across bacteria and are used to rapidly sense and respond to changing environmental conditions. The human pathogen *Staphylococcus aureus* uses the *S. aureus* exoprotein expression (*sae*) TCS to sense host signals and activate transcription of virulence factors essential to pathogenesis. Despite its importance, the mechanism by which the sensor kinase (SaeS) recognizes a stimulus and activates its cognate response regulator (SaeR) to regulate transcription of virulence genes is incompletely defined. However, findings from our lab suggest that SaeR/S mediated transcription is unique-to and dependent-on specific host stimuli. Studies outlined in this dissertation suggest that residues in the extracellular loop may be involved in refinement of the *sae* regulated targets at the single amino acid level. By generating single amino acid replacement mutants in the response regulator SaeR, we identified a key aspartate residue at position 46 (D46) on SaeR to be important in SaeR mediated signaling as mutation D46A prevented the recombinant protein from binding promoter recognition sequence and subsequently influenced virulence regulation. Current studies are aimed to define the phosphorylation patterns in SaeR using SDS-PAGE analysis and mass spectrometry. Overall, these structure-function studies provide insight into the Sae- signal transduction mechanism and raise some new questions regarding the role the Sae system in the larger regulatory network *S. aureus* uses to control expression of its secreted virulence factors.

CHAPTER ONE

INTRODUCTION

Staphylococcus aureus

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) has been identified as a human pathogen for centuries with first reported as “incurable boils in 1882 by Sir Alexander Ogston (1,2). *S. aureus* is a prominent cause of skin and soft-tissue infections worldwide and can also cause life-threatening diseases like septicemia and necrotizing pneumonia. *S. aureus* is a leading cause of both native valve and prosthetic valve infective endocarditis (3–5) and is associated with higher mortality rates relative to other microbes. Infectious endocarditis caused by *S. aureus* has a 100% fatality rate if left untreated (3,6,7). *S. aureus* is also the number one cause of skin and soft-tissue infections and in the United States causes approximately 12-13 million outpatient visits per year (3). *S. aureus* is an opportunistic pathogen colonizing almost every area of the human body; including the nose, pharynx, and skin (8,9). However, its preferred site of colonization is the anterior nares (9,10). About 30% of healthy individuals are colonized by *S. aureus* in the nares, where nasal carriage is substantially higher in children (45-70%) (9,11). Emerging strains of *S. aureus* have also become a major cause of human morbidity and mortality in healthy individuals. The exact mechanisms responsible for the increased ability to infect healthy hosts are not well understood.

The ability to express appropriate virulence factors in response to specific environmental cues is likely essential for pathogenesis (12–16). Bacterial recognition of

environmental signals and subsequent alterations in gene transcription are directed by two-component gene-regulatory systems, 16 of which have been putatively identified in the *S. aureus* genome (14).

S. aureus is also known to develop antibiotic resistance rapidly. Shortly after the first use of penicillin, *S. aureus* developed resistance via acquisition of a plasmid encoded penicillinase, causing hospitals to be plagued with penicillin-resistant *S. aureus* (17). Currently, due to the pressure to use vancomycin to treat methicillin resistant *S. aureus*, the pathogen has developed intermediate and full resistance to vancomycin (18,19). Recently, two lineages of vancomycin resistant *S. aureus* have been identified (18,19). Until recently, *S. aureus* was primarily a concern in hospitals and long-term care facilities, but the emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains that are able to kill healthy individuals has changed this trend (20).

The Bacterial Two-Component System

Since the discovery ~30 years ago, bacterial two-component systems have been extensively studied due to their complex multistep nature. Bacterial two-component systems are a way for bacteria to adaptively respond to environmental cues (21). More than 50,000 two-component systems have been identified with each system having regulatory features to guide diverse functions in their respective species (22,23).

Typical bacterial two-component systems consist of a trans-membrane histidine kinase and cytoplasmic response regulator (21,22) (Fig 1.1). Through structural studies, histidine kinases are classified into 3 broad categories based on their ability to sense

environmental cues. The first kind has the sensing domain in the extra-cytoplasmic region between the two trans-membrane helices. The second category has multiple membrane spanning helices and typically sense membrane-associated stimuli. The third group has a cytoplasmic sensing domain for sensing diffused internal stimuli (22,24). Most response regulators share about 26% sequence homology and have about 120 amino acid residues, typically comprised of two α/β folds (21,22).

Upon detection of environmental signal(s) the sensor kinase undergoes a conformational change, resulting in the auto-phosphorylation of the conserved histidine residue. This histidine residue typically resides in the dimerization and histidine phosphotransfer domain (DHp). The distal catalytic domain catalyzes the dimerization event. The phosphoryl group is then transferred to a conserved aspartate residue on the cytoplasmic response regulator (Fig 1.1). The phosphorylation of the response regulator typically leads to some structural changes (dimerization/oligomerization) in most response regulators leading to DNA binding thereby regulating gene expression (23,25). Dimerization brings the DNA binding sites in close proximity, enabling the response regulator to bind to the recognition sequence. Bacterial response regulators are highly sensitive and bind only to their recognized consensus promoter sequence, thereby adding another level of regulation of transcription of only specific genes (21,22). Some bacterial response regulators have diverse modes of regulation. For example, some response regulators induce open complex formation in RNA polymerases, thereby leading to inhibitory signals (22).

In order to prevent cross-talk between other two-component systems, most bacterial histidine kinases also have the capability to dephosphorylate the response regulator (22). Complex gene regulation is further achieved by having multiple histidine kinases and response regulators resulting in a phosphorelay system (22) (Fig 1.1d). For example in the hybrid kinase system the histidine kinase ArcA can be phosphorylated by ATP at either His containing domain, which further transfers the phosphate group to the response regulator ArcB. The different His-domain phosphorylations are utilized in aerobic versus anaerobic conditions (26) (Fig 1.1d). Typical phosphorelay systems undergo five phosphoryl transfer reactions and contain four phosphoprotein intermediates (histidine-aspartate) that are mechanistically linked by the phosphor-transfer event (21). There are many well characterized phosphorelay systems including the sporulation control system *spoOF* in *B. subtilis* (27), virulence control system *bvgSA* in *Bordetella pertusi* (28), and osmoregulation system SLN1/YPD1/SSK1 in *S. cerevisiae* (29).

In addition to the prototypical histidine-aspartate phosphorylation, bacterial two-component systems have evolved eukaryote like serine-threonine phosphorylation patterns as well (30–36). For example, the CovRS TCS in Group A and Group B *Streptococcus* is involved in regulating virulence gene expression, production of capsule and other factors essential to escape host immune system (37–39). *Streptococcus* has a eukaryote-like serine threonine kinase, namely serine threonine kinase-1 (stk-1). Studies have shown that the response regulator CovR can be phosphorylated by its cognate histidine kinase at aspartate 53 (Asp 53) resulting in the expression of virulence genes. In addition, CovR can also be phosphorylated via Stk-1 at threonine 65 (Thr 65) resulting in

decreased DNA binding capacity of CovR- thereby inhibiting virulence gene expression (39). Taken together, these data suggest that CovR Asp 53 activating phosphorylation and Thr 65 inhibiting phosphorylation have a complex interplay in Streptococcal host-pathogen interaction. Thus gene regulation by bacterial two-component systems is intricate and occurs at the single amino acid level.

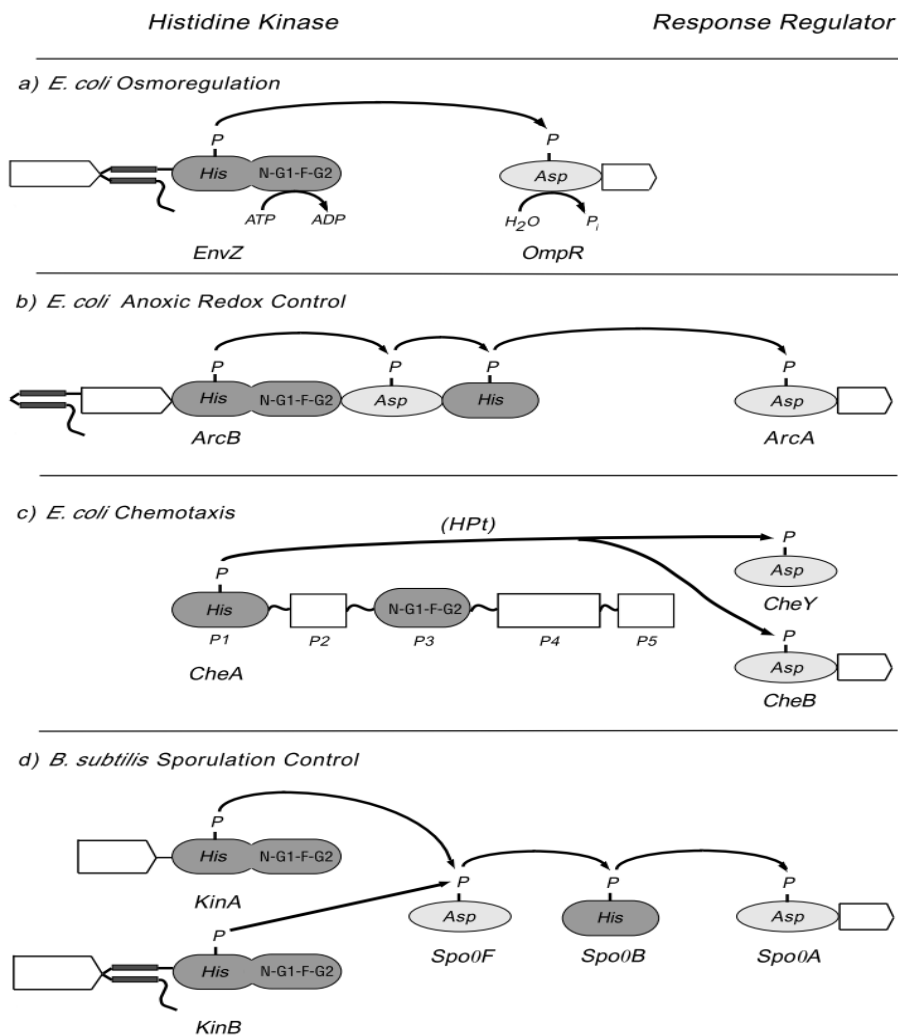


Figure 1.1: Schematic diagram depicting the modular organization of representative two-component systems. Asp-containing domains are colored dark gray. His-containing domains are colored light gray, and variable auxillary domains are colored white. (a) The

prototypical two-component pathway exemplified by the *E. coli* osmoregulatory system uses a single phosphoryl transfer event between the orthodox histidine protein kinase (HK) EnvZ and its cognate response regulator protein (RR) OmpR, (b) The *E. coli* Arc system illustrates a phosphorelay involving the hybrid HK ArcB. Depending on aerobic conditions, ArcA is capable of receiving a phosphoryl group from either the catalytic core or the His-containing phosphotransfer (HPt) domain of ArcB. (c) The *E. coli* chemotaxis pathway involves an atypical soluble HK CheA that phosphorylates either of the two RRs, the single domain RR CheY and the methylesterase CheB. (d) The *B. subtilis* sporulation control system is a multicomponent His-Asp-His-Asp phosphorelay system in which all of the signaling domains are independent proteins. SpoOF revives phosphoryl moiety from either KinA or KinB and subsequently transfers it to the HPt SpoOB, which then phosphorylates the terminal RR SpoOA. *Adapted from* (40).

Gene Regulation in *S. aureus*

S. aureus is capable of causing a variety of infections at multiple sites in the body due to its tissue tropism and the capacity to develop antibiotic resistance soon after the first use of a particular antibiotic.

Regulation of virulence by *S. aureus* contributes to the versatility of this pathogen. Virulence is regulated by many systems highlighting the importance of gene regulation for pathogen success. *S. aureus* has single component systems, small regulatory RNAs, two-component systems (TCS), mobile genetic elements, winged helix turn helix DNA binding proteins and alternative sigma factors (41–44) that form a complex gene regulatory network essential for successful pathogenesis. These regulation mechanisms refine when virulence factors are expressed during the axenic growth and in vivo during infection. Thus, timely expression of many factors contribute towards *S. aureus* pathogenesis

SarA System

SarA protein family members are a set of global regulators that affect transcription of staphylococcal genes (45). SarA can directly bind P2 and P3 promoters of the Agr system (discussed later) with different affinities thereby increasing transcription of *agrBDCA* and increases RNA III levels (45,46). In addition, SarA can affect virulence expression independently of the Agr system by directly binding promoter regions of virulence genes (47–50). The SarA system in *S. aureus* regulates a variety of virulence factors including alpha-toxin, Spa, hemolysins and proteolytic exotoxins.

In vivo therapeutic studies reveal the hypersusceptibility to oxacillin treatment in an infective endocarditis model by inactivation of *sarA*, which further reduces oxacillin resistance and thereby biofilm formation (51). Thus, *sarA* regulates oxacillin resistance in *mecA* positive MRSA.

S. aureus also possess eukaryote like serine threonine kinase (Stk) and phosphatases (Stp) that play a crucial role in cell wall metabolism, glycolysis, citrate cycle, purine-pyrimidine synthesis and translation. In addition they play a major role in virulence regulation. *S. aureus* has two Stk namely Stk-1 and Stk-2 and two Stp: Stp-1, Stp-2 (32–34,52).

Stk-1 and stk-2 both phosphorylate SarA to regulate gene expression. Studies suggest that Stk-1 phosphorylates SarA at a threonine residue in the N-terminus thereby enhancing the ability of SarA to bind promoter regions of target genes (53). On the contrary, stk-2 phosphorylates SarA at serine residues in the N-terminus, thereby

reducing the ability of SarA to bind promoter regions (53). Taken together, these studies reveal the intricate nature of gene regulation, wherein the residue that gets phosphorylated by the kinase dictates mode of positive versus negative regulation. In vitro studies have thus revealed the complex nature of *S. aureus* virulence gene regulation. Further research is needed to investigate gene regulation via SarA- stk1/stk-2 in the presence of an infection using an in vivo model.

Another example of complex gene regulation is that of a DNA binding protein, MgrA that regulates the expression of eight surface proteins that affect clumping, biofilm formation and virulence (54). The SarA system further cross talks with MgrA through dimerization, regulating virulence factors like alpha-toxin, coagulase, protein A, autolysins and synthesis of capsular polysaccharides (33,52). Stk-1 is shown to phosphorylate SarA/MgrA dimer at N-terminus cysteine residues to regulate virulence gene expression and also regulate genes important to confer antimicrobial resistance to antibiotics like vancomycin (55).

Two-Component Systems in *S. aureus*

S. aureus has sixteen different two-component systems (TCS) that survey the extracellular milieu for cues from environments encountered within the host. Several of the 16 two-component systems in *S. aureus* have been extensively studied including but not limited to Agr, WalRK, GraSR, and VraR/S. Many of these systems cross talk among each other resulting in several virulence genes regulated by multiple methods. Several vaccine and therapeutic trials have been unsuccessful due to our limited understanding of

in vivo regulation of virulence factors. The redundant nature of virulence regulation; wherein many signaling pathways contribute to the expression of a single essential virulence factor add to the complexity to our understanding of virulence gene regulation (10,56). For example, in response to limited oxygen availability by the host; increased virulence regulation is observed by *srrAB*, *nreBC*, *airSR* TCS. Another example is the regulation of hemolysins including alpha, beta and gamma hemolysins by Agr, SarA, WalKR, and Sae systems (10,57–60) contributing to the complex gene regulatory network, essential for the successful colonization and pathogenesis of the bacteria.

GraRS System

The GraRS system is involved in resistance to cationic antimicrobial peptides, virulence regulation, cell wall metabolism and vancomycin resistance (32,61,62).

Studies have shown that the response regulator GraR can be directly phosphorylated by its cognate histidine kinase GraS (36,52,63). In addition, eukaryote like stk-1 (discussed above) also phosphorylates GraR, thereby increasing its DNA binding capacity leading to increased gene expression for genes like *mprF* (phosphatidylglycerol synthetase), *dlt* (teichoic acid D-alanylation) (62).

VraRS System

The vancomycin-resistance-associated TCS responds to several antibiotics targeting the cell wall. The VraSR system dictates the regulation of over 40 genes in *S. aureus* including genes involved in biosynthesis of peptidoglycan in response to cell wall damage (64). Thus, VraS senses sub-lytic concentrations of β -lactams and vancomycin-

antibiotics that inhibit cell wall synthesis (19,65,66). VraRS system regulates transcription of genes involved in peptidoglycan synthesis both at early and late stages. For example the VraRS system regulates the transcription of *murF* involved in the synthesis of peptidoglycan precursor and *pbpB* involved in the incorporation of the precursor in the growing peptidoglycan which is essential to response against cell wall damage caused by antibiotics including β -lactams and vancomycin (67).

The response regulator VraR can be phosphorylated by its cognate histidine kinase VraS at an aspartate residue, resulting in DNA binding through a conformational change in VraR (64,67). In addition, *stk-1* phosphorylates VraR at Threonine 106, 119, 175 and 178 (Thr 106, Thr 119, Thr 175 and Thr 178), negatively affecting the ability of VraR to bind necessary promoter regions (31,36). Thus, the sensing and response mechanism in the VraRS TCS is highly complex and is regulated at the single amino acid level.

Agr System

Quorum sensing is population density dependent and environment dependent gene regulation that occurs through cell-cell communication. The Agr system in *S. aureus* is the quorum sensing four component system comprising of AgrC as the histidine kinase and AgrA as the cognate response regulator. In addition, the operon encodes AgrD, the precursor of the quorum signal that is processed and exported as a thiolactone-containing oligopeptide autoinducer (autoinducing peptide, AIP) by AgrB. Upon binding to the extracellular sensory domain of AgrC, AIP activates the kinase activity of AgrC, subsequently leading to phosphorylation of the response regulator AgrA. The

phosphorylated AgrA binds to the promoters (P2 and P3) and ultimately activates the transcription of *agrBDCA* and RNAIII. (68).

RNAIII is an intracellular molecule of the Agr system (69,70). RNAIII controls gene expression by binding the 5' untranslated region forming RNA duplexes, inhibiting the production of surface proteins (71). In addition, RNAIII blocks the translation of repressor of toxin (Rot) protein, thereby blocking the transcription of exoproteins and toxins (72–74).

The expression of the Agr system is altered in response to different stimuli. Acidic and basic pH, antimicrobial peptide gramicidin inhibit *agr* expression (75,76). However, subinhibitory concentrations of antibiotics like tetracycline and clindamycin increase expression of RNAIII and Agr-regulated genes (76). Transcriptional studies reveal that blood and blood components including: serum, and neutrophils result in a decrease in *agr* expression. The mechanism of inhibition is likely due to interference of AIP signaling by the direct binding of apolipoprotein and metalloproteins to AIP, thereby disrupting AgrC-AIP interactions (11,77–81).

Besides quorum sensing, the DNA-binding domain of the response regulator AgrA contains a redox-active cysteine (Cys-199), which forms an intramolecular disulfide bond with Cys-228 under oxidative stress. The oxidized AgrA dissociates from its cognate DNA, leading to down-regulation of the expression of RNAIII and up-regulation of the glutathione peroxidase gene (*bsaA*), which is shown to be important for bacterial resistance to oxidative stress (82). The Agr system regulates virulence expression in

response to host environment by several different mechanisms, thereby making a multifaceted gene regulation process.

WalRK system

The WalRK system is a TCS in *S. aureus* that affects virulence and cell wall metabolism genes. Mutational studies in the response regulator WalR- making a phosphomimetic response regulator (D55E), led to an increase in virulence gene expression that are regulated by the Sae system. Genes that were found to have increase in the transcription were *hla*, *hlgABC*, *fnbA*, etc suggesting that the WalRK system positively regulates the SaeR/S system (59,61,83).

The SaeR/S System

During the characterization of the Tn551 mutant for its defect in the production of exoproteins, Giraudo *et al* discovered the *Staphylococcus aureus* exoprotein, or SaeR/S system in 1994 (14). Since then numerous studies have been conducted to understand the role of the SaeR/S system in pathogenesis and its role in regulating the transcription of virulence factors. (12,14–16,60,84–90).

The *sae* operon encodes 4 genes: *saeP* encodes a lipoprotein; *saeQ* encodes a trans-membrane protein; *saeS* encodes a trans-membrane histidine kinase; and *saeR* encodes a cytoplasmic response regulator (87,90,91) (Fig 1.2). The *sae* operon further consists of 2 promoters referred to as P1 and P3 (12,88). The P3 promoter is located within *saeQ* and thereby regulates transcription of only *saeS* and *saeR* (12,14,87,92) .

While the P3 promoter is located upstream of *saeP* and regulates transcription of all four *sae* genes. The P3 promoter has 2 SaeR-recognized binding sequences and thereby is auto-induced by the SaeR/S two-component system (12,60,84,86).

SigB is a repressor of the Sae system via the P1 promoter (88,93). Repressor of toxins (Rot) has been shown to decrease Sae P1 promoter activity, thereby repressing the expression of *hla* via the SaeR/S system (58,94). Recently, fatty acid kinase (*fakA/fakB*) has also been shown to be associated to the Sae system by positively regulating the Sae-target genes including *hla*. In these studies, *fakB* was proposed to be the phosphoryl donor for SaeR (95,96).

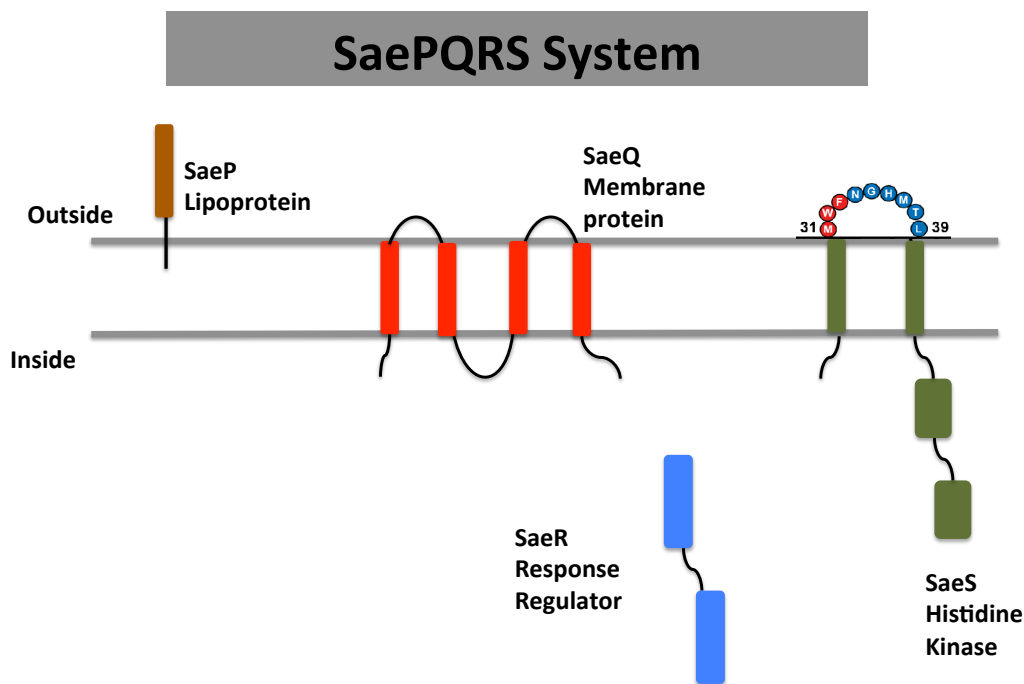


Figure 1.2: Model for the SaePQRS system. The Sae system consists of a lipoprotein SaeP, a trans-membrane protein SaeQ, a sensor kinase SaeS and a cytoplasmic response regulator SaeR (14,102). SaeS is predicted to have a nine amino acid extracellular loop, which is shown to be essential in sensing host-derived stimuli (91, 108). SaeR is known to bind a consensus promoter sequence, thereby regulating the transcription of genes, also known as SaeR-targets (61,86).

The interaction between SaeR/S and the Agr system is a topic of debate in the *S. aureus* field of research. Several experimental studies have suggested that the SaeR/S system lies downstream of the Agr system in the exoprotein production pathway (97,98). Mutation of *agr* has been shown to reduce *sae* P1 promoter activity (12,97). On the contrary, several studies suggest that *agr* and *sae* have opposite effects on the transcription of several virulence genes. For example, *coa* and *fnbA* are repressed by the Agr system, but activated by the SaeR/S system (14,90,99,100). The current studies suggest that Agr affects the transcription of *sae* via the P1 promoter, but does not affect the overall activity of the SaeR/S system.

The expression of alpha toxin gene *hla* is regulated by multiple regulatory systems in *S. aureus* including *agr*, *sae*, *sar*, etc (57,74,97). Studies suggest that *hla* expression is controlled by the Sae system at the transcriptional level (14,101) and at the translational level by the Agr system (70). There are several proposed mechanisms on the complex gene regulation achieved by the Sae system. An existing hypothesis in the research field suggests that SaeR has different binding affinities to SaeR- recognized DNA binding sequence, thereby leading to complex gene regulation. The variations in the sensitivity of SaeR binding to promoter region dictate some of the regulation patterns. Of the known promoter sequences, six have 1.5 SaeR binding sequence (i.e., three half binding sites). Since it is unlikely that all three half-binding sites are occupied by SaeR simultaneously, one of the half-binding sites at the boundary might be non-functional, leaving only one SaeR binding sequence (102,103).

The other hypothesis in the field explaining the mechanism of differential

regulation of virulence genes is the expression of 2 classes of genes dictated via levels of SaeR phosphorylation. Studies by Wolz et al have defined two types of phosphorylation in the SaeR/S system thereby regulating two classes of virulence genes i.e. type I and II (104). Transcription of type I virulence genes takes place irrespective of SaeS and requires low levels of SaeR phosphorylation while transcription of type II virulence genes requires SaeS and an increased amount of SaeR phosphorylation (104). The exact site of phosphorylation and the precise levels of phosphorylation have not been characterized molecularly.

The SaeR/S system senses a variety of host derived stimuli including changes in pH, temperature, and glucose levels (12,91). In addition, SaeS detects modifications in the membrane that are often related to phagocytosis related effector molecules like hydrogen peroxide, human neutrophil peptide-1 (HNP-1), mouse and human neutrophils (12,77,85,88,105). Sub-inhibitory concentrations of both hydrogen peroxide and alpha-defensins trigger the SaeR/S system to regulate virulence expression as early as 5 minutes post-infection. (105). Moreover, the response regulator SaeR recognizes a consensus promoter binding sequence and thereby regulates the transcription of varied toxins, adhesions and other virulence factors essential to host innate immune evasion (60,84).

SaeS is the sensor histidine kinase consisting of 351 amino acid residues. SaeS consists of two trans-membrane helices, a 9 amino acid extracellular domain (85,106). The extracellular domain plays an essential role in recognizing stimuli (discussed below). The C-terminus of the protein is comprised of a HAMP domain and a histidine kinase domain (Fig 1.2) (14,85,106). SaeS by itself only performs kinase

activity and is thought to form a protein complex with auxiliary protein SaeP and trans-membrane protein SaeQ to perform its phosphatase activities (107).

The response regulator SaeR belongs to the OmpR family of response regulators. The protein SaeR is a 228 amino acid long protein, comprising of a N-terminus phospho-receiving domain, which is predicted to be aspartate 51 (D51) and a C-terminus DNA-binding domain (104). Upon detection of an environmental signal, SaeS is thought to phosphorylate SaeR, which ultimately binds a consensus promoter sequence. SaeR recognizes a 17 base pair partially palindromic sequence comprised of 2 conserved domains (60,84). Binding of SaeR to its recognition sequence within the promoter region initiates transcription of target genes. These SaeR-regulated genes include toxins and immunomodulatory factors, such as *hla*, *hly*, *lukA*, *lukS-PVL*, *sbi* and *efb* (60).

The mechanism of sensing and thereby differentially regulating gene expression via the SaeR/S two-component system is intricate and operates at a single amino acid level. The nine amino acid extracellular loop of SaeS plays an important role in sensing different neutrophil-derived stimuli (85,106). Highly conserved residues M31, W32, and F33 on SaeS are essential to the appropriate activation of Sae-target genes (Fig1.2). Residue M31 is essential to the activation of *sae* regulon and plays an important role in sensing neutrophil-derived stimuli. Strains with mutations in aromatic residues W32 and F33 have disrupted normal basal signaling of SaeS in the absence of inducing signals, yet both mutant kinases have appropriate activation of effector genes following exposure to neutrophil-derived stimuli (85). These results suggest that the sensing and response mechanism of the SaeR/S system is more sophisticated than the mere on/off signal and

that the response is refined at the single amino acid level, thereby making *S. aureus* a successful pathogen.

In addition to the 9 amino acid extracellular loop sensing domain, the trans-membrane domain of *S. aureus* is thought to be necessary to respond to HNP-1 and thereby inducing SaeS activity (85,105,106). This hypothesis is based on the observation that HNP-1 causes membrane damage, thereby causing a conformational change in the trans-membrane helices. *S. aureus* strain Newman has a constitutively on SaeS. Upon characterization it was revealed that strain Newman has only one amino acid replacement from USA300 in SaeS that is within the first trans-membrane domain. The substitution of a leucine at position 18 to a proline appears to result in constitutive expression of SaeS (12,104). Taken together, these studies indicate that the sensing domain and thereby the kinase activity of SaeS is modulated at the single amino acid level by the extracellular sensing domain and that the trans-membrane domain likely plays a role in sensing due to conformational changes; giving the pathogen the advantage to sense and respond to host stimuli and being a successful pathogen.

Regulation of SaeR/S

Sae-regulated genes are expressed differentially during different growth phases (90). Genes including *chp*, *scn*, *eap*, *emp*, *coa* and *fnbA* are expressed in early exponential phase, while *hla*, *hlb* and *sae* P1 are expressed in the early stationary phase (90,92,104). Additionally, unlike other two-component systems, the Sae system is sophisticated at sensing and responding to its environmental cues differentially (85,104,105).

Neutrophils are the most abundant leukocytes in blood and are the first line of defense against bacterial infection. Upon phagocytosis, neutrophils expose bacteria to a variety of antimicrobial peptides like HNP-1, LL-37; reactive oxygen species (108–110). The Sae system plays an essential role to evade neutrophil phagocytosis (77) by sensing and responding to neutrophil and its components (77,85,105,109). For example, upon phagocytosis, the Sae system regulates the transcription of pore forming toxins like *hlgABC*, *hla*, *hly*, leukocidins, etc (77,85,105). Sub-inhibitory concentrations of hydrogen peroxide and beta-lactam antibiotics have been reported to activate the Sae system (12,105,111,112). The mechanism of activation is unknown, but studies suggest the extracellular sensing domain is involved (85,106). There are some other well studied host-factors that activate the Sae system which include neutrophils, human neutrophil peptide 1-3, and calprotectin.

Human neutrophil peptide 1, 2, and 3 (HNP1-3) are cationic antimicrobial peptides produced by human neutrophils that constitute 30% to 50% of the total protein in azurophilic granules and have potent antimicrobial properties (113). HNP1-3 activates the Sae system at sub-inhibitory concentrations of 0.5-2 $\mu\text{g/mL}$ (12,85,105). The activation is specific to HNP1-3 as other cationic antimicrobial peptides as LL-37, daptomycin, are unable to activate the Sae system (12). Interestingly, the response to neutrophils and its components via the Sae system is unique and that only a subset of genes are turned on in response to each trigger (85,105). For example, only hemolysins are turned on in response to HNP-1 and leukotoxins are not (85). The mechanism of

activation is unknown, however the existing hypothesis suggests that the cationic antimicrobial peptide cause a membrane damage in *S. aureus*, which leads to a conformational change in SaeS leading to initiate its kinase activity (93). However, the actual experiments testing this hypothesis need to be performed. Intriguingly, murine neutrophils that do not produce HNP1-3 also activates the Sae system, indicating that murine neutrophils contain other Sae-activating molecules (105,114,115).

Recently, the role of calprotectin in activating the Sae system has been demonstrated (115). Calprotectin is the most abundant cytoplasmic protein in neutrophils and decreases bacterial burden by sequestering zinc and manganese. Calprotectin turns on the Sae system and lead to the production of proinflammatory cytokines like IL-1 β and TNF- α (115), thereby increasing bacterial virulence and promoting host mortality.

As described, the Sae system senses and activates its kinase activity by a variety of host-derived stimuli. Certain host-derived stimuli also turn down *sae* activity including acidic pH, NaCl (12,75,101); 18 β -glycyrrhetic acid (116); silk apolipoprotein (117,118); metals like copper, zinc and manganese, florfenicol (119); corilagin (89); licochalcone A (120); thymol (121); the enoyl-acyl carrier protein reductase inhibitor AFN-1252 (121); cerulenin (122); subinhibitory concentration of linezolid (123); Manuka honey (124); and flavone (125).

S. aureus and The Host Innate Immune System

The SaeR/S system regulates virulence expression and controls the transcription of about 2-8% of genes (60,77). Deletion mutant strains of *saeRS* are unable to express adhesion genes including *fnbA*, *eap*, *emp*; toxins like leukotoxins, hemolysins etc (91,92,126). Gene expression is not only different during different phases of bacterial growth (92) but the Sae system controls virulence expression in a sophisticated way in response to different host-derived stimuli (85,105). For example, the SaeR/S system increases the transcription of pore forming toxins like *hla*, *hly*, and *lukAB* in response to neutrophil phagocytosis (85,105,127,128).

Both neutrophils and *S. aureus* have factors that make the sensing and phagocytosis and thereby escape from phagocytosis possible. *S. aureus* affects neutrophil response at various stages like neutrophil priming, activation and adhesion, recruitment, ability to produce reactive oxygen species, and its ability to kill bacteria (details can be found in appendix A). The bacteria affects neutrophil activation, chemotaxis and thereby adhesion by transcribing genes involved in immunomodulation. For example, the chemotaxis inhibitory protein of *S. aureus* inhibits neutrophil migration and activation thereby preventing neutrophils from reaching the site of infection (129).

S. aureus produces two proteins that bind and inhibit the activation of formyl peptide receptor-1-binding to which leads to mobilization and chemotaxis leading to superoxide production (109). Staphylococcal infections induce chemoattractants like CXCL-1, CXCL-2, and CXCL-8 that are essential in neutrophil recruitment (130,131). In order to prevent neutrophil recruitment, *S. aureus* produces cysteine protease

Staphopain A that cleaves CXCR-2 at the N-terminus, thereby preventing ligand binding essential for neutrophil recruitment (132). Thus, *S. aureus* employs a variety of strategies to prevent neutrophil recruitment essential for successful pathogenesis.

S. aureus also uses a multitude of strategies to inhibit neutrophil phagocytic activity and killing. It has the ability to produce several virulence factors that target different aspects of phagocytic activity of the neutrophil. For example, *S. aureus* complement inhibitor (SCIN) binds C3 convertase preventing opsonization (133). It also has the ability to inhibit immunoglobulin recognition by transcribing genes that bind immunoglobulins directly. Membrane bound Protein A binds the constant region of FC γ region of IgG preventing proper recognition of FC γ receptors on the neutrophil (134–136). SaeRS regulated gene *sbi* is transcribed to prevent neutrophil mediated phagocytosis by binding IgG (60,137,138). Taken together, these studies suggest that *S. aureus* uses an array of tricks to prevent phagocytosis by the neutrophil. In addition to avoiding recognition by neutrophils, *S. aureus* can evade neutrophil killing as well. *S. aureus* cause cell membrane modifications to decrease efficacy of antimicrobial peptides by electrostatic repulsion. For example genes containing *dltABCD* increases *S. aureus*'s tolerance to cationic peptides like HNP-1 by incorporating positively charged D-alanine into teichoic acids (139,140). *S. aureus* regulated staphylokinase binds HNP-1 directly and inhibit its bactericidal activity (141).

Upon phagocytosis, the NADPH oxidase complex is assembled within the neutrophil that leads to the production of reactive oxygen species and hypochlorous acid that are essential to kill phagocytosed bacteria (142–145). *S. aureus* produces several

enzymes in response to neutrophils that detoxify reactive oxygen species generated by the assembly of NADPH oxidase. For example, it produces two superoxide dismutases encoded by *sodA* and *sodM* genes that convert superoxide to hydrogen peroxide and molecular oxygen (146). In addition, SaeRS regulated virulence factors decrease neutrophil peroxide and hypochlorous acid production following phagocytosis through mechanisms unknown (147). In addition to oxidative-mediated killing, neutrophils are capable of extruding DNA decorated with antimicrobial peptide- Neutrophil extracellular traps (NET) (148–150). *S. aureus* is able to express SaeRS regulated *nuc* to escape NETs (151). Collectively these studies suggest that *S. aureus* produces a variety of factors that affect the neutrophil's ability to kill bacteria.

Virulence factors regulated by the SaeR/S system modulate the host response to promote infection. In response to host immune response, the Sae system leads to the production of TNF- α , IFN- γ , IL-1 β and IL-6, thereby leading to a robust proinflammatory response. In a mouse model of invasive infection, increased bacterial burdens, followed by increased mouse mortality were observed (152). These results were confirmed in human whole blood assays that showed increased production of TNF- α , IFN- γ , IL-6 and IL-2 in blood treated with secreted factors from Δ *saeR/S* versus wild-type *S. aureus* (152). In congruence with these studies a mouse model of peritonitis demonstrated Sae system promoted IFN γ production in neutrophils that correlated with exacerbated disease (153). Several additional mouse and rabbit models of skin infection, osteomyelitis, infective endocarditis and pneumonia have confirmed the importance of the Sae system in pathogenesis (6,60,128,154–156). It is the ability of the Sae system to sense and

respond to environmental cues in a timely manner as witnessed by the expression of only a subset of genes in response to PMNs and other host derived-stimuli(85,105), that makes it essential to pathogenesis. The precise mechanism of this differential gene regulation is unknown.

Hypothesis

The SaeR/S system regulates a variety of toxins and immunomodulatory factors (15,60). Studies from our lab (60) and Bae et al (84) identified a similar 17 base pair palindromic sequence (GTTAAN₆GTAA) and confirmed binding affinities using EMSAs (84). One major difference between the two studies was the requirement to phosphorylate recombinant SaeR-His for EMSA assays. Our group was able to show SaeR binding to SaeR-recognized DNA-binding sequences without having to artificially phosphorylate the protein (60). However, Bae et al showed increased binding of DNA-binding sequence with increasing levels of SaeR phosphorylation (84). These observations further demand the need to investigate the role of phosphorylation of SaeR in Sae-mediated virulence gene expression. In addition, precise mechanisms of how SaeR/S regulates virulence in physiological conditions remain unknown and the impact of host-derived stimuli on phosphorylation and virulence gene expression is incompletely defined. Structural studies of SaeR reveal that the SaeR DNA binding domain is a winged helix turn helix motif and exists as a monomer. Residues lysine 174, histidine 198, arginine 199, arginine 201 and tryptophan 218 are critical for DNA binding activity and thereby transcription of Sae-regulated genes (157,158). Interestingly, the DNA binding

domain binds to target recognition DNA sequence without phosphorylation, suggesting that an unphosphorylated receiver domain (D51) prevents DNA binding and that SaeR undergoes a conformational change upon phosphorylation (158). Additional structural studies including full length SaeR are needed to confirm this hypothesis and to understand the importance of residue D51 in the signaling mechanism of SaeR. This study tests the hypothesis that host-derived signals sensed by SaeS influence phosphorylation patterns in SaeR to regulate virulence gene expression. We first generated plasmid based single amino acid mutant strains in the predicted nine amino acid extracellular loop of SaeS. Through site-directed mutagenesis, we identified key residues in SaeS essential to sensing human neutrophils and neutrophil-derived stimuli (Highlighted in chapter 2). Following, we investigate the mechanism of signaling in the response regulator SaeR specifically looking at role of phosphorylation in gene regulation. In order to determine the role of phosphorylation in gene regulation, we generated plasmid-based single amino acid mutant strains at predicted phosphorylation sites. These sites included aspartate (D) residues 61 (D61) and D46. Through a series of molecular methods using these strains and a clinical isolate (18805) that contains a natural mutation in D51 (159) we investigated the role of phosphorylation during neutrophil interaction to define its impact on gene expression. In addition, we tested the ability of recombinant proteins from wild-type and mutant strains to bind known SaeR-recognized DNA binding sequences and identified phosphorylation patterns on our recombinant proteins using mass spectrometry. Our results suggest the SaeR/S system can take advantage of other putative phosphorylation sites in order to generate a specific

response and that phosphorylation of D51 may not be necessary to confer gene expression.

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CHAPTER TWO

DIFFERENTIAL REGULATION OF STAPHYLOCOCCAL VIRULENCE BY THE
SENSOR KINASE SaeS IN RESPONSE TO NEUTROPHIL-DERIVED STIMULIContribution of Authors and Co-authors

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Contributions: Conceived the study, provided funding and edited/critiqued manuscript in every stage of preparation.

Manuscript Information Page

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Introduction

Staphylococcus aureus (*S. aureus*) is a successful pathogen due to its ability to adapt to its host environment by sensing and responding to its host derived stimuli using varied two-component gene-regulatory systems (TCS). The *Staphylococcus aureus* exoprotein (Sae) TCS encodes 4 genes namely *saeP* which coded for a lipoprotein, *saeQ* which codes for a transmembrane protein, *saeS* which codes for a histidine kinase and *saeR* which codes for a cytoplasmic response regulator. Recently, SaePQ were shown to form a protein complex with SaeS to turn off kinase activity, thereby repressing the Sae system (1). Previous studies from the lab have shown that *S. aureus* can turn on specific subsets of genes in response to neutrophil and neutrophil-derived stimuli, and that the response for each stimulus is different (2). In this study, we mutagenized the nine amino acids in the putative extracellular loop of SaeS and determined that the highly conserved methionine residue (M31) is essential for sensing human neutrophils and human neutrophil peptide-1 (HNP-1). We also demonstrate that the aromatic amino acid residues tryptophan (W32) and phenylalanine (F33) appear to refine the sensory capacity of SaeS. Collectively, our observations suggest that the reciprocal communication between host and the pathogen is complex and is not simply an on/off signal.

Materials and Methods

Substituted Cysteine Accessibility Method (SCAM)

SCAM was performed in *S. aureus* strain AH 2216 (Δ saePQRS) containing individual SaeS cysteine constructs (3). In brief, overnight cultures were diluted 1:50 in 25mL TSB containing Cam and 0.1% xylose. Cultures were grown in a 37°C shaker to an OD₆₀₀=2.0, pelleted and resuspended to an OD₆₀₀=25 in buffer A (100mM HEPES, 250mM sucrose, 25mM MgCl₂, 0.1mM KCl pH 7.5). Duplicate sets of 100 µl aliquots were made of each strain. One set of samples was pre-treated with 20 µg lysostaphin (AMBI products) and 2U DNase I (Fermentas) at 37°C for 30 min prior to labeling with 50 µM MBP (Nα-(3-maleimidylpropionyl)biocytin, Invitrogen) at room temperature for 10 min in the dark. Labeling was quenched by adding 73mM BME. The second set of samples were treated with 50µm MBP, quenched with 73mM BME and washed with buffer A containing 20mM BME before incubating with lysostaphin and DNase I. Both sets of samples were solubilized by adding 400 µl T7 binding/wash buffer (4.29mM Na₂HPO₄, 2.7mM KCl, 137mM NaCl, 1.% NO-40 substitute, pH 7.3).

To immunoprecipitate labeled SaeS constructs, 40 µl anti-T7 agarose (Novagen) was added to each sample and incubated at room temperature for one hour, followed by three 20min washes with 600 µl T7 binding/wash buffer. Samples were eluted with 60 µl T7 elution buffer (100mM citric acid, pH 2.2, 0.5% SDS) at room temperature for 15 min. Following elution, 55 µl sample was collected to avoid disturbing the agarose. Samples were neutralized with 5 µl 2M Tris, pH 10.4. Since boiling membrane proteins can cause

aggregation, samples were heated at 60°C for 10 min following addition of 2X SDS-PAGE loading buffer containing 5% BME.

Analysis of Gene Expression

Overnight cultures of *S. aureus* were diluted 1:100 in TSB and grown to an OD₆₀₀ = 1.5. One mL of each culture was pelleted and resuspended in 750 µl RLT buffer plus BME from RNA purification kit RNeasy (Qiagen) as described in (2,4)(77,105) .

Expression was analyzed by TaqMan qRT-PCR in triplicate and *saeS* expression was normalized to *gyrB* as described (2,4). Relative quantification of *saeS* was expressed as the log₁₀ fold change relative to the wild type bacteria.

PMN Membrane Permeability Assay

Polymorphonuclear leukocytes (PMNs or neutrophils) were isolated from healthy human donors following procedures described elsewhere (4). All procedures were performed in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. In order to perform PMN membrane permeability assays, Human PMNs at 1×10^6 cells/µL were added to 96 well round bottom plates, prior coated with 20% normal human serum in DPBS. Bacteria at 1×10^7 CFU were added to human PMNs. Infections were synchronized ($400 \times g$, 8 min, 4 °C), and plates were incubated statically at 37 °C with 5% CO₂ for 3 hrs. Membrane permeability was measured by flow cytometry using propidium iodide (Invitrogen). To

measure PMN membrane permeability caused by bacterial supernatants, overnight bacteria were diluted 1:100 and grown for 5hrs. Spent supernatants were filter sterilized and further diluted 1:10 in RPMI with 5mM HEPES. Diluted supernatants were added to human PMNs. Intoxication was synchronized (as described above), and PMN cytotoxicity was measured using flow cytometry (5).

Identifying Sensing Domain of SaeS

In order to identify the topology of SaeS we used substituted cysteine accessibility method (SCAM) (6,7). Briefly, cysteine containing proteins are labeled with a maleimide ring conjugated to biotin. SCAM screens suggested that C50 and C56 were in the transmembrane region and that C226 was in the cytoplasmic region. We combined SCAM with TOPCONS (8,9) (a consensus topology prediction program) to get a more complete topology of SaeS. The combination of these methods predicted a nine amino acid extracellular loop as the sensing domain connected with two transmembrane helices (Fig 2.1). We further aligned SaeS sequence against all staphylococcal species and found that first 3 residues of the sensing loop- methionine residue (M31), tryptophan residue (W32) and phenylalanine residue (F33) are highly conserved across all staphylococcal species (Fig 2.1).

We next mutagenized the nine amino acids in the putative extracellular loop of SaeS to alanine residues using site-directed mutagenesis (9). Briefly, using two-step overlap PCR, we generated a markerless *ΔsaeS* strain. Using primers specific to each

mutation, we generated single amino acid replacement mutant strains in pEPSA5 plasmid. Each strain was further C-terminally T7 tagged and each of the plasmids containing the *saeS* with each of the single amino acid substitutions was transformed into the *ΔsaeS* strain.

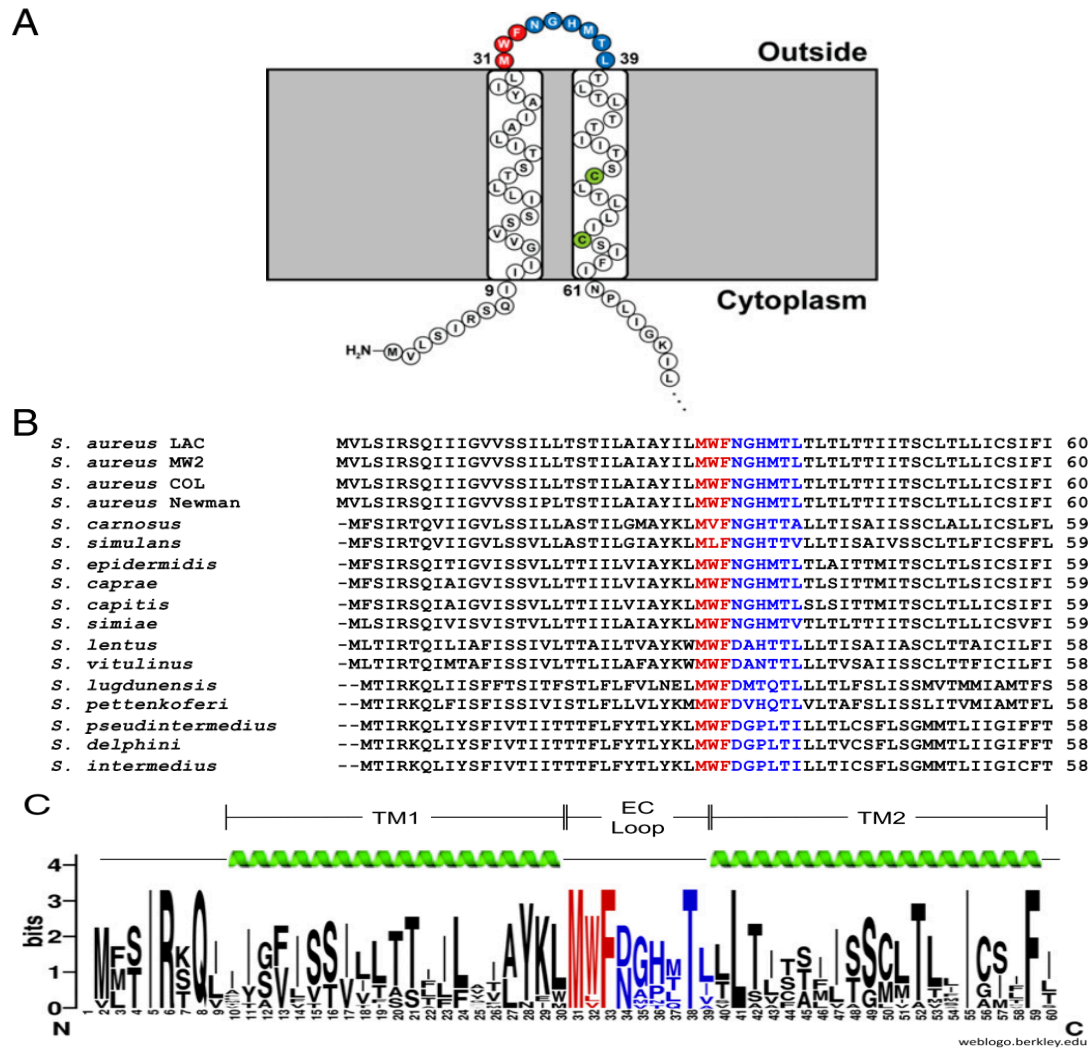


Figure 2.1: Residues in the EC loop of SaeS are conserved across staphylococcal strains and species. (A) Cartoon representation of the predicted topology of LAC's SaeS sensing domain from TOPCONS. (B) A sequence alignment of the sensing domain of SaeS from all staphylococcal species and strains of *S. aureus* identified as having Sae systems shows a high level of conservation across the entire domain. SaeS sequences from species other

than *S. aureus* were identified by comparison with the *S. aureus* LAC sequence using BLAST. The first three predicted extracellular residues (depicted in red) are completely conserved with the exception of W32 in *S. carnosus* and *S. simulans*. Strain sequences used for each species are as follows: *S. carnosus*, TM300; *S. simulans*, ACS-120-V-Sch1; *S. epidermidis*, M23864:W1; *S. caprae*, C87; *S. capitis*, SK14; *S. simiae*, CCM 7213; *S. lugdunensis*, M23590; *S. pettenkoferi*, VCU012; *S. pseudintermedius*, HKU10-03. Other species not listed did not have strains designated. (C) An SaeS Weblogo constructed from the sequences listed in A. For *S. aureus*, only the LAC sequence was used. TM1, transmembrane domain 1; TM2, transmembrane domain 2.E2038.

The Expression of *saeS* and *saeR* Are Not Affected By Single Amino Acid Replacements

To determine if single amino acid substitutions influenced expression of *saeS* and *saeR*, bacteria were harvested at mid-exponential growth phase and RNA was extracted from bacterial cells using previously described methods (2,4). Relative quantitation of *saeS* and *saeR* was done using TaqManTM RT-PCR with primers and probes specific to *saeS* and *saeR* and *gyrB* (endogenous control). Results demonstrated that mutations in the extracellular loop of *saeS* did not influence transcript abundance of *saeS* or *saeR* (Fig 2.2).

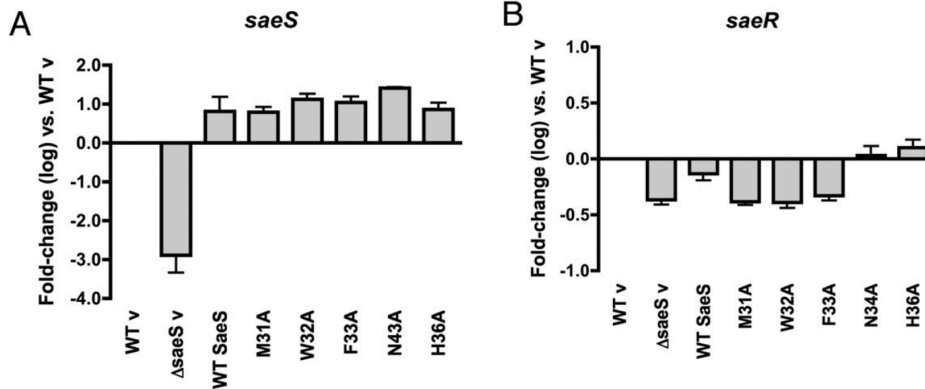


Figure 2.2: In-vitro basal transcription of *saeS* and *saeR* verified by qRT-PCR. RNA was extracted from bacteria harvested at midlog (OD600 = 1.5) and examined by TaqMan quantitative real-time PCR. Normalizing to *gyrB* and expressing mean values as log₁₀ changes relative to the WT control determined relative quantification of transcripts. Two to four biological replicates of each experiment were analyzed in triplicate. (A) Expression of *saeS*. (B) Expression of *saeR*.

SaeS Mutant M31A Is Unable to Induce Cytotoxicity in Human PMNs Following Phagocytosis.

In order to assess the effect of amino acid substitutions on pathogenesis, we infected human polymorphonuclear leukocytes (PMNs or neutrophils) with wild-type (USA 300) and isogenic *saeS* mutant strains and assessed PMN cytotoxicity by evaluating plasma membrane damage using propidium iodide uptake as described in (77).

We have previously demonstrated that the SaeR/S system is essential for *S. aureus* survival following neutrophil phagocytosis (4). The SaeR/S system regulates an abundance of virulence factors known to cause neutrophil lysis and membrane damage (2,4). To determine if specific residues in the putative extracellular loop influenced production of cytolytic factors, we assessed plasma membrane damage following

phagocytosis of mutant and wild type *S. aureus* strains. Wild type bacterial supernatants caused >90% plasma membrane damage whereas the M31A mutant demonstrated significantly less (~50%) and was similar to the $\Delta saeS$ strain (Fig 2.3 A). These data suggested that the mutant M31A is deficient in sensing human PMNs and thereby producing virulence factors necessary to evade neutrophil phagocytosis.

Additionally, we subjected human PMNs to wild-type bacteria at a 10:1 (bacteria: human PMNs) multiplicity of infection (MOI) for 3 hours. Wild-type bacteria were able to induce ~85% plasma membrane damage whereas the mutant M31A was unable to induce any (~1%) plasma membrane damage and was similar to the $\Delta saeS$ strain (Fig 2.3B). Interestingly, our plasma membrane damage assays with bacterial supernatants and whole bacteria revealed an interesting aspect of the sensing extracellular loop of SaeS. Bacterial supernatants from strain M31A were unable to cause membrane damage, suggesting that strain M31A like $\Delta saeS$ strain is deficient in producing toxins that are *saeR/S* regulated. On the contrary, whole bacteria from strains M31A, W32A and F33A like $\Delta saeS$ strain are unable to sense human PMNs and thereby deficient in generating a response i.e. releasing toxins essential to evade PMN phagocytosis. Moreover, these, suggest that perhaps residue M31 is essential to sensing host-derived stimuli-specifically human neutrophils and that residue M31 is deficient in making *sae*-regulated toxins, thereby unable to respond to neutrophil phagocytosis.

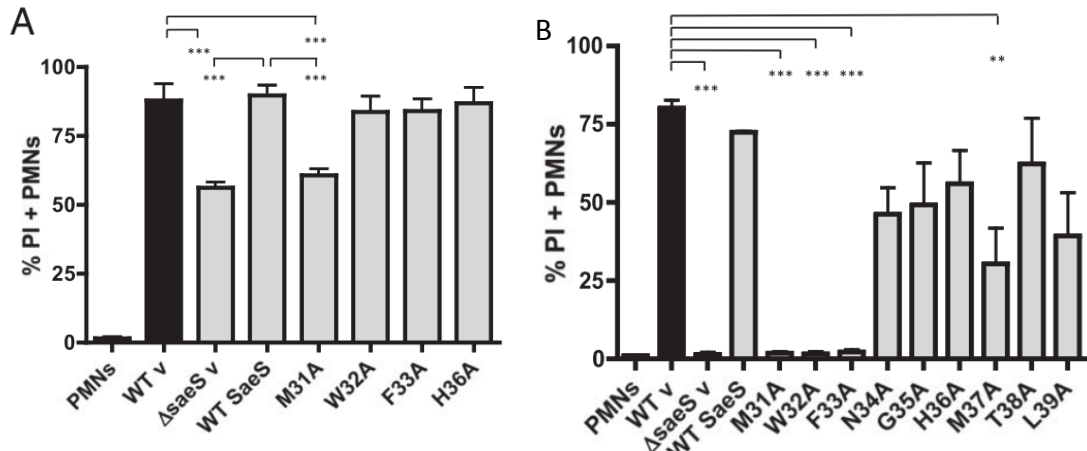


Figure 2.3: Residue M31 in the SaeS sensing domain is essential for SaeS-induced PMN plasma membrane permeability and lysis. (A) PMN plasma membrane permeability at 1 hour after incubation with bacterial supernatants. (B) Compiled results representing three separate experiments investigating PMN plasma membrane permeability 3 hours after infection with SaeS EC mutants (10:1 MOI). Results are the mean $\pm$ SEM of three separate PMN donors. Asterisks denote statistical significance evaluated by a one-way ANOVA with Tukey's posttest based on the following P values: *0.01–0.05, **0.001–0.01, and ***<0.001. E2040.

Transcription Analysis Confirms Influence of Residue M31 on Virulence Factors

Wild type and *saeS* mutant bacteria were further subjected to transcriptional assays following exposure to human PMNs and HNP-1. Transcription of *hlgA* was reduced in strains M31A, W32A and F33A like Δ *saeS* strain in the presence of media only compared to wild-type bacteria. On the contrary upon exposure to either human PMNs or HNP-1 for 30 minutes induced expression of *hlgA* in strains W32A and F33A. Interestingly like Δ *saeS* strain, M31A was unable to induce transcription of *hlgA* in the presence of host-derived stimuli [For figures see reference (10)]. Additionally, we used Quantigene 2.0, to determine the transcription of virulence genes *hlgA*, *lukA*, and *hla* increased in the presence of host stimulus (human PMNs and HNP-1) for wild type, W32A and F33A strains. No change in gene expression was observed following exposure

to host stimulus in *ΔsaeS* strain and M31A mutant strains [For figures see reference (10)]. Taken together, these data demonstrate that although the aromatic point mutations W32A and F33A are able to activate sae-dependent toxins in a signal specific manner, basal expression of several *sae*-regulated toxins is reduced in these mutants. These data suggest a role of aromatic residues in providing structural stability to SaeS during sensing of host-derived stimuli (11,12). Recent biochemical studies on *Escherichia coli* and *Saccharomyces cerevisiae* have identified the importance of aromatic residues in stabilizing the overall structure of trans-membrane helices and thereby essential in further ligand binding and protein activity (11-15). Collectively these results suggest that the SaeR/S system utilizes complex sensing mechanism that refine the transcriptional response based on the specific stimulus.

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CHAPTER THREE

ROLE OF SaeR PHOSPHORYLATION IN REGULATION OF *Staphylococcus aureus*
VIRULENCE GENES

Contribution of Authors and Co-authors

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Contributions: Conceived the study, provided funding and edited/critiqued manuscript in every stage of preparation.

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Abstract

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is a leading cause of skin and soft-tissue infections and infective endocarditis worldwide. *S. aureus* uses two- component gene regulatory systems (TCSs) to sense and respond to varied environmental conditions. The pathogen's success is due in part to the *S. aureus* exoprotein expression (SaeR/S) system. Upon detection of host-derived stimuli sensed by the sensor kinase SaeS, the response regulator SaeR is thought to be phosphorylated and binds a consensus DNA binding sequence, thereby regulating virulence factor expression. We have recently demonstrated that virulence expression in *S. aureus* is much more sophisticated than the simple on/off signal. Through this study, we aimed to determine the influence of aspartate residue 46 of SaeR (D46) on *S. aureus* gene expression and neutrophil evasion. We use site directed mutagenesis to substitute an alanine for aspartate (D46A) on SaeR. We demonstrate this residue to be important in virulence gene expression following neutrophil interaction. Additionally, we observe that recombinant SaeR-D46A protein was unable to bind target genes containing the SaeR-DNA binding sequence. The molecular mechanism dictating modulation of pathogenesis at the host-pathogen interface. Our study has identified a role for D46A in SaeR- mediated signaling. The findings from this study add to the understanding of complex regulatory networks contributing to *S. aureus* pathogenesis.

Introduction

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is a prominent cause of skin and soft-tissue infections worldwide and can also cause life-threatening diseases like septicemia and necrotizing pneumonia. It is a leading cause of both native valve and prosthetic valve infective endocarditis (1-3) and is associated with higher mortality rates relative to other microbes. Infectious endocarditis caused by *S. aureus* has a 100% fatality rate if left untreated (1,4,5). The ability to express appropriate virulence factors in response to specific environmental cues is likely essential for pathogenesis (6-10). The clinical *S. aureus* strain USA300 genome encodes a diverse assortment of virulence factors that promote survival. Bacterial recognition of environmental signals and subsequent alterations in gene transcription are directed by two-component gene-regulatory systems, 16 of which have been putatively identified in the *S. aureus* genome (8).

The innate ability of *S. aureus* to adapt to varied environments encountered during interaction with the human host is dependent on tightly controlled regulation of virulence accomplished by transcriptional regulatory networks. One of the best-studied two-component systems is the *Staphylococcus aureus* exoprotein (SaeR/S) two-component system (6,8-11,13-16,18-22). Sae is actually composed of 4 genes that include a histidine kinase *saeS*, a response regulator *saeR*, and two accessory genes *saeP* and *saeQ* (8,23). Deletion mutants in *saeR/S* have been shown to be important in pathogenesis during invasive and skin infections (9). SaeR/S senses neutrophil-derived

stimuli and regulates the transcription of virulence genes by binding the SaeR- DNA binding sequence thereby regulating virulence genes essential to evade neutrophil killing (15,16). Additionally, the SaeR/S system regulates a variety of toxins and adhesins (9,15) Studies also demonstrate that the regulation of virulence genes is refined by single amino acids in the putative extracellular loop of SaeS (17-21).

Phosphorylation of SaeR also contributes to regulation of virulence (24). Studies by Wolz et al have described the importance of levels of phosphorylation in virulence regulation wherein transcription of type I genes require low levels of SaeR phosphorylation and can occur independent of SaeS and transcription of type II genes require higher levels of SaeR phosphorylation and need SaeS (24). However the impact of host-derived stimuli on phosphorylation and virulence gene expression is incompletely defined. Previous studies have suggested that aspartate 51 (D51) residue of SaeR is the amino acid phosphorylated by SaeS (8,24). In the current study we use a clinically relevant strain of USA 300 (strain 18805) that was originally isolated from a patient with infective endocarditis (7) and compared it to newly generated plasmid-based single amino acid mutants in nearby aspartate residues D46 and D61. Strain 18805 has a 15bp deletion in SaeR encoding the VLDIM region, of which D is at position 51 (7). Our results suggest that D46 is important in SaeR-mediated signaling following phagocytosis by human neutrophils and suggest that phosphorylation at different sites on SaeR may be important for virulence regulation.

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli (*E. coli*) with USA300 background plasmids pEPSA5 or DH10 vector were grown in Luria-Broth (LB) at 37°C with shaking and 100µg/mL of ampicillin was used for plasmid maintenance. *E. coli* strains for protein purification were generated as previously described in (88). The SaeR region of strain 18805 was generated in *E. coli* as previously described in (7).

S. aureus were grown in tryptic soy broth (TSB) at 37°C with shaking and 10 µg/mL of chloramphenicol was used for plasmid maintenance. Bacterial growth was monitored using NanoDrop 2000c Spectrophotometer (Thermo Scientific). All plasmid-based single amino acid mutant strains were generated as previously described in (17,25) (Fig S3.1). Briefly, single amino acid mutations were generated using ligase independent PCR reactions where the primer sets introduce an alanine mutation. Additionally a N-terminal 6-his tag was introduced so strains could be detected by Western blot. All strains were confirmed using DNA sequencing. All plasmids were complemented back to USA300Δ*saePQRS* strain. A detailed list of strains used in this study is listed in table 3.1 and a list of oligonucleotides used in this study is listed in table 3.2.

Table 3.1: Strains used in this study

Strain	Genotype/description	Resistance	Source
<i>Escherichia coli</i>			
SaeR-His	recombinant protein expression strain	ampicillin	[11]
SaeR-D51A-His	recombinant protein expression strain	ampicillin	this study
SaeR-D46A-His	recombinant protein expression strain	ampicillin	this study
18805-His	recombinant protein expression strain	Kanamycin, ampicillin	this study
<i>Staphylococcus aureus</i>			
CFS111	AH1263::LL29-hla-sGFP-pEPSA5	None	[12]
AH2216	AH1263 Δ saePQRS	None	[12]
CFS114	AH1263::LL29-hla-sGFP- Δ saeRS-pEPSA5	chloramphenicol	[12]
CFS100	AH1263::LL29-hla-sGFP- Δ saeS-pEPSA5	chloramphenicol	this study
USA300 strain 18805	USA300LAC:18805	Tetracycline	[7]
SaeR-D51A	AH2216:SaeR-D51A	chloramphenicol	this study
SaeR-D46A	AH2216:SaeR-D46A	chloramphenicol	this study
SaeR-D61A	AH2216:SaeR-D61A	chloramphenicol	this study

Table 3.1: Strains used in this study. All *E. coli* and *S. aureus* plasmid-based strains used in this study are listed with appropriate genotypes and antibiotics used to maintain plasmid.

Table 3.2: Primers and probes used in this study

Primer or probe	Sequence	description
DNA primers		
SaeR_6xHis_fwd-1	5'- CAC CAT CAC CAT CAC CAT ACC CAC TTA CTG ATC GTG -3'	N-terminal His-tag insertion
SaeR_6xHis_fwd-2	5'- ACC CAC TTA CTG ATC GTG GAT G -3'	N-terminal His-tag insertion
SaeR_6xHis_rvs-1	5'- ATG GTG ATG GTG ATG GTG CAT CTA TTT TTT CAC CTC TGT TC -3'	N-terminal His-tag insertion
SaeR_6xHis_rvs-2	5'- CAT CTA TTT TTT CAC CTC TGT TCT TAC GAC -3'	N-terminal His-tag insertion
saeR_D46A_Fwd	5'- CTT TAC TAT CAA ATG ATA TTG CGA TCA TGG TAC TTG ATA TCA TG -3'	Mutating SaeR D46A fwd
saeR_D46A_Rvs	5'- CAT GAT ATC AAG TAC CAT GAT CGC AAT ATC ATT TGA TAG TAA AG -3'	Mutating SaeR D46A Rvs
saeR_D61A_Fwd	5'- GAT GCC AGA AGT TAA TGG TTA CAA UAT TGT CAA AGA AAT GAA AAG -3'	Mutating SaeR D61A fwd
saeR_D61A_Rvs	5'- CTT TTC ATT TCT TTG ACA AT GCG TAA CCA TTA ACT TCT GGC ATC -3'	Mutating SaeR D61A Rvs

Real time RT-PCR Primers and probe

<i>gyrB</i> fwd	.CAAATGATCACAGCTTTGGTACAG-3'
<i>gyrB</i> rvs	.CGGCATCAGTCATAATGACGAT-3'
<i>gyrB</i> probe	.AATCGGTGGCGACTTTGATCTAGCGAAAG-3'
	.GTACATTCAGAGTAGAAAACCTCTCGTAATAC-
<i>saeS</i> fwd	.GTTGCGCGAGTTCATTAGCTATATAT-3'
<i>saeS</i> rvs	.AGCCTAATCCAGAACCACCCGTT-3'
<i>saeS</i> probe	.CTGCCAAAACACAAGAACATGATAC -3'
<i>saeR</i> fwd	.ATTTACGCCTTAACTTTAGGTGCAGAT -3'
<i>saeR</i> rvs	.ATTTACGCCTTAACTTTAGGTGCAGAT -3'
<i>saeR</i> probe	.ATTTACGCCTTAACTTTAGGTGCAGAT-3'
<i>sbi</i> fwd	.ATACATCAAAACATTACGCGAACAC-3'

<i>sbi</i> rvs	·CTGGGTTCTTGCTGTCTTTAAGTG-3'
<i>sbi</i> probe	·CAGGTAGCTTTATGGTTGCTACAAAAAT-3'
<i>hla</i> fwd	·CAACAACACTATTGCTAGGTTCATATT-3'
<i>hla</i> rvs	·CCTGTTTTTACTGTAGTATTGCTTCCA-3'
<i>hla</i> probe	·ATGAATCCTGTCGCTAATGCCGCAGA-3'
<i>efb</i> fwd	·CTACAATTGCGTCAACAGCAGAT-3'
<i>efb</i> rvs	·ACCATCATTGTACTCTACGATATTGTGA-3'
<i>efb</i> probe	·CGAGCGAAGGATACGGTCCAAGAGAAA-3'
<i>fnbA</i> fwd	CGTCAAACGCAACACAAGTAA-3'
<i>fnbA</i> rvs	- CGCTTCTTCCTTAACTACCTCTT-3'
<i>fnbA</i> probe	- AAGCACCACAAACTGCACAACCAG-3'
<i>hlgA</i> fwd	- ACTTATTT GCACAAGACCCAAGTG-3'
<i>hlgA</i> rvs	- CCACTTTGAATTAAAGGA GGTAATTGAT-3'
<i>hlgA</i> probe	- CAGCAGCAAGAGACTA TTTCGTCCCAG-3'

Table 3.2: Primers and probes used in this study. All PCR primers used to generate mutant strains are listed under DNA primers. All qRT-PCR primers and fluorescent probe sequence are listed under real-time RT-PCR primers and probes.

Purification of SaeR and Single Amino Acid Mutants of SaeR and EMSAs

Single amino acid mutation pET100D plasmids with X6 His tag on the N-terminal were transformed into BL21-DE3 *E. coli* cells and strains expressing wild type SaeR and SaeR-D46A were grown in LB broth medium at 37°C and induced with 0.5mM IPTG at OD₆₀₀ 0.8. Bacteria were further allowed to grow for an additional 6 hours at 37°C. Bacteria were lysed via sonication in 20mM Tris-HCl, pH 8.0 on ice for 15 minutes. Lysed cells were pelleted and the pellets were further resuspended in lysis buffer (8M urea, 0.5M NaCl, 20mM Tris-HCl, pH 8.0) followed by sonication again on ice for 15 minutes. The clarified lysate post centrifugation was subjected to a nickel-

nitrilotriacetic acid affinity chromatography (Qiagen) and diethylaminoethyl cellulose anion exchange chromatography (GE Healthcare) using a BioLogic LP Chromatography system (Biorad). Purified protein was reconstituted in 20mM Tris, pH 8.0 using dialysis and concentrated using a Centricon Plus-20 centrifugal filter (Millipore).

All DNA-binding assays were conducted using purified recombinant protein that was not artificially phosphorylated. Varied concentrations of recombinant wild type SaeR and SaeR-D46A (such as 0,6, 8, 10 and 15 μ M) was combined with 0.25 pmol of *hla* or *sbi* DNA-binding sequence (60) in DNA binding buffer (100mM KCl, 1mM EDTA, 0.1mM DTT, 5% vol/vol glycerol and 10ng/mL bovine serum albumin) and incubated at room temperature for 15 mins. Control samples were had DNA binding sequence combined with PBS. Samples were separated on a 10% native gel, stained with SYBR green and viewed using Alpha Innotech Alpha view.

Analysis of Gene Expression Following Exposure to Host Stimuli

Overnight *S. aureus* cultures were diluted 1:100 and grown to an $OD_{600} = 1.5$. Bacteria were washed in DPBS and then plated in 96-well round bottom plates at 1×10^6 CFU/ μ L per well making a 10:1 bacteria:PMN ratio. Infection was synchronized by centrifugation (400 X g, 8min, 4°C), and plates were incubated statically at 37°C for 30 mins. After 30mins, bacterial RNA was isolated using methods as previously described in (17,26,27).

For blood-induced gene expression, 100% human heparinized venous blood was added to bacteria (1×10^8 CFU/mL). Bacteria were incubated with gentle rotation for

30mins at 37°C with 5% CO₂. At 30 mins, bacteria were pelleted and washed with water 3 times (8000 X g, 5 mins, 4°C). RNA was isolated from RNeasy kits as previously described in (17,26,27). The primers and probes used are listed in table 3.2.

Gene expression was measured using TaqMan™ real-time PCR using methods previously described in (17,26,27). Gyrase B (*gyrB*) was used as an endogenous control to normalize gene expression (17,26,27).

PMN Membrane Permeability Assay

Polymorphonuclear leukocytes (PMNs or neutrophils) were isolated from healthy human donors following procedures described elsewhere (27). All procedures were performed in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. In order to perform PMN membrane permeability assays, Human PMNs at 1×10^6 cells/ μ L were added to 96 well round bottom plates, prior coated with 20% normal human serum in DPBS. Bacteria at 1×10^7 CFU were added to human PMNs. Infections were synchronized ($400 \times g$, 8 min, 4 °C), and plates were incubated statically at 37 °C with 5% CO₂ for 3 hrs. Membrane permeability was measured by flow cytometry using propidium iodide (Invitrogen). To measure PMN membrane permeability caused by bacterial supernatants, overnight bacteria were diluted 1:100 and grown for 5hrs. Spent supernatants were filter sterilized and further diluted 1:10 in RPMI with 5mM HEPES. Diluted supernatants were added to human PMNs. Intoxication was synchronized (as described above), and PMN cytotoxicity was measured using flow cytometry (17).

Human PMN Phagocytosis Bactericidal Activity Assays

Human PMN bactericidal activity assays were performed by adding 1×10^6 PMNs to 96 well round bottom plates pre-coated with 20% normal human serum in DPBS. 1×10^7 CFU of mid-exponentially harvested bacteria were added to the human PMNs. Infections were synchronized ($400 \times g$, 8 min, $4^\circ C$), and plates were incubated statically at $37^\circ C$. At designated time points, 0.1% saponin was added and the plates were allowed to rest on ice for 20 mins. CFUs were plated on tryptic soy agar (TSA) at 0 and 3 hour time points, after overnight incubation of TSA plates at $37^\circ C$ with 5% CO_2 CFUs were enumerated. Bacterial survival was calculated using the formula: $(CFU_{+PMN} \text{ at } t=3\text{hrs} / CFU_{+PMN} \text{ at } t=0) \times 100$ (25,27).

SDS-PAGE Analysis of Purified Recombinant Protein

Recombinant protein (20 μ g of SaeR and SaeR-D46A as determined by Bradford protein quantification assay) in 20mM Tris, pH 8.0 were combined with 2X -SDS-PAGE Laemmli loading buffer with 5% beta-mercaptoethanol. Bradford Assay was conducted using manufacturer's guidelines (Peirce). Samples were then boiled at $99^\circ C$ for 5 minutes and loaded on a 12% polyacrylamide gel. Following the gel run, the gel was stained with Coomassie blue stain.

Results:SaeR Mutant D46A Has Reduced Survival Post Human PMN Phagocytosis

The Sae system in *S. aureus* shares sequence homology to the bacterial OmpR/PhoB sensory systems. SaeR shares sequence homology with the bacterial OmpR family of cytosolic response regulators (8). As per sequence alignment studies, D51 is the predicted phosphorylation residue (Fig3.1). However, experiments demonstrating the importance of D51 in binding to SaeR recognized consensus DNA-binding sequence have not been done. Moreover, a naturally occurring human infective endocarditis strain USA300-strain 18805 has been shown to cause infection despite having a deletion mutation of 15bp—coding the VLDIM region of SaeR, including the aspartate residue 51 (7,29). These studies suggest that there are other phosphorylation sites in SaeR. In order to identify other putative phosphorylation sites, we performed multiple sequence alignment of SaeR (GenBank ID ABD22748.1) and found that aspartate residue 46 (D46) is also a conserved aspartate residue across all staphylococcal species (Fig 3.1). Using this information, we generated plasmid-based point mutations in SaeR at residue D46. We also generated a mutation in D61. These strains were generated by substituting the aspartate GAC for alanine GCG. Mutant strains were checked for growth abnormalities using growth curve assays (Fig S3.2). In addition, the transcript levels of *saeR* and *saeS* did not show measurable differences when compared to wild type SaeR (Fig S3.3).

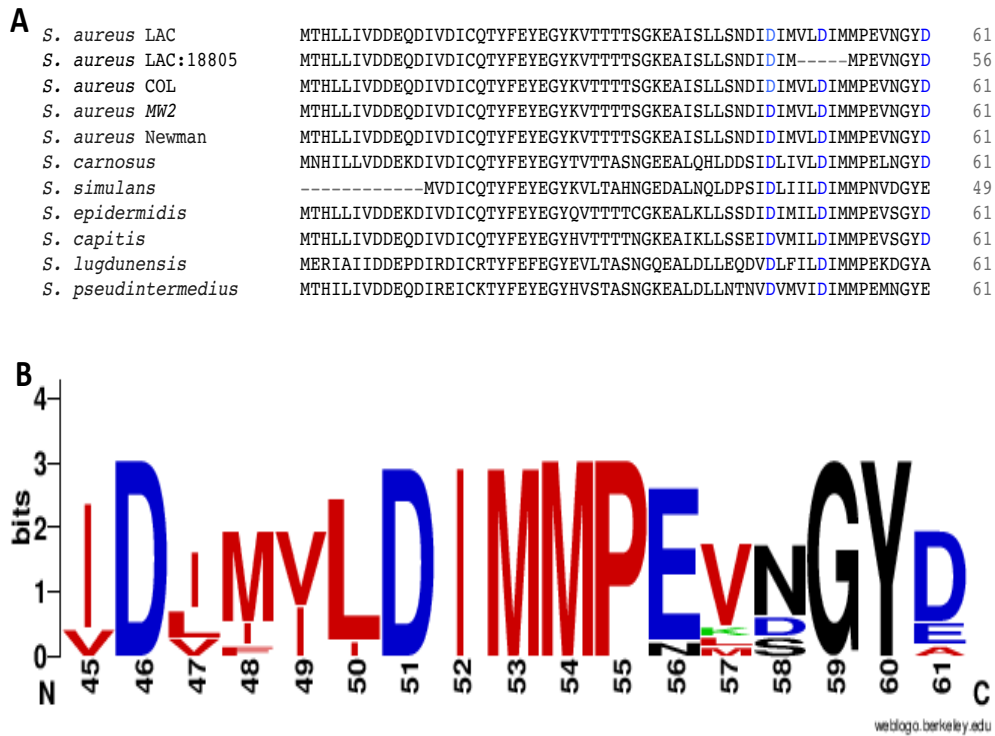


Figure 3.1: Conserved aspartate residues near the putative phosphorylation site of SaeR. SaeR sequences across all staphylococcal species aligned (A) revealed that aspartate 46 (D46) and D51 are highly conserved and that D61 is partially conserved. A weblogo (<https://weblogo.berkeley.edu/logo.cgi>) was created using information from (A) and is depicted in (B). Note that the red arrow for residue 51 indicates that it is the predicted phosphorylation site.

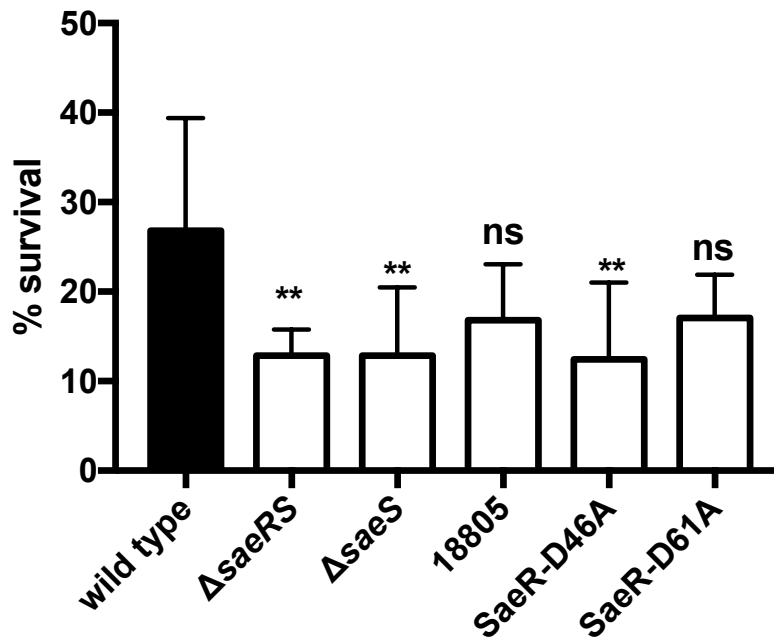


Figure 3.2: SaeR mutant D46A has reduced survival post PMN phagocytosis. SaeR mutant strains were used to infect PMNs at a 10:1 bacteria: PMN ratio. Bacterial survival was measured at 3 hours post-phagocytosis by enumerating CFUs at time 0 and 3 hours respectively. Results are the mean $\pm$ of seven separate experiments. Asterisks denote statistical significance evaluated by one-way ANOVA with Tukey's posttest compared to wild-type strain based on the following P-values: ** 0.001-0.01. ns denotes non-significant.

SaeR Mutant D46A Has Reduced Cytotoxicity In Human PMNs Following Phagocytosis

SaeR mutant D46A is unable to regulate virulence factors, despite the presence of host-derived stimuli (Fig 3.2 and 3.5) and its ability to interact with human PMNs is also altered (Fig3.6). In order to investigate the effect of PMN-bacterial interaction on the bacteria we looked at bacterial survival three hours post human PMN phagocytosis. As seen in Fig 3.2, strain 18805 and SaeR-D61A had a better survival rate comparable to that of wild type. These results confirm the in vivo studies conducted for strain 18805 as described in (29). However, mutant SaeR-D46A had reduced survival (~12%) compared

to wild type (P-value: <0.001) was comparable to *saeR/S* and *saeS* mutant strains.

(Fig3.2).

In order to further assess our observation of reduced survival of mutant D46A in response to PMN phagocytosis, we observed the ability of our putative phosphorylation site mutants to cause PMN membrane damage following PMN phagocytosis using cytotoxicity assays. Previous studies have shown that sensing and responding to PMNs is a key function of the Sae system (9,17,26,27,30-32). Moreover, this response is specific to its signal and that only a subset of *sae* regulated genes are transcribed in response to PMN exposure (17,26). In order to investigate PMN cytotoxicity in our putative phosphorylation site mutants, we exposed supernatants from our SaeR mutant strains to human PMNs followed by phagocytosis and assessed cytotoxicity by measuring membrane permeability at 1-hour post exposure (Fig 3.3). As compared to ~85% membrane permeability caused by wild type and complemented strains, mutant SaeR-D61A induced ~40% cytotoxicity. Culture supernatants from strain SaeR-D46A and 18805 had significantly reduced ($<2\%$, p-value <0.0001) membrane damage, levels comparable to $\Delta saeRS$ and $\Delta saeS$ strains (Fig 3.3).

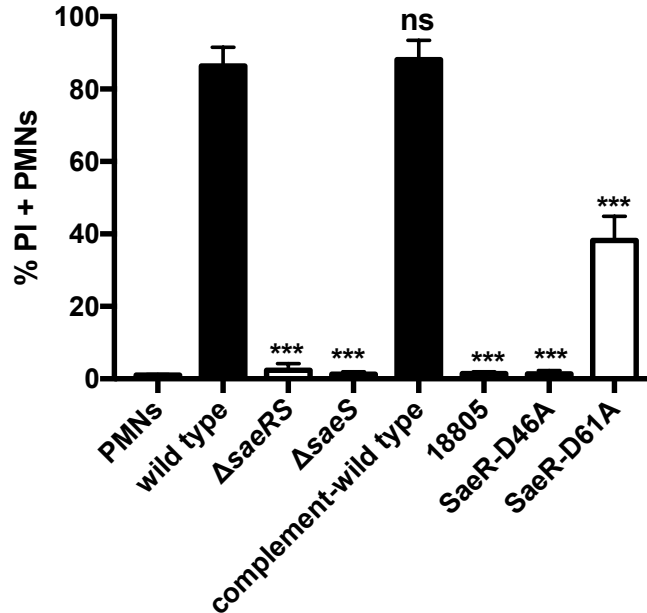


Figure 3.3: SaeR mutant strains are deficient in producing secreted toxins essential to induce PMN cytotoxicity. PMN plasma membrane permeability following exposure to bacterial supernatant for 1 hour from wild-type, Δ saeR/S, SaeR single amino acid mutant strains and strain 18805. Results are the mean $\pm$ of five separate experiments. Asterisks denote statistical significance evaluated by one-way ANOVA with Tukey's posttest compared to wild type based on the following P-values: ** 0.001-0.01, and *** <0.0001. ns denotes non-significant.

In order to assess the ability of bacteria to respond to specific host-derived stimuli (human PMNs), we infected human PMNs with bacteria from our *saeR* mutant strains and assessed PMN cytotoxicity via membrane permeability at 3 hours post- infection (Fig 3.4). As compared to ~60% and ~45% membrane permeability caused by wild type and complemented strains respectively, mutant SaeR-D61A induced ~50% membrane permeability. Surprisingly, strain 18805 induced ~50% membrane permeability, levels comparable to that of wild type bacteria. Mutant SaeR-D46A induced ~30% ,similar to

that of $\Delta saeRS$ and $\Delta saeS$ strains (fig 3.4). It is interesting that strain 18805 is able to induce membrane damage following exposure to host-derived stimuli-human PMNs (Fig 3.4) but that basal toxin production was significantly reduced (Fig 3.3 and Fig 3.4). These data suggest that *S. aureus* can take advantage of other aspartate residues besides D51 to accomplish SaeR-mediated signaling when presented with a host stimuli and indicate that residue D46 is important in responding to PMN components.

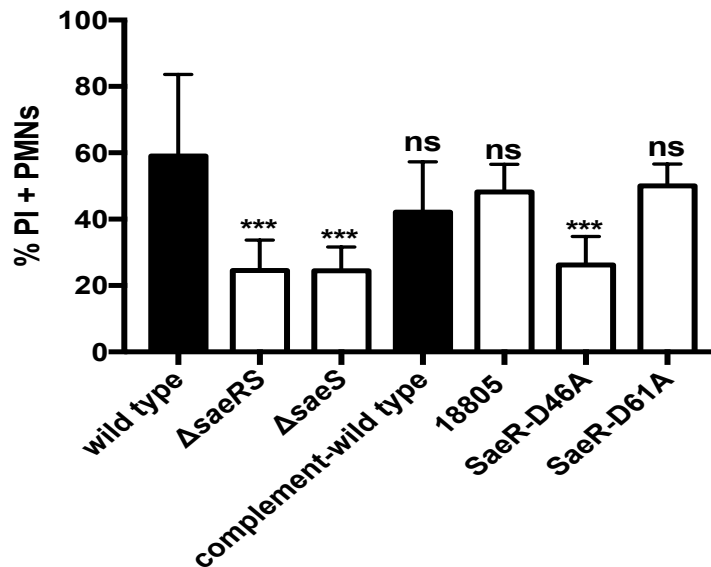


Figure 3.4: SaeR mutant D46A has reduced cytotoxicity in human PMNs following phagocytosis. PMN plasma membrane permeability following exposure to whole bacteria for 3 hours at a 10 bacteria:1PMN ratio from wild-type, $\Delta saeR/S$, SaeR single amino acid mutant strains and strain 18805. Results are the mean $\pm$ of five separate experiments. Asterisks denote statistical significance evaluated by one-way ANOVA with Tukey's posttest compared to wild type based on the following P-values: ** 0.001-0.01, and *** <0.0001. ns denotes non-significant.

Virulence Gene Expression in Mutant SaeR-D46A During Host Challenge

The SaeR/S system is essential to sensing and responding to different host derived stimuli (9,17,26-28,30-32). Moreover, the extracellular sensing domain of the sensor kinase SaeS plays an important role in sensing different host derived stimuli and thereby regulating differential gene expression (17,21). However, the downstream mechanisms used by SaeR to regulate virulence gene expression is incompletely defined. In order to address this, putative SaeR phosphorylation site mutant strains and wild type *S. aureus* strains were challenged with human PMNs and human whole blood. At designated times RNA was isolated from *S. aureus* to investigate gene expression in select genes that contain the SaeR binding domain (*hlgA*, *fnbA* and *lukAB*). As seen in Fig 3.5, strain 18805 had a transcriptional profile similar to the wild type *S. aureus* strain and demonstrated increased transcription of *hlgA* and *fnbA* during challenge with human PMNs. (No significant changes were observed with *lukAB* transcription-data not shown) However, SaeR-D46A had demonstrated diminished transcription of these genes even in the presence of human PMNs. Previous studies have shown that only a subset of genes are turned on in response to PMN stimulus (17,26). Consistent with our previous findings (17,26), we found that *sae*-regulated genes *hla* and *efb* were not induced in response to PMN phagocytosis (fig S3.4).

Strain 18805 was able to respond to human whole blood by increasing the transcription of *hlgA*, *lukAB* and *fnbA* (fig 3.6). Mutant SaeR-D46A had reduced transcription of these genes (Fig 3.6). Collectively, data suggest that, strain 18805 can transcribe virulence genes under host-derived pressure. However, SaeR-D46A had

diminished expression of the genes investigated, suggesting that residue D46 may be important for regulating genes during host challenge.

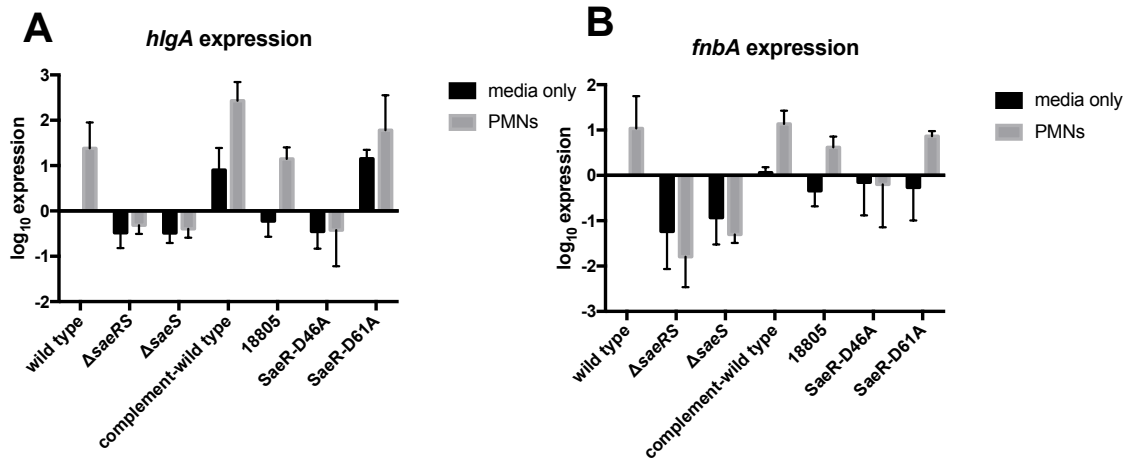


Figure 3.5: Transcription of genes containing the *sae-binding* domain following PMN phagocytosis in *SaeR* mutant strains. Relative gene expression of *hlgA* (A), *fnbA* (B) was analyzed after a 30 min exposure to human PMNs (1 PMN:10 bacteria). Data displayed as fold-change and transcript levels were normalized to *gyrB* and calibrated to wild type in media only (time matched). Data shown is mean fold change of 2 separate experiments.

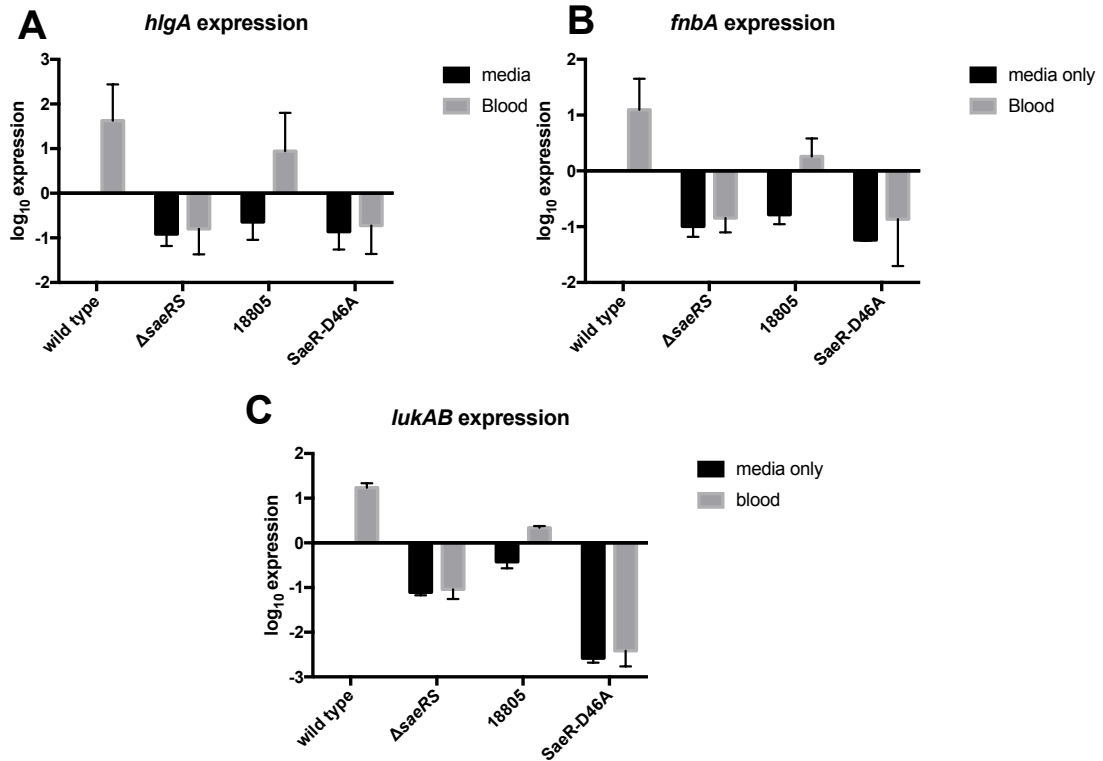


Figure 3.6: Transcription of genes containing the *sae-binding* domain following challenge with human whole blood in SaeR mutant strains. Relative gene expression of *hlgA* (A), *fnbA* (B) and *lukAB* (C) was analyzed after a 30 min exposure to human whole blood. Data displayed as fold-change and transcript levels were normalized to *gyrB* and calibrated to wild type in media only (time matched). Data shown is mean fold change of 2 separate experiments.

Aspartate 46 (D46) Impacts Binding of Recombinant SaeR- Protein to DNA-Binding Sequence

EMSA were performed to determine the influence of single amino acids on the binding ability of recombinant SaeR to bind its consensus DNA-binding sequence (15,16). Purity of protein was determined using SDS-PAGE analysis (Fig S3.5). Wild-type recombinant SaeR was able to bind the SaeR-recognized DNA sequence at the designated concentration (Fig 3.6A), confirming our prior observations (15).

Surprisingly, we found that substituting aspartate 46 (D46) to alanine completely inhibited binding activity of recombinant SaeR-D46A, suggesting that D46 may be critical for SaeR to bind its consensus DNA-binding sequence (Fig 3.7B). However we only tested two genes that contain the SaeR-binding domain so additional studies are needed to determine the impact of D46 on gene regulation.

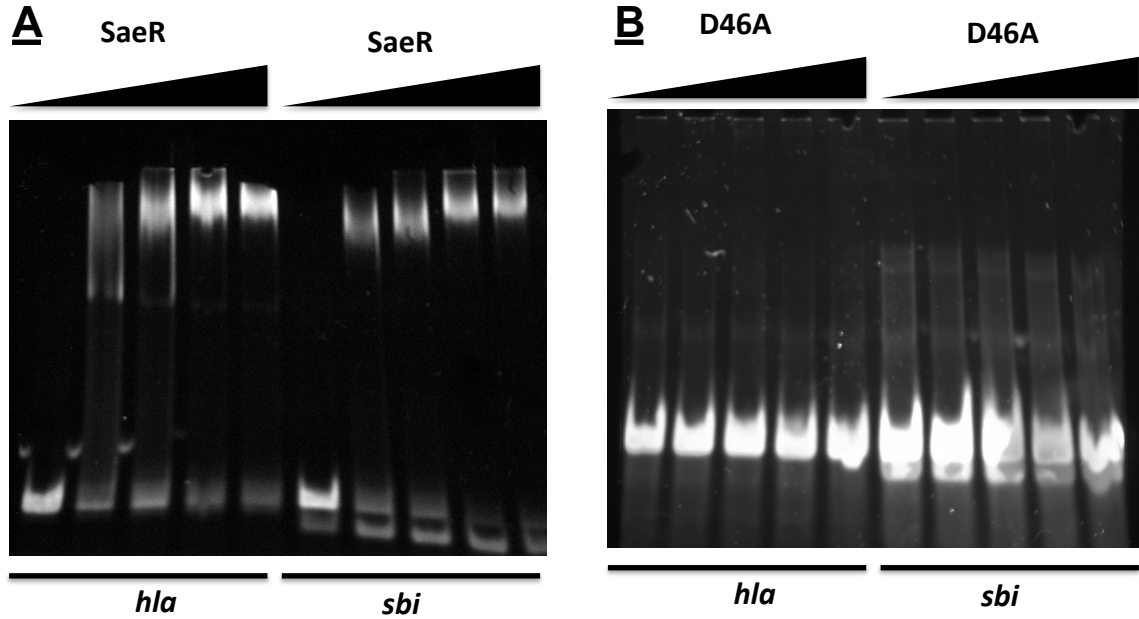


Figure 3.7: Aspartate residue 46 in SaeR is important for binding of recombinant SaeR to known SaeR recognized DNA-binding sequences. Varied amounts(μ M) of recombinant SaeR(A), SaeR-D46A(B) (as indicated) were incubated with 0.25pmol of *hla* and *sbi* DNA containing the SaeR-binding domain. Gels were visualized using SYBR Green stain(Sigma-Aldrich)with a Alpha Innotech Alpha view imaging system. Data representative of 3 separate experiments.

Discussion:

The SaeR/S two-component system in *S. aureus* plays an important role in virulence regulation (15,17,21,26,27,33). Our previous studies indicated the importance of single amino acids in the sensing domain of SaeS to sense host-derived stimuli. However, the signaling mechanism that occurs downstream by the response regulator SaeR is incompletely defined. In this investigation, we examined the role of putative phosphorylation amino acid residues in the response regulator SaeR. Our studies indicate that aspartate residue 46 may be important to the functioning of SaeR during host challenge as plasmid-based mutation to alanine (D46A) rendered this mutant similar to the $\Delta saeRS$ and $\Delta saeS$ strains. Moreover, we found that mutation in the predicted phosphorylation site, aspartate residue 51 (strain 18005) (7,18,28) did not completely prevent transcription of SaeR-target genes under host-derived stimuli. Substituting aspartate 61 to alanine did not show any significant differences from wild type USA 300 in any of our phenotypic assays.

The Sae system relies on phosphorylation modifications to regulate gene expression (23,33,34) and it has been hypothesized that different levels of phosphorylation of SaeR dictate the different virulence factors transcribed by the system (24). Through our in vitro assays, we suggest that in addition to different levels of phosphorylation, different sites (amino acid residues) may play an important role in refining the response to each host-derived stimuli.

Previous studies from our lab and Bae, et al have identified the consensus DNA-

binding sequence that is shared by virulence factors that are recognized by SaeR, thereby regulating gene expression (15,16). One interesting finding from our study was that mutant SaeR-D46A is unable to bind the consensus DNA-binding sequence of *hla* and *sbi* genes (Fig 3.6). Although more experiments are needed, these data suggest an important role for residue D46 in SaeR-mediated signaling.

The ability to induce human PMN cytotoxicity was diminished in our SaeR-D46A mutant (Fig 3.4). Basal level toxin production was diminished in all our mutant strains (Fig 3.3). However, strain 18805 and SaeR-D61A were able to regulate cytolytic factors in the presence of human PMNs (Fig 3.4). In the D46A mutant strain we observed that the transcription of *sae* –regulated genes *fnbA*, *hlgA* and *lukAB* were reduced (Fig 3.5 and 3.6). In the presences of host-derived stimuli like human PMNs and whole human blood, strain 18805 was able to transcribe virulence genes, suggesting that there are alternative mechanisms employed by SaeR to transcribe virulence genes during challenge with host components.

Our observation that strain 18805 (i) has comparable survival post PMN phagocytosis to wild type bacteria and (ii) can transcribe selected virulence genes in response to human PMNs and whole blood and that the mutant SaeR-D46A is deficient in either of the above suggest that perhaps residue D46 could be the *S. aureus* activating residue. This investigation is a step towards the understanding of molecular mechanisms employed by the SaeR/S system to sense and respond to different host-derived stimuli. Like the sensor kinase, our data suggest the response regulator also refines its signal at the single amino acid level.

Whether residue D46 is the actual phosphorylation site is a question that needs to be answered through molecular and proteomic studies.

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SUPPLEMENTAL INFORMATION

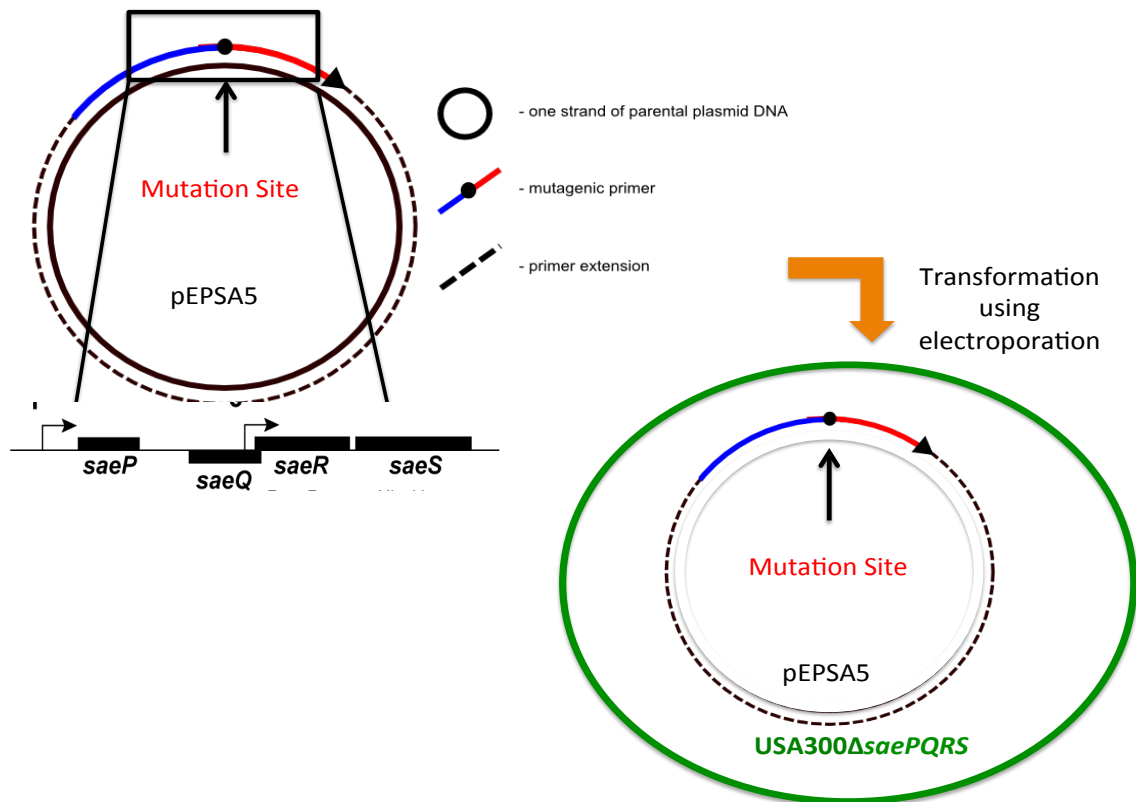


Figure S3.1: Schematic of generating single amino acid substitutions in SaeR. Briefly, primers specific to generating particular single amino acid replacement were designed as described in table 3.2 and PCR was conducted using pEPSA5 vector and DH10 *E. coli* cells. The resulting plasmid was then transformed into RN4220 strain followed by transforming into the USA300Δ*saePQRS* strain using electroporation.

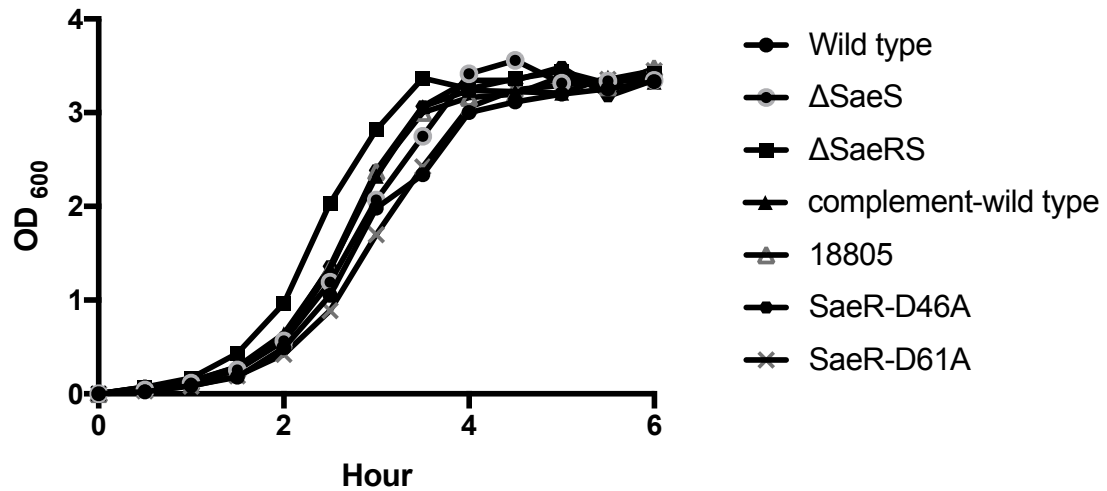


Figure S3.2: Growth curves in tryptic soy broth for wild type and mutant SaeR strains. Y-axis represents 600nm optical density (logarithmic scale) and X-axis the growth time in hours. Optical density measurements were taken every 30 minutes for 6 hours.

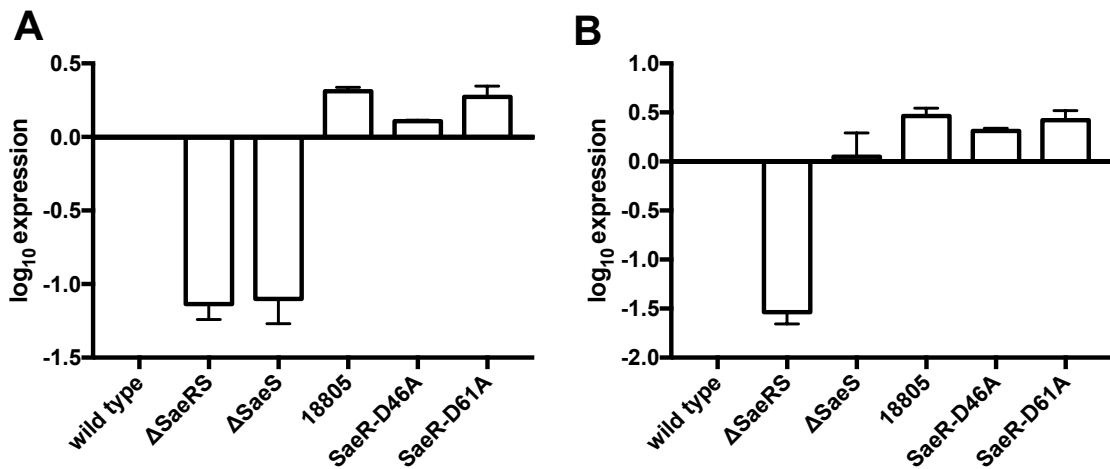


Figure S3.3: Transcription of *saeS* (A) and *saeR* (B) using qRT-PCR. RNA was extracted from bacteria harvested at midlog (OD₆₀₀ = 1.5) grown in tryptic soy broth and examined by qRT-PCR. Relative quantification of transcripts was determined by normalizing to *gyrB* and calibrated to wild type strain and expressing mean values as log₁₀ changes. Two biological replicates of each experiment were analyzed in triplicate.

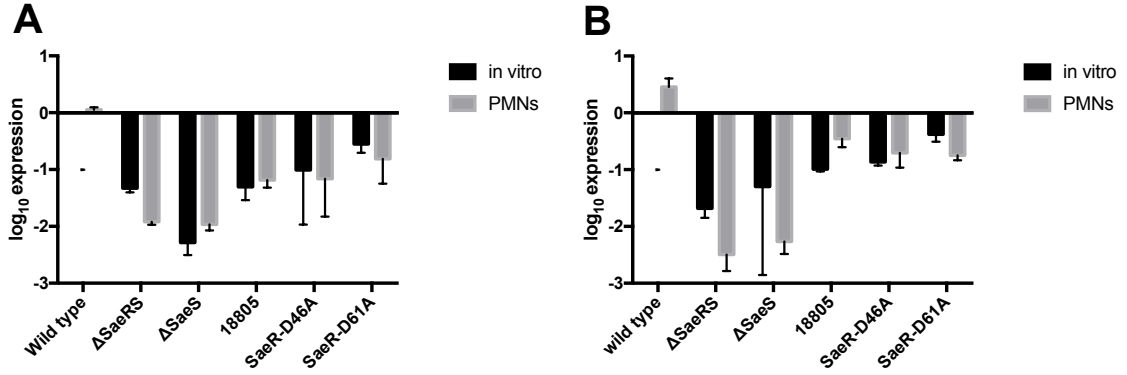


Figure S3.4: Relative gene expression of *hla* (A), and *efb* (B) was analyzed after a 30 min exposure to human PMNs(10^7) at a ratio of 1 neutrophil:10 bacteria. Data displayed as fold-change and transcript levels were normalized to *gyrB* and calibrated to wild-type in media only (time matched). Data shown is mean fold change of 2 separate experiments.

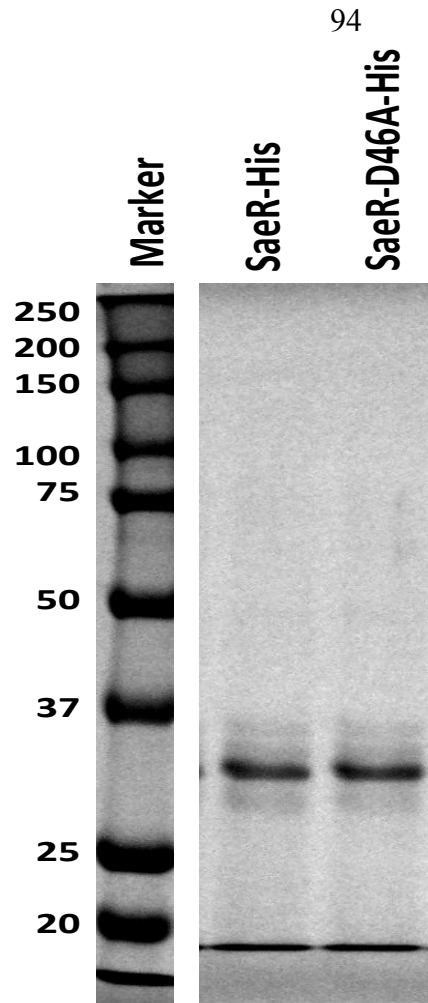


Figure S3.5: SDS-PAGE analysis demonstrates purity of recombinant SaeR from wild-type and SaeR-D46A strains. Recombinant protein (20 μ g) was loaded on a 12% SDS-PAGE gel. Gels were run at 100V for 1.5 hrs, followed by Coomassie blue staining. Gels were scanned using Alpha Innotech Alpha view imaging system. Each experiment was conducted 3 times.

CHAPTER FOUR

FUTURE STUDIES AND CONCLUSION

Identifying The Phosphorylation Sites on SaeR

The Sae system utilizes the extracellular sensing domain to sense and thereby respond to varied environmental stimuli (1-3). The response is further refined at the response regulator level through phosphorylation. Fundamental aspects of the SaeR/S system remain undefined. For example, the role of phosphorylation in regulation of SaeR-dependent virulence gene expression is incompletely defined and the dependence on SaeS for SaeR phosphorylation has only been confirmed for a few target genes suggesting either phosphorylation occurs independent of SaeS or that phosphorylation of SaeR is not necessary for expression of some SaeR-target genes (4). Studies by Wolz et al have defined two types of phosphorylation in the Sae system thereby regulating two classes of virulence genes i.e. type I and II (4). Transcription of type I virulence genes can take place irrespective of SaeS and requires low levels of SaeR phosphorylation while transcription of type II virulence genes requires SaeS and an increased amount of SaeR phosphorylation (4). Additionally, evidence for the role of phosphorylation has only been studied in vitro with SaeR protein purified from *E. coli*, and investigation into how host stimulus influences phosphorylation is lacking. Through this investigation, we aim to determine the phosphorylation sites on both SaeS and SaeR and to confirm that phosphorylation occurs in vivo.

Materials and Methods

Protein Identification

a) In Solution Protocol 3.5ug of SaeR, SaeR-D51A, and SaeR-D46A were diluted in to 100mM ammonium bicarbonate solution, pH8 and digested with a trypsin (Promega) protease:protein ratio of 1:10 for 3h at 37°C. Resulting mixtures were filtered through 3k MWCO filter (Pal Corporation) to remove intact proteins before LC-MS analysis (spun at 5000xg for 15min, room temperature).

b) In-Gel Protocol Protein identification from gel bands was performed according to standard protocols recommended by the manufacturer using a trypsin (Promega) protease:protein ratio of 1:10 at 37°C overnight.

Peptides Identification

LC-MS/MS measurements were performed using a maXis Impact UHR-QTOF instrument (Bruker Daltonics) interfaced with a Dionex 3000 nano-uHPLC. Peptide mixtures were desalted and then separated on a Dionex column (20mm × 100µm, 5µm, 100Å, Acclaim PepMap100, C18, Dionex) at 40°C with a flow rate of 800nL/min using the step gradient: 3% for 2min, 3 to 30%B over 18min, followed by 30 to 80%B over 2min and a 2min 80%B column wash step. The column was equilibrated with 3%B for 3 min prior to the next injection. Solvent A = 0.1% formic acid (FA, Sigma) in water (ThermoFisher) and solvent B = 0.1% FA in acetonitrile (ACN, ThermoFisher). Electrospray conditions in both MS and auto MS/MS modes were as follows: drying gas flow of 5.0L/min at 180°C, capillary voltage of 1600V. Data was collected in positive

mode over the m/z range of 150–2200 at an acquisition rate of 2Hz for both MS and MS/MS. A linear voltage gradient depending on the m/z ratio was applied for peptides fragmented in auto MS/MS experiments with a decreasing order of preference (+2 > +3 > +4 > +1) for charged parent ions. Raw data were converted to mgf format using MS Convert and uploaded to SearchGUI (version 3.2.20.) for sequence identification and visualized/validated in PeptideShaker (version 1.16.14.)

Intact Protein Analysis

Intact protein samples were analyzed using a 1290 ultrahigh pressure (UPLC) series chromatography stack (Agilent Technologies) coupled directly to an electrospray-time of flight (ESI-TOF) mass spectrometer (Bruker Micro-TOF). Before infusion to ESI source samples were separated on Onyx monolithic C18 column (1.0 x 100mm, Phenomenex) at 50°C using a flow rate of 400ul/min and the following program: 1.0min, 10% B; 1.0-7.0min, 10-70% B; 7.0-8.0min, 70-90%B; 8.0-9.0min, 90%B; 9.0-9.1min, 90-10%B; 9.1-10.0min, 10%B; solvent A = 0.1% formic acid (FA, Sigma) in water (ThermoFisher) and solvent B = 0.1% FA in acetonitrile (ACN, ThermoFisher). Electrospray conditions were as follows: nebulizer 5bar, drying gas at a flow rate of 7.0L/min, drying temperature at 200°C, capillary voltage at 4.5kV, and capillary exit voltage at 150V. Data were collected in positive mode at a rate of 2Hz over the 200–3000 m/z scan range. Data processing and analysis were performed using the Bruker Data Analysis package 4.0 (Bruker Daltonics). Charge deconvolution was performed using a maximum entropy algorithm for H^+ adducts only and 0.1 m/z data

point spacing. The low mass end was defined by the mass of the SaeR monomer while the high mass end was defined as 3.3x the dimer mass.

Results and Discussion

Molecular Architecture of SaeR During Intact Protein Analysis Using Mass Spectrometry

Previous studies have demonstrated that binding to promoter sequences recognized by SaeR only occurs when phosphorylation is induced in SaeR by the kinase SaeS (5). However, in our hands, recombinant SaeR is able to bind its promoter sequence without artificial phosphorylation of the protein (Fig 3.6). In order to assess the phosphorylation state of the protein, we loaded 20 µg of recombinant SaeR, or SaeR-D51A, or SaeR-D46A on a 12% SDS-Page gel. This gel was stained with PRO-Q diamond stain-which only stains phosphorylated protein followed coomassie stain. The percent of phosphorylated protein was then measured using the formula

$$\frac{\text{intensity of phosphorylated protein}}{\text{intensity of total protein}} \times 100.$$
 Using the above procedure, we found that our method of recombinant protein purification yields about 15% phosphorylated protein (Fig 4.1), thereby eliminating the need to artificially phosphorylate the protein for our binding assays (Fig 3.6). However, since PRO-Q diamond staining can have false positive results (non-specific binding to acidic proteins), we chose mass spectrometry to identify actual phosphorylation sites on recombinant protein from SaeR and SaeR mutant strains.

Through our mass spectrometry analysis of intact recombinant proteins, we found that most proteins and protein complexes when injected on reverse phase LC column and exposed to mobile phase (organic solvent/acidic conditions) lose their native structure

and undergo complete or at least partial denaturation. Expectedly, WT and SaeR-D46A are present almost exclusively in monomeric forms in intact protein experiments. In addition WT and SaeR-D46A contain two and one phosphorylation sites, respectively (Fig 4.1 and table 4.1). Interestingly, no phosphorylation was detected in case of SaeR-D51A mutant (table 4.1). In contrast to WT and SaeR-D46A mutant, SaeR-D51A is present as a mixture of monomer and dimer in LCMS data (Fig 4.2). Additionally, SaeR-D51A monomer is always heavier by 32Da (table 4.1), which suggests double oxidation. Interestingly, the dimer of SaeR-D51A does not appear to be modified. The exact oxidation sites have yet to be determined. Collectively, these data suggest that important role for residue D46 and the molecular architecture needed for SaeR-mediated signaling.

Interestingly, our mass spectrometric analysis of recombinant intact SaeR revealed that the wild type SaeR has 2 phosphate groups attached to it, whereas mutant SaeR-D46A has only one and both exist mainly as monomeric units (Fig 4.2). In contrast, mutant SaeR-D51A exists as mixture of monomer and dimer and that there is no phosphorylation associated with the protein (Fig 4.2). This suggests that decreasing the net negative charge around residue 51 caused a dimer to be more stable. Moreover, the oxidation state observation for SaeR-D51A (13.2) suggests that the monomer of mutant SaeR-D51A is more prone to oxidation than the dimer. However, since we have not identified oxidation sites we can only speculate, potentially there is oxidation (double) of the only cysteine residue in SaeR sequence. This type of oxidation is irreversible.

Evidence 1: If SaeR-D51A dimerization occurs via a disulfide (-S-S-) bond this double oxidation would prevent dimer formation. Our observation confirms higher dimer

stability excluding dimerization by formation of disulfide bridge between oxidized monomers. Evidence 2: -S-S- bond is stable in LC conditions therefore our results (monomer observed in intact protein experiments) suggest different dimerization mechanism. Evidence 3: Also a docking of 2 SaeR molecules as rigid bodies does not return any solution, which would satisfy -S-S- bond distance (around 2Å). Increasing the distance to 15Å resulted in series of potential dimer structures (Fig 4.3). This suggests two exclusive scenarios: significant structural rearrangement during -S-S- bond formation or different mechanism. Oxidation can also be a reason why phosphate was not detected. If a methionine residue near D46 (7) is double oxidized it could work as on/off switch, in a similar fashion as was proposed for serine threonine and tyrosine phosphorylation.

Table 4.1: Modifications of SaeR detected in intact protein experiments

Protein	Calculated, Da	Observed, Da	Δ mass, Da	Modification type (calculated, Da)
SaeR	28,747.9	28,906.0	+159.94	Double phosphorylation (28,907.83)
SaeR-D46A	30,939.2	31,022.7	+79.98	Single phosphorylation (31,019.2)
SaeR-D51A	30,939.2	30,971.6	+ 31.97	Double oxidation (30,971.2)
	61,878.4	61,878.4	+0.0	No oxidation

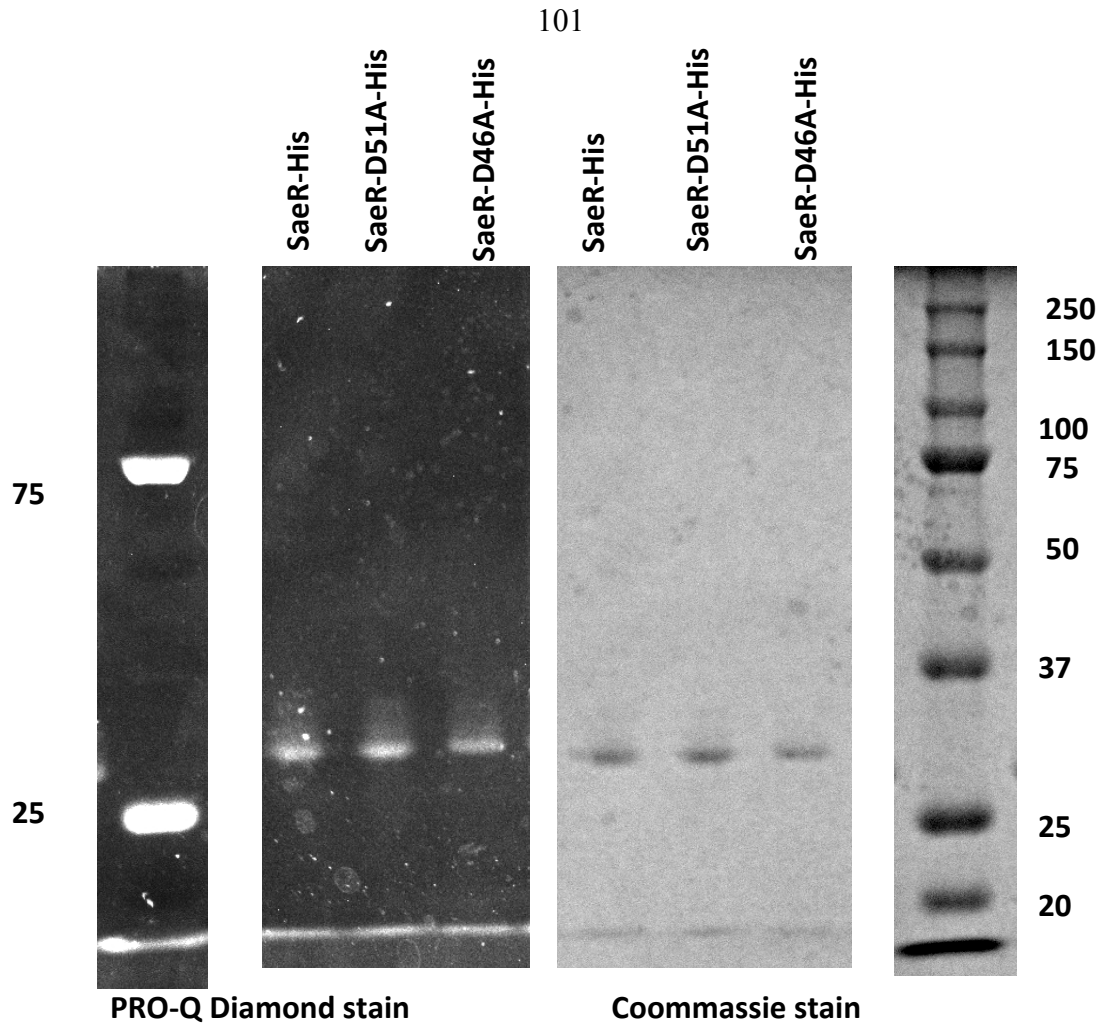


Figure 4.1: SDS-PAGE representing the phosphorylation state of the recombinant proteins from wild-type SaeR and point mutant strains of SaeR. 20µg of recombinant SaeR-His, or SaeR-D51A His or SaeR-D46A His were loaded on a 12% gel and stained with PRO-Q diamond stain following manufacturer's guidelines, followed by coomassie staining. Gels were visualized using Alpha Innotech Alpha view imaging system. Band intensities were determined using ImageJ analysis and the percent of phosphorylated protein was then measured using the formula $\frac{\text{intensity of phosphorylated protein}}{\text{intensity of total protein}} \times 100$. Intensity of phosphorylated protein was determined by band intensities from PRO-Q Diamond stained gel and intensity of total protein was determined by band intensities from Coomassie stained gel.

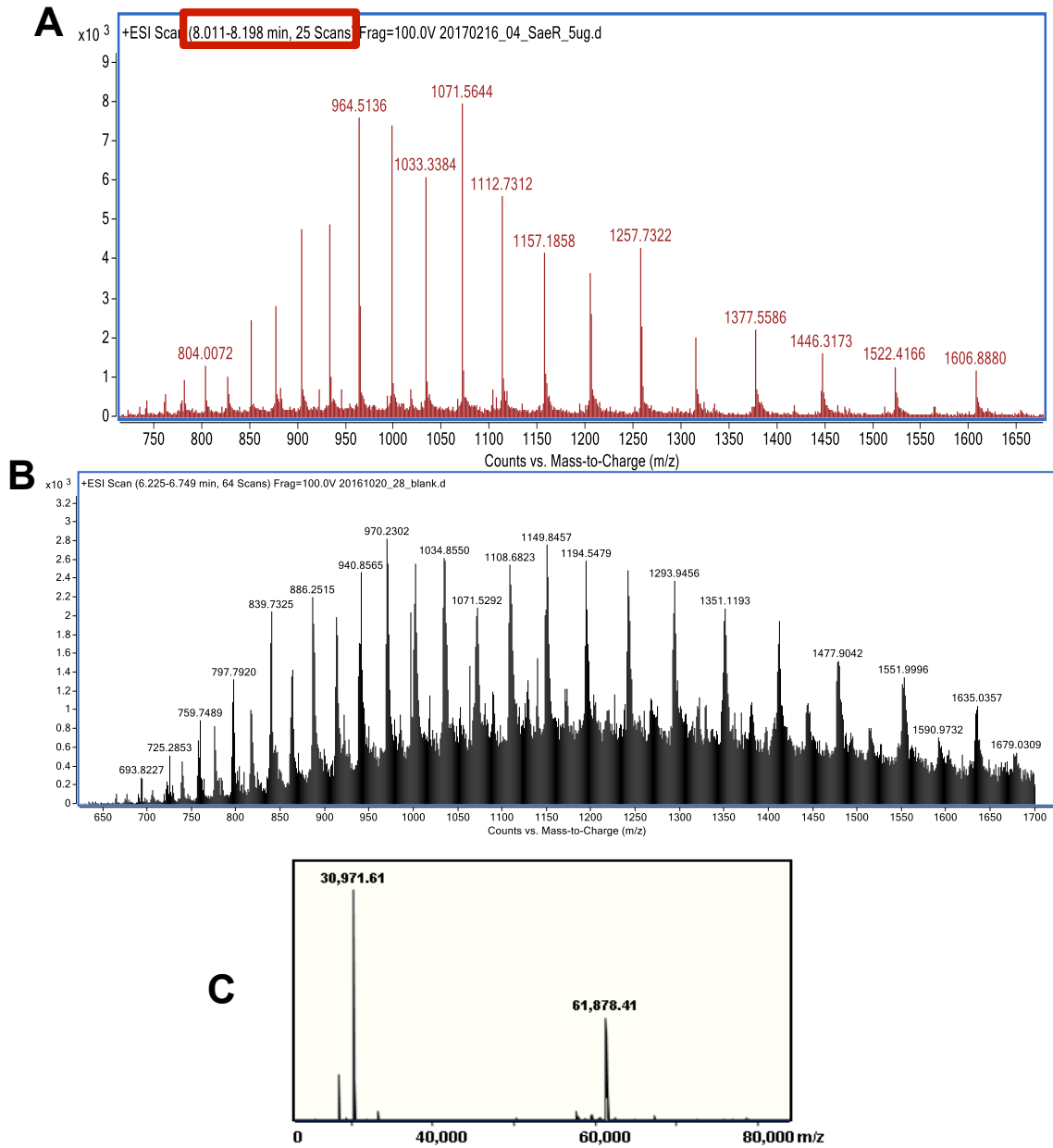


Figure 4.2: Post-translational modifications in recombinant protein from wild-type SaeR and point mutant strains of SaeR. Deconvoluted mass spectrum of recombinant SaeR (A), SaeR-D46A (B) and SaeR-D51A (C).

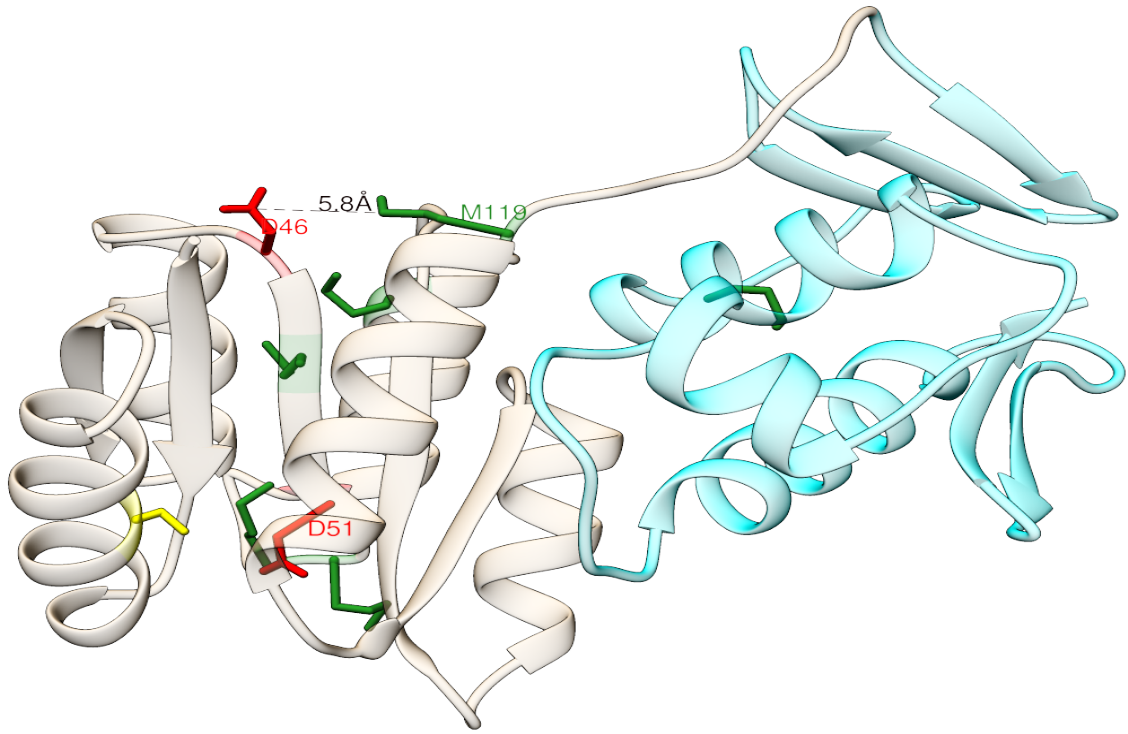


Figure 4.3: Homology model of the response regulator SaeR from *Staphylococcus aureus*. The homology model was created using a combination of PHYRE2 and Chimera softwares. DNA-binding domain in cyan, Cysteine residue in yellow, Aspartate residues in red, Methionine residues in green. Each residue is indicated in order to gauge the spatial distance between them and the likelihood of interaction occurring, giving a hint towards the molecular architecture of SaeR.

CONCLUSION

Molecular studies highlighted in this dissertation, identified key amino acid residues in SaeS and SaeR important for signaling in the Sae system. We have identified a nine amino acid extracellular loop in the sensor kinase SaeS that is essential to sensing human neutrophil and neutrophil-derived stimuli (3) which was further confirmed by Bae et al (2). In addition, we identified a key aspartate residue D46 in the response regulator SaeR that influences virulence gene expression and survival following interaction with human PMNs (chapter 3). Our studies have put into question the accepted role of aspartate 51 in the phosphorylation mediated signaling of the Sae system. Moreover, through both our studies we highlight the importance of host-derived stimuli in modulating the response generated by the Sae system. The critical observation that SaeR can regulate virulence expression in strain 18805 despite missing, D51 in the presence of host derived stimuli, suggest that the Sae system can take advantage of phosphorylation sites or that perhaps phosphorylation is not necessary during stress.

Our studies have provided insights and filled knowledge gaps into the intricate molecular signaling that occurs via the Sae system in response to specific host-derived stimuli. However, many questions remain. Future studies will combine molecular biology and mass spectrometry to determine if phosphorylation occurs *in vivo*. To this extent, we have conducted preliminary *in vitro* studies with recombinant proteins and identified the phosphorylation state of each of our putative phosphorylation site mutant strains (discussed above). If phosphorylation occurs *in vivo*, next step will be to define the particular site and levels of phosphorylation in response to host-derived stimuli. Our

preliminary data suggest that recombinant protein from strains SaeR, and SaeR-D46A are phosphorylated (Table 4.1) and that mutant SaeR-D51A exists as a dimer. These data suggest that if D51 is perhaps the phosphorylation site, then SaeR forms a dimer like other response regulators to bind DNA (8,9). Collectively, our data suggests that D46 impacts the functioning of SaeR-during interaction with the human host hinting towards it being the labile phosphorylation site under host trigger, and that D51 is for the overall structural stability of the monomer SaeR.

Other Gram positive bacteria like Group A Streptococcus (GAS) optimize their response to host-derived stimuli by modulating the sites of phosphorylation (10). In the control of virulence system (CovR/S), phosphorylation at aspartate 53 (Asp 53) by cognate histidine kinase CovS resulted in the expression of virulence genes. However, phosphorylation at threonine 65 (Thr 65) via serine threonine kinase-1 resulted in decreased DNA binding capacity of CovR- thereby inhibiting virulence gene expression (10). Taken together, these data suggest that CovR Asp 53 activating phosphorylation and Thr 65 inhibiting phosphorylation have a complex interplay in GAS gene expression during host-pathogen interaction.

The observation that strain 18805 is able to respond to host-derived stimuli by transcribing essential virulence genes (chapter 3) and that recombinant SaeR-D51A has no phosphate group attached (Table 4.1), suggest that perhaps phosphorylation is not the only mechanism used by SaeR to respond to host-derived stimuli. Our very preliminary observation that recombinant protein is phosphorylated (Fig 4.1), suggests that perhaps the SaeR/S system is always phosphorylated and it is rather the dephosphorylation event

that turns the system on. Research in the field has shown that the Sae system has a feedback mechanism, wherein SaeP and SaeQ forms a protein complex with SaeS and leads to a phosphatase signal (11). One hypothesis that we propose through our findings is that the phosphatase signal is likely responsible for turning the SaeR/S system on and thereby regulate virulence gene expression.

Through this dissertation and other findings from our lab, we show that the bacterial response via the SaeR/S system is dependent on host-derived factors. The existing hypothesis in the field with differential gene regulation is that different levels of SaeR phosphorylation influence the transcription of different classes of virulence genes [typeI and type II] (4). Based on our observations, we propose the hypothesis that different host-derived stimuli induce different phosphorylation patterns (site of phosphorylation and levels of phosphorylation), thereby regulating complex gene regulation. Future studies aimed at testing this hypothesis will involve the use of mass spectrometry on bacteria stimulated with known host-derived stimuli like hydrogen peroxide and HNP-1. In addition, future studies will aim to determine the transcription of virulence genes in response to host-derived stimuli in the Type I and Type II category (4).

Despite advances, a comprehensive understanding of virulence regulation in *S. aureus* remains incompletely defined. Considering SaeR/S system controls virulence factors that have been the focus of studies aimed at therapeutics and vaccines it is necessary to understand how/when these virulence factors are induced in response to the host. Findings outlined in this dissertation address knowledge gaps of virulence regulation in *S. aureus* and provide mechanistic insights into the importance of single

amino acid residues in both the kinase and the response regulator in the overall regulation of virulence factors via the Sae system.

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APPENDIX A

EPIC IMMUNE BATTLES OF HISTORY: NEUTROPHILS VS *Staphylococcus aureus*

APPENDIX A

EPIC IMMUNE BATTLES OF HISTORY: NEUTROPHILS VS *Staphylococcus aureus*

Contribution of Authors and Co-authors

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Contributions: Wrote sections of the manuscript.

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Epic Immune Battles of History: Neutrophils vs. *Staphylococcus aureus*

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Neutrophils are the most abundant leukocytes in human blood and the first line of defense after bacteria have breached the epithelial barriers. After migration to a site of infection, neutrophils engage and expose invading microorganisms to antimicrobial peptides and proteins, as well as reactive oxygen species, as part of their bactericidal arsenal. Ideally, neutrophils ingest bacteria to prevent damage to surrounding cells and tissues, kill invading microorganisms with antimicrobial mechanisms, undergo programmed cell death to minimize inflammation, and are cleared away by macrophages. *Staphylococcus aureus* (*S. aureus*) is a prevalent Gram-positive bacterium that is a common commensal and causes a wide range of diseases from skin infections to endocarditis. Since its discovery, *S. aureus* has been a formidable neutrophil foe that has challenged the efficacy of this professional assassin. Indeed, proper clearance of *S. aureus* by neutrophils is essential to positive infection outcome, and *S. aureus* has developed mechanisms to evade neutrophil killing. Herein, we will review mechanisms used by *S. aureus* to modulate and evade neutrophil bactericidal mechanisms including priming, activation, chemotaxis, production of reactive oxygen species, and resolution of infection. We will also highlight how *S. aureus* uses sensory/regulatory systems to tailor production of virulence factors specifically to the triggering signal, e.g., neutrophils and defensins. To conclude, we will provide an overview of therapeutic approaches that may potentially enhance neutrophil antimicrobial functions.

Keywords: host-pathogen interactions, innate immunity, immune evasion, host defense, phagocytosis, chemotaxis, *Staphylococcus aureus*

INTRODUCTION

Polymorphonuclear leukocytes (PMNs or neutrophils) are the first line of defense against bacterial pathogens that have breached epithelial barriers. Within minutes of bacterial invasion, neutrophils respond to soluble factors including chemokines and cytokines and are recruited to the site of infection where they ingest microbes. Subsequently, neutrophils expose microorganisms to antimicrobial proteins, peptides, and reactive oxygen species to kill the invading pathogen. This is a delicate process that must eliminate the pathogen while controlling excessive inflammation. Concurrently, neutrophils secrete cytokines and chemokines to continue to recruit neutrophils

and enhance other host responses to infection. Finally, neutrophil death is essential for proper resolution of infection and must be regulated to minimize bystander damage while continuing to signal if more immune response is needed or if tissue repair should begin. These potent mechanisms are effective at eliminating most fungal and bacterial microorganisms. However, successful pathogens have developed strategies to disrupt various neutrophil functions to cause infection.

S. aureus is a highly-adaptable Gram-positive pathogen estimated to colonize 50–60% of the population (Wertheim et al., 2005; Gorwitz et al., 2008). It is also a leading cause of infections ranging from superficial skin abscesses to life-threatening diseases, including septicemia and necrotizing pneumonia (Klevens et al., 2007; Kobayashi et al., 2015). The ability of *S. aureus* to cause human disease is based in part on its ability to evade the innate immune response, thereby circumventing rapid elimination. Many factors contribute to *S. aureus* pathogenesis. These include production of numerous toxins, such as the barrel forming two-component toxins capable of directly lysing host immune cells (Menestrina et al., 2003), and tissue destroying enzymes including protease, lipase, and hyaluronidase, as well as many surface proteins and adhesins linked to virulence (Lowy, 1998). In this review, we will focus on evasion strategies used by *S. aureus* to disrupt neutrophil functions essential for bacterial clearance. First, we will highlight virulence factors produced by *S. aureus* to alter neutrophil priming, activation, chemotaxis, and adhesion. Then, we will discuss strategies used by *S. aureus* to subvert neutrophil killing by antimicrobial peptides and proteins and reactive oxygen species. Additionally, we will examine recent literature investigating mechanisms used by *S. aureus* to modulate neutrophil cell death programs. Finally, we will highlight the reciprocal communication between *S. aureus* and the neutrophil emphasizing sensing and adaptive responses used by *S. aureus* to recognize and respond to neutrophil challenge. The review will conclude with an overview of potential therapeutic approaches aimed at disrupting bacterial sensing and signaling to decrease production of virulence factors during neutrophil interaction and discuss putative immunotherapies to boost immune responses to *S. aureus* while limiting inflammatory damage caused by neutrophils.

STRATEGIES USED BY *S. AUREUS* TO DISRUPT NEUTROPHIL PRIMING, ACTIVATION, CHEMOTAXIS AND ADHESION

Neutrophils are initially recruited to a site of infection by following chemokine gradients in a process termed chemotaxis. Taking cues from activated endothelium, neutrophils slow their movement through blood vessels by selectin-mediated tethering to the endothelium followed by complete movement arrest through interaction with integrins on the endothelium. Extravasation from the blood vessels through the endothelial barrier is required for neutrophils to access interstitial fluid and migrate via a chemotactic gradient to the site of infection

where ingestion of bacteria can take place. For detailed reviews of neutrophil chemotaxis, adhesion to the epithelium, and transmigration, please refer to (Kolaczowska and Kubes, 2013; de Oliveira et al., 2016). Herein, we will focus on virulence factors produced by *S. aureus* to inhibit specific neutrophil receptors from binding host and bacterial derived ligands, which results in impaired neutrophil priming, activation, chemotaxis, and adhesion to the endothelium.

NEUTROPHIL PRIMING: A POTENTIAL TARGET OF *S. AUREUS*?

Priming refers to the ability of a primary agonist to enhance a neutrophil's response to a secondary stimulus (Swain et al., 2002). There are many known neutrophil priming agents including: complement components C3a and C5a (Skjelle et al., 2014), interferon- γ (IFN- γ) (Edwards et al., 1988), interleukin-8 (IL-8) (Mitchell et al., 2003), and tumor-necrosis factor- α (TNF- α ; Rainard et al., 2000). Bacterial derived products such as N-formyl methionyl peptide, formyl-methionyl-leucyl phenylalanine (fMLF), peptidoglycan, and *S. aureus* cytolytic toxins also demonstrate an ability to prime neutrophils (Elbim et al., 1994; El-Benna et al., 2008; Clarke et al., 2010; Malachowa et al., 2012). The ability of these agents to prime neutrophils is typically not universal in that concentration and neutrophil response can vary drastically (Swain et al., 2002). Primed neutrophil responses influence many neutrophil functions including increases in adhesion, phagocytosis, superoxide production, and degranulation (Ellis and Beaman, 2004). It can also influence neutrophil apoptosis (Wright et al., 2013). Thus, priming can set the stage for subsequent neutrophil-pathogen interactions and influences outcome of this interaction.

Not much is known about the impact of *S. aureus* on neutrophil priming. Earlier studies investigated priming of neutrophils with conditioned medium from peripheral blood mononuclear cells (PBMC) challenged with killed *S. aureus* (Ferrante et al., 1989; Bates et al., 1991). These studies demonstrated increased neutrophil staphylocidal activity following priming with conditioned media from PBMCs challenged with killed *S. aureus* vs. priming with medium from unstimulated PBMCs. This enhanced bactericidal activity was shown to be dependent on TNF- α produced by PBMCs in response to killed *S. aureus* (Ferrante et al., 1989, 1993; Bates et al., 1991). These studies suggest that the response of PBMCs and resident cells to *S. aureus* may strongly impact the outcome of neutrophil-*S. aureus* interactions. Supporting this idea are studies demonstrating that *S. aureus* promotes production of IFN- γ and this production has been linked to poorer outcome of infection in mouse models (Watkins et al., 2011, 2013). IFN- γ impacts neutrophil function in many ways, including priming of oxidative burst and degranulation mechanisms (Ellis and Beaman, 2004). Using a murine wound model of *S. aureus* infection, IFN- γ -mediated CXC chemokine production by T cells promoted a robust recruitment of neutrophils that resulted in elevated *S. aureus* burdens at the infectious foci (McLoughlin et al., 2008). Subsequent studies using a mouse peritonitis model

also demonstrated IFN- γ was associated with higher bacterial burdens but that the source of IFN- γ was neutrophils (Watkins et al., 2013). Potentially, priming by IFN- γ causes overactivation and inflammation in neutrophils making them less effective at clearing *S. aureus*. The role of IFN- γ and neutrophil bactericidal activity against *S. aureus* warrants further investigation especially considering that IFN- γ is used to treat patients with chronic granulomatous disease (Gallin et al., 1991). CGD patients receiving recombinant IFN- γ show a decrease in *S. aureus* infections (Gallin et al., 1991). Furthermore, *in vitro* studies have demonstrated that priming of human neutrophils with IFN- γ increased bactericidal activity against *S. aureus* (Edwards et al., 1988). Thus, more studies are needed to determine under what conditions IFN- γ promotes an effective neutrophil response against clinically relevant strains of *S. aureus*. Clearly, source, timing and amount of IFN- γ play an important role in outcome of *S. aureus*-neutrophil interactions.

S. aureus produces hemolysins, and bi-component leukocidins that directly impact immune cell function (Seilie and Bubeck-Wardenburg, 2017). Many of these toxins act by forming pores in the membrane of immune cells causing lysis. Among these, the bi-component toxins leukotoxin GH (LukGH, also known as LukAB) and Panton-Valentine leukocidin (PVL) have the ability to cause neutrophil pore-formation when present at high concentrations. However, at sublytic concentrations, these factors can promote enhanced binding, uptake and killing of *S. aureus* through priming of neutrophils (Graves et al., 2012). This is also consistent with reports that have demonstrated the ability of PVL to enhance neutrophil superoxide, granule exocytosis, and release of leukotriene B₄ and IL-8 (König et al., 1995; Colin and Monteil, 2003). In addition, low doses of alpha-toxin, a single component toxin encoded by *hla*, has been shown to promote transcriptional activity leading to production of inflammatory mediators that can prime neutrophils including IL-8 (Dragneva et al., 2001).

As mentioned above, most of the studies thus far have not directly investigated how modulation of known neutrophil priming agents (such as cytokines and chemokines) by *S. aureus* actually influences neutrophil function. Also, the impact of *S. aureus* toxins and immunomodulatory proteins on neutrophil priming has not been thoroughly investigated. Taken together, targeting neutrophil priming may be very important for *S. aureus* to gain an edge on evading the neutrophil. However, additional studies are needed to understand the impact of *S. aureus* on neutrophil priming.

KEY FACTORS USED BY *S. AUREUS* TO DISRUPT NEUTROPHIL ACTIVATION, CHEMOTAXIS AND ADHESION

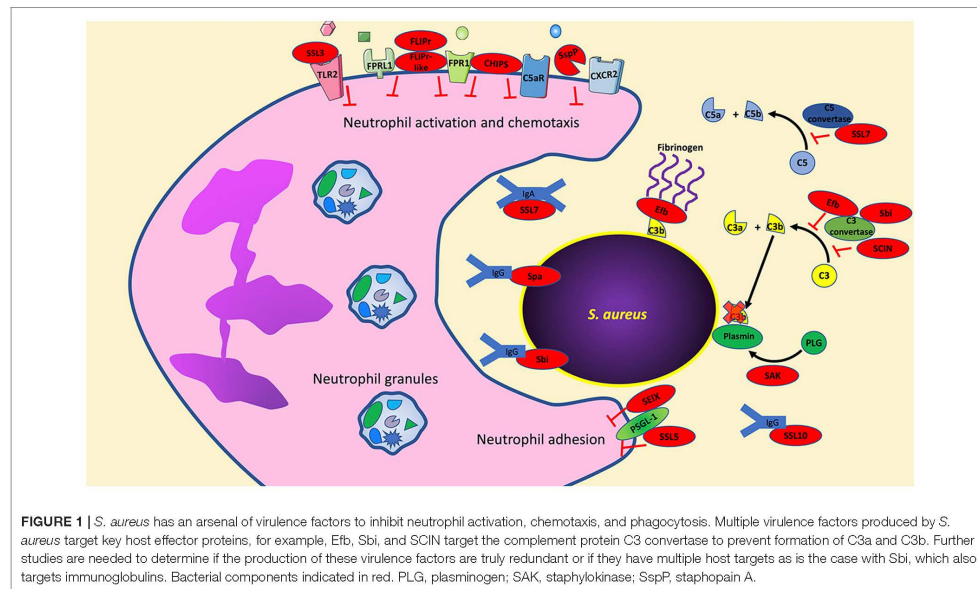
In contrast to our understanding of how *S. aureus* may impact priming, there is an abundance of data that highlight how specific *S. aureus* factors impact neutrophil activation, chemotaxis and adhesion. In this section, we highlight individual virulence factors that have been studied for their ability to disrupt these key mechanisms (Figure 1).

CHIPS

The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1-kDa exoprotein that inhibits neutrophil migration and activation (De Haas et al., 2004). CHIPS prevents neutrophils from responding to both host- and bacteria-derived chemoattractants. Neutrophil activation by host-derived C5a is inhibited by CHIPS binding to the C5a receptor (C5aR). Similarly, neutrophil detection of bacteria-derived formylated peptides is inhibited by CHIPS binding to the formyl peptide receptor (FPR). CHIPS shows high affinity to both C5aR and FPR with K_D -values of 1.1 ± 0.2 and 35.4 ± 7.7 nM, respectively; the affinity of C5aR and FPR for their natural ligands is within the CHIPS binding affinity to C5aR and FPR (Falk et al., 1982; Huey and Hugli, 1985; Postma et al., 2004). While C5aR and FPR are G-protein coupled receptors (GPCRs), the active blocking domain in CHIPS is distinct for inhibiting C5aR and FPR (Haas et al., 2005). By using FITC-labeled CHIPS, Postma et al. demonstrated that CHIPS is not internalized following binding to neutrophil C5aR and FPR and its activity is ATP and cell-signaling independent (Postma et al., 2004). It has also been demonstrated that CHIPS binds to C5aR and FPR extracellularly and does not act as an agonist since receptor binding does not induce calcium mobilization (De Haas et al., 2004). It was further demonstrated that intravenously administered CHIPS was able to inhibit mouse neutrophil influx following intraperitoneal administration of C5a despite a 30-fold lower affinity of CHIPS for mouse C5aR (De Haas et al., 2004). Finally, neutralizing antibodies to CHIPS have been observed in human sera isolated from both normal donor controls and *S. aureus* infected samples, implying CHIPS plays an active role during staphylococcal infection (Wright et al., 2007).

FLIPR AND FLIPR-LIKE

Neutrophils express formyl peptide receptor-like-1 (FPRL1). This receptor is activated by diverse peptides and proteins including: the synthetic peptides Trp-Lys-Tyr-Met-Val-D-Met-NH₂ (WKYMVM), and L-conformer WKYMVM, lipoxin A₄, the T21/DP107 leucine zipper-like domain of the HIV-1 envelope protein gp41, serum amyloid A, the mitochondrial peptide fragment MYFINLTL, the antimicrobial peptide LL-37, and prion peptide fragments (Fiore et al., 1994; Su et al., 1999a,b; Chiang et al., 2000; De Yang et al., 2000; Christophe et al., 2001; Le et al., 2001). Activation of FPRL1 on neutrophils leads to intracellular calcium mobilization and chemotaxis, while superoxide production through FPRL1 is ligand dependent (Bae et al., 2003). *S. aureus* produces two proteins that bind FPRL1 and inhibit its activation. FPRL1 inhibitory protein (FLIPr) is a secreted protein that binds and antagonizes both FPRL1 and FPR, but FPRL1 antagonism by FLIPr is much stronger than on FPR (Prat et al., 2006). Similarly, a second FPRL1 antagonist termed FLIPr-like shares 73% overall homology with FLIPr (Prat et al., 2009). FLIPr and FLIPr-like show similar inhibitory activity toward FPRL1 following stimulation with the FPRL1 agonist MMK-1; however, FLIPr-like antagonism toward FPR is 100-fold stronger than that of FLIPr following stimulation with fMLF (Prat et al., 2009). As expected, neutrophil exposure to purified



FLIPr and FLIPr-like completely inhibits neutrophil chemotaxis toward the FPRL1 agonist MMK-1 (Prat et al., 2006, 2009).

FLIPr and FLIPr-like have also been shown to bind different FcγR isoforms to block IgG binding (Stemberding et al., 2013). Stemberding et al. showed that FLIPr preferentially binds to FcγR class II receptors while FLIPr-like can bind to FcγR class I, II, and III receptors. As expected, neutrophil pretreatment with FLIPr or FLIPr-like significantly reduced phagocytosis of *S. aureus* opsonized with purified IgG (Stemberding et al., 2013). However, it should be noted that phagocytic inhibition of neutrophils toward *S. aureus* opsonized with human serum by FLIPr and FLIPr-like was only observed at low serum concentrations (<1% serum). At higher serum concentrations (1–10% serum), FLIPr and FLIPr-like do not inhibit neutrophil-mediated phagocytosis of opsonized *S. aureus*. Under these higher serum conditions, neutrophil phagocytosis of opsonized *S. aureus* is likely mediated by complement receptors, which are not antagonized by FLIPr and FLIPr-like proteins. It follows that experiments using neutrophils treated with FLIPr and FLIPr-like had reduced uptake of *S. aureus* only when opsonized with complement inactivated serum thus showing the specific inhibition of FLIPr and FLIPr-like toward serum immunoglobulins (Stemberding et al., 2013).

STAPHOPAIN A

The CXCR2 chemokine receptor (also known as IL8RB) is highly expressed on human neutrophils and has high specificity for

the potent neutrophil chemoattractants CXCL1, CXCL2, and CXCL7, as well as CXCL8 (IL-8, which can also bind to CXCR1) (Yoshimura et al., 1987; Baggolini et al., 1989; Ben-Baruch et al., 1997; Ritzman et al., 2010). *S. aureus* infection induces CXCL1, CXCL2, CXCL8 production resulting in neutrophil recruitment (Mempel et al., 2003; Sasaki et al., 2003; Oлару and Jensen, 2010). To prevent neutrophil recruitment induced by CXCR2 ligands, *S. aureus* secretes the cysteine protease staphopain A which cleaves the N-terminus of CXCR2 required for ligand binding (Laarman et al., 2012). Neutrophils pretreated with staphopain A blocked calcium mobilization following stimulation with CXCL1 and CXCL7. Importantly, these chemokines only bind CXCR2 (and not CXCR1). In contrast, staphopain A did not inhibit neutrophil calcium mobilization following stimulation with CXCL8 since it can also signal through CXCR1. The impacts of staphopain A *in vivo* are lacking since staphopain A is specific for human CXCR2 and does not cleave murine CXCR2 (Laarman et al., 2012).

SEIX AND SSL5

Neutrophils express P-selectin glycoprotein ligand 1 (PSGL-1) on the cellular surface to bind P- and E-selectin on activated endothelial cells or platelets and L-selectin expressed on leukocytes (Moore et al., 1995; Guyer et al., 1996; Hidalgo et al., 2007; Huo and Xia, 2009; Stadtmann et al., 2013). PSGL-1 binding to selectins tethers neutrophils to activated endothelial cells and is a first step in the process of transmigration. *S. aureus* secretes two proteins that inhibit PSGL-1 binding to

selectins. The staphylococcal enterotoxin-like toxin X (SEIX) is a highly conserved superantigen that binds glycosylated PSGL-1 on neutrophils and inhibits binding to P-selectin (Wilson et al., 2011; Fevre et al., 2014). Similarly, the staphylococcal superantigen-like 5 (SSL5) protein directly binds PSGL-1 on neutrophils inhibiting rolling on endothelial cells (Bestebroer et al., 2007). In addition to binding PSGL-1, SSL5 also binds to other GPCRs but only inhibits the ligands that require the N-terminal domain of their respective receptors for activation (Bestebroer et al., 2009). Thus, SSL5 pretreatment of neutrophils inhibited activation induced by C3a, C5a, CXCL1, and CXCL8 (Bestebroer et al., 2009).

SSL3

Toll-like receptor 2 (TLR2) recognizes staphylococcal peptidoglycan-associated lipoproteins (Fournier and Philpott, 2005; Kurokawa et al., 2009). TLR2 can also discriminate between diacylated and triacylated lipoproteins by associating with TLR6 or TLR1, respectively (Takeuchi et al., 2001, 2002). The recognition of staphylococcal lipoproteins such as the staphylococcal iron transporter C (SitC) are exclusively dependent on TLR2 to induce immune activation (Stoll et al., 2005; Kurokawa et al., 2009; Müller et al., 2010). The importance of TLR2 during *S. aureus* infection is highlighted by TLR2^{-/-} mice that show increased susceptibility and nasal colonization to *S. aureus* compared to wild-type mice (Takeuchi et al., 2000; González-Zorn et al., 2005; Hoebe et al., 2005). In addition, TLR2^{-/-} mice infected with *S. aureus* show alterations in cytokines that modulate neutrophil function including TNF- α and IL-1 β (Knuefermann et al., 2004). Engagement of TLR2 influences many neutrophil functions including: adhesion molecule expression, reactive oxygen species production (following stimulation with fMLF), and modulates CXCL8, and chemokine receptor expression (Sabroe et al., 2003). *S. aureus* produces the staphylococcal superantigen-like 3 protein that binds TLR2 to inhibit activation in neutrophils and other cell types expressing TLR2 (Bardoel et al., 2012; Yokoyama et al., 2012). Crystal structures showed that SSL3 binding to TLR2 reduced the lipopeptide binding pocket by ~50%, which inhibited binding of the TLR2 agonist Pam₂CSK₄ (Koymans et al., 2015). The same study demonstrated SSL3 can bind a preformed TLR2- Pam₂CSK₄ complex and this blocked TLR2-TLR1 and TLR2-TLR6 heterodimerization thereby inhibiting downstream signaling.

STRATEGIES USED BY *S. AUREUS* TO INHIBIT NEUTROPHIL PHAGOCYTOSIS

Phagocytosis is a process by which neutrophils, and other phagocytes, ingest particles from their extracellular environment including bacteria and host cells. In neutrophils, this sequestration results in the formation of an intracellular compartment termed the phagosome following invagination of the cellular membrane. Neutrophil granules fuse with the phagosome to release antimicrobial peptides and proteins, as well as produce reactive oxygen species to kill invading

microorganisms. Neutrophils bind to pathogen-associated molecular patterns on the *S. aureus* surface to initiate the phagocytic process. Furthermore, binding of immunoglobulins to *S. aureus* and complement activation enhances phagocytosis, as well as engages different neutrophil receptors resulting in branching downstream signaling. *S. aureus* employs strategies and produces a wide range of virulence factors to disrupt neutrophil phagocytosis. Herein, we will highlight select strategies used by *S. aureus* to disrupt neutrophil phagocytosis. Since a complete review on the strategies used by *S. aureus* to avoid phagocytosis is outside the scope of this publication, we recommend previous detailed reviews focusing on immunoglobulin and complement evasion by *S. aureus* to inhibit phagocytosis (Lambris et al., 2008; Serruto et al., 2010; van Kesse et al., 2014).

CAPSULE SYNTHESIS

The production of capsular polysaccharide by *S. aureus* has been proposed as an antiphagocytic evasion strategy but its actual role in inhibiting neutrophil phagocytosis remains controversial. Indeed, previous studies have shown that *S. aureus* strains producing high levels of capsular polysaccharide withstand neutrophil killing better than microencapsulated strains (Xu et al., 1992; Thakker et al., 1998). However, it should be noted that under these experimental conditions neutrophil killing of *S. aureus* was correlated to the ability of neutrophils to associate with bacteria without clearly showing ingestion. Furthermore, the experimental conditions used suspended instead of adherent neutrophils which can greatly influence neutrophil ingestion of *S. aureus* (Lu et al., 2014). Also, an intraperitoneal *S. aureus* infection model showed no differences in neutrophil intracellular staphylococcal survival between a highly and microencapsulated strain (Gresham et al., 2000). Notably, the predominant clinical isolate USA300 is unencapsulated, further questioning the role of capsule synthesis in *S. aureus* pathogenicity (Montgomery et al., 2008; Carrel et al., 2015).

COMPLEMENT INHIBITION

Human pathogens have developed strategies to evade complement, thus inhibiting immune recognition, cytokine production, and neutrophil uptake (Lambris et al., 2008; Serruto et al., 2010). *S. aureus* produces several virulence factors that target different machinery in the complement system. The staphylococcal complement inhibitor (SCIN), is a 9.8-kDa exoprotein that specifically binds and inhibits human C3 convertases (Rooijakkers et al., 2005a, 2006). SCIN binding to C3 convertases does not prevent C3 binding but completely inhibits generation of C3b and thus opsonization of *S. aureus* by C3b (Rooijakkers et al., 2005a; Ricklin et al., 2009; Garcia et al., 2010). In addition, SCIN stabilizes C3 convertases by competing with factor H which accelerates decay of C3 convertases (Ricklin et al., 2009). *In vitro*, recombinant SCIN can significantly inhibit neutrophil phagocytosis of human serum opsonized *S. aureus* resulting in increased bacterial survival (Rooijakkers et al.,

2005a). *In vivo* studies with SCIN are lacking since it is human specific, but SCIN is immunogenic since a limited study with 80 healthy people and 20 individuals with recurring staphylococcal infections showed all produced antibodies to SCIN (Rooijakkers et al., 2005a).

S. aureus is also equipped with virulence factors that target complement without direct binding to C3 convertase. Staphylokinase is a secreted protein that binds human plasminogen converting it into its active form plasmin (Parry et al., 2000; Mölken et al., 2002; Rooijakkers et al., 2005b). Plasmin, a serine protease, is bound to the *S. aureus* surface and degrades C3 convertase-dependent C3b to prevent deposition on the bacterial surface. Human neutrophils show decreased phagocytic activity toward human serum opsonized *S. aureus* (depleted of IgG and IgM to rule out immunoglobulin mediated phagocytosis) and pretreated with recombinant staphylokinase (Rooijakkers et al., 2005b). In addition, *S. aureus* secretes the extracellular fibrinogen-binding (Efb) protein that binds to C3 to prevent cleavage to C3b and can also directly bind C3b deposited on the extracellular bacterial membrane (Lee et al., 2004; Ko et al., 2013). As its name implies, the extracellular fibrinogen-binding protein also contains a fibrinogen binding domain that recruits fibrinogen and inhibits neutrophil phagocytosis (Ko et al., 2013). Complement receptor recognition of C3b is blocked by a thick layer of fibrinogen linked by Efb on the bacterial surface (Ko et al., 2013). *Ex vivo* studies with human blood exposed to Efb and *S. aureus* showed a significant reduction in neutrophil phagocytosis compared to *S. aureus* alone. Similarly, supernatants from wild-type *S. aureus* reduced neutrophil phagocytosis of opsonized *S. aureus* compared to supernatants from Efb-deficient *S. aureus* in the presence of fibrinogen. Finally, neutrophils from mice intraperitoneally infected with GFP-expressing wild-type *S. aureus* or an Efb-deficient mutant showed significantly higher ingestion of the Efb mutant compared to the wild-type (Ko et al., 2013). While this observation suggests that sufficient levels of Efb are produced by *S. aureus* to have an *in vivo* effect, it should be noted that intraperitoneal injection was performed with *S. aureus* grown to late exponential phase without washing culture toxins. Thus, increased and unknown levels of Efb were likely present from the beginning giving an advantage to *S. aureus* to prevent neutrophil phagocytosis.

INHIBITION OF IMMUNOGLOBULIN RECOGNITION

Complement and immunoglobulins function as opsonins to enhance *S. aureus* recognition and ingestion by neutrophils. While *S. aureus* produces virulence factors that inhibit complement activation and deposition on the bacterial surface, the efficacy of these virulence factors *in vivo* is controversial since immunoglobulin opsonization plays a redundant role in opsonophagocytosis. In fact, opsonophagocytosis assays investigating the role of *S. aureus* virulence factors that disrupt complement must be done in the absence of immunoglobulins to observe an inhibitory effect on phagocytosis because *S.*

aureus produces virulence factors that inhibit opsonization by immunoglobulins. Protein A, encoded by the *spa* gene, is one of the better characterized virulence factors produced by *S. aureus* that inhibits neutrophil phagocytosis. Protein A is a 42-kDa secreted and membrane bound protein that binds the constant Fc γ region of IgG thus preventing proper engagement of Fc γ receptors on neutrophils and antigen recognition (Forsgren and Sjöquist, 1966; Kronvall et al., 1970; Sjödahl, 1977). Indeed, early studies clearly showed that purified protein A inhibited neutrophil phagocytosis of *S. aureus* independent of capsule polysaccharide on the bacterial surface (Dossett et al., 1969; King and Wilkinson, 1981). More recent studies have demonstrated protein A induces B cell proliferation and production of V $_H$ 3 serum IgG and IgM (Pauli et al., 2014; Kim et al., 2016). Although abundant, these antibodies do not provide protection against *S. aureus*. Generation of protein A variants that were unable to bind immunoglobulin induce a protective antibody response presumably via allowing Fc mediated uptake of opsonized *S. aureus* (Falugi et al., 2013). These data suggest that mechanisms of neutrophil recognition and uptake of *S. aureus* are likely critical to resolution of infection.

S. aureus produces a second binding protein of immunoglobulin (termed Sbi) that also binds IgG to inhibit neutrophil phagocytosis (Jacobsson and Frykberg, 1995; Zhang et al., 1998, 1999; Smith et al., 2011). Human neutrophils can phagocytose significantly more opsonized Sbi-deficient *S. aureus* compared to wild-type, and Sbi plays a protective role in *ex vivo* whole human blood killing assays (Smith et al., 2011). Interestingly, Sbi binds to lipoteichoic acid in the bacterial cell surface to remain anchored, while it can also exist in a secreted form that binds complement protein C3 to induce its degradation away from the bacterial surface (Burman et al., 2008; Upadhyay et al., 2008). Sbi also plays another immunomodulatory role that was demonstrated to impact neutrophil function. In a mouse model of peritonitis Sbi induced production of IL-6 and CXCL-1, which resulted in neutrophil recruitment and subsequently an exacerbated inflammatory response (Gonzalez et al., 2015).

In addition to Sbi, *S. aureus* secretes other proteins that bind both immunoglobulins and complement proteins to disrupt neutrophil phagocytosis. The 23-kDa staphylococcal superantigen-like 7 (SSL7) exoprotein binds to human IgA with high affinity (K_D of 1.1 nM) and inhibits Fc α R binding on the neutrophil surface (Langley et al., 2005). Using an *ex vivo* whole blood infection model it was demonstrated that binding of IgA by SSL7 reduced neutrophil phagocytosis of *S. aureus* (Bestebroer et al., 2010). SSL7 also binds to the C5 complement protein (K_D of 18 nM) and inhibits C5a generation in an IgA binding-dependent manner, as well as formation of the C5b-9 membrane attack complex (Langley et al., 2005; Bestebroer et al., 2010; Laursen et al., 2010; Lorenz et al., 2013). *In vivo* mouse studies showed that administration of purified SSL7 inhibited neutrophil recruitment following exposure to heat-killed *S. aureus*. Thus, SSL7 serves diverse functions that include inhibiting neutrophil phagocytosis, recruitment, and complement activation (Bestebroer et al., 2010; Lorenz et al., 2013). Another staphylococcal superantigen-like protein termed SSL10 also binds to IgG (K_D of 220 nM) and to the complement protein C1q (Itoh et al., 2010). SSL10 significantly

inhibited neutrophil ingestion of IgG-opsonized *S. aureus* by blocking binding of FcγR (Patel et al., 2010).

Taken together, it is clear that *S. aureus* produces virulence factors that decrease neutrophil phagocytosis via production of physical barriers (capsule polysaccharide), inhibition of complement activation leading to decreased deposition of opsonins, and disruption of immunoglobulin binding to bacterial antigens. However, the efficacy of these virulence factors has been difficult to determine. Neutrophil-mediated phagocytosis of *S. aureus* is efficient in the absence of opsonins. *In vitro* studies have shown that adherent neutrophils rapidly ingest both opsonized and unopsonized *S. aureus* (Lu et al., 2014). Thus, neutrophils contain pattern-recognition receptors that are sufficient for phagocytosis of *S. aureus* independent of opsonins. Clearly, the redundancy in neutrophil receptor-mediated phagocytosis mechanisms compounds studies and strongly suggests the combination of *S. aureus* virulence factors are needed to thwart the innate ability of neutrophils to recognize this pathogen.

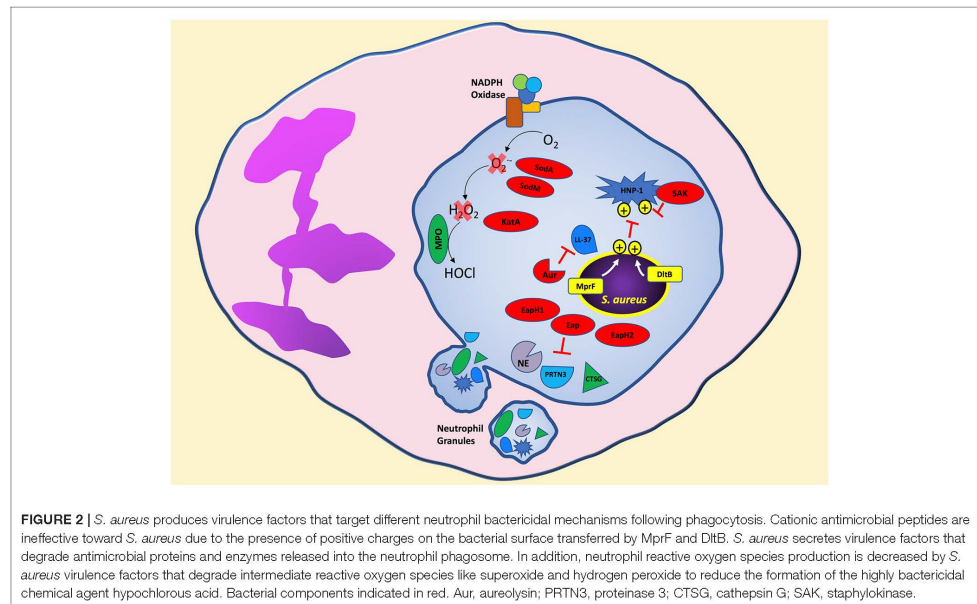
EVASION STRATEGIES USED BY *S. AUREUS* TO SURVIVE NEUTROPHIL KILLING

S. aureus is clearly decorated to avoid ingestion by neutrophils. Additionally, *S. aureus* is loaded with mechanisms to disarm potent neutrophil bactericidal mechanisms. Soon after initiation of the phagocytic process, and possibly before complete sealing

of the phagosome, neutrophil granules mobilize to fuse and release antimicrobial agents into the phagosome containing *S. aureus* (Flannagan et al., 2009). Following phagocytosis, neutrophils expose *S. aureus* to antimicrobial peptides, proteins, and reactive oxygen species to degrade essential bacterial proteins and disrupt homeostasis resulting in bacterial death (Figure 2). In this section, we will highlight strategies used by *S. aureus* to avoid oxidative and non-oxidative mechanisms of neutrophil killing. Although we separate *S. aureus* evasion of oxidative and non-oxidative killing, it is important to remember that these mechanisms likely have synergistic antimicrobial activity in a physiological setting.

S. AUREUS EVASION OF NON-OXIDATIVE KILLING

The *dlt* operon in *S. aureus*, containing genes *dltABCD* is involved in activation and transfer of D-alanine, and increases tolerance to cationic antimicrobial peptides (AMPs), including human neutrophil peptide-1 (HNP-1, also known as alpha-defensin), by incorporation of positively charged D-alanine into teichoic acids (Peschel et al., 1999; Collins et al., 2002). Teichoic acids are highly negatively charged by deprotonized phosphate groups that electrostatically interact with cationic peptides. Thus, a *S. aureus dlt* mutant strain retains a negatively charged teichoic acid backbone and is significantly more susceptible to killing by cationic peptides (Peschel et al., 1999). *In vitro*, *S. aureus*



dlt mutants are highly susceptible to neutrophil non-oxidative killing mechanisms whereas wild-type *S. aureus* is resistant and mainly susceptible to neutrophil oxidative killing (Collins et al., 2002). The importance of the *dlt* operon to increase *S. aureus* survival was observed in an *in vivo* mouse model of sepsis, which showed increased host mortality infected with wild-type *S. aureus* compared to the non-lethal *S. aureus dlt* mutant (Collins et al., 2002). Similarly, the *mprF* gene in *S. aureus* encoding lysylphosphatidylglycerol (LPG) synthetase confers resistance to cationic AMPs by transferring the positively charged L-lysine to the negatively charged lipid phosphatidylglycerol creating LPG (Peschel et al., 2001; Oku et al., 2004; Ernst et al., 2009). Electrostatic repulsion between cationic AMPs and the attached lysine prevents binding of cationic AMPs and disruption of the bacterial membrane. Wild-type *S. aureus* is more resistant to killing by human neutrophils compared to a *S. aureus mprF* mutant (Peschel et al., 2001). Thus, *S. aureus* modifies its cell surface as an evasion strategy by increasing electrostatic repulsion of neutrophil antimicrobial peptides leading to decreased bacterial killing.

In addition to cell membrane modifications to decrease efficacy of antimicrobial peptides by electrostatic repulsion, *S. aureus* produces proteins that directly bind, inhibit, and degrade antimicrobial peptides. Staphylokinase, which activates plasminogen to the active serine protease plasmin leading to degradation of IgG and C3b (Roosjakkers et al., 2005b), also binds HNP-1 and inhibits its bactericidal activity (Jin et al., 2004). The cathelicidin LL-37, which has potent staphylococcal bactericidal activity, is degraded by the *S. aureus* secreted metalloprotease aureolysin (Sieprawaska-Lupa et al., 2004). Thus, *S. aureus* strains producing aureolysin are significantly more resistant to LL-37 than aureolysin-negative strains. Furthermore, the extracellular adherence protein (Eap) and the Eap-homologs 1 and 2 (EapH1 and EapH2) are neutrophil serine protease inhibitors (Harraghy et al., 2005). Purified Eap, EapH1, or EapH2 inhibit the activity of neutrophil elastase, proteinase 3, and cathepsin G which are found in neutrophil azurophilic granules and are bactericidal to *S. aureus* (Papayannopoulos et al., 2010; Stapels et al., 2014).

S. AUREUS EVASION OF OXIDATIVE KILLING

Neutrophil activation leads to assembly of the NADPH oxidase resulting in production of reactive oxygen species (ROS; DeLeo and Quinn, 1996; DeLeo et al., 1999; Bréchar and Tschirhart, 2008; Nunes et al., 2013). Assembly of the membrane and cytoplasmic NADPH oxidase components results in electrons shuttled from NADPH to oxygen producing superoxide. Spontaneous and myeloperoxidase (MPO) catalyzed dismutation of superoxide produces hydrogen peroxide, which in the presence of MPO and chloride results in the highly bactericidal agent hypochlorous acid/hypochlorite anion (Kettle et al., 2007; Klebanoff et al., 2013). Neutrophil ingestion exposes *S. aureus* to concentrated ROS in the phagosome resulting in oxidation and chlorination of bacterial and host proteins (Green et al., 2014). Inhibition of neutrophil ROS production by

diphenyleneiodonium significantly increases *S. aureus* survival following neutrophil phagocytosis even with functioning non-oxidative killing mechanisms (Hampton and Winterbourn, 1995; Hampton et al., 1996). Individuals with chronic granulomatous disease carry mutations in components of NADPH oxidase preventing formation of superoxide and hydrogen peroxide and suffer from recurring bacterial infections including *S. aureus* (Lekstrom-Himes and Gallin, 2000; Assari, 2006). Thus, uninhibited neutrophil ROS production is essential for *S. aureus* clearance.

Bacterial pathogens have developed strategies to inhibit ROS killing (Flannagan et al., 2009). *S. aureus* produces several enzymes in response to neutrophils to degrade and detoxify ROS. The ROS chain of production begins with the formation of superoxide from oxygen. *S. aureus* produces two superoxide dismutases, encoded by *sodA* and *sodM*, that convert superoxide into hydrogen peroxide and molecular oxygen (Karavolos et al., 2003). Superoxide dismutase activity assessed on nondenaturing polyacrylamide gel electrophoresis from the *S. aureus* cytoplasm shows three distinct bands consisting of SodA and SodM homodimers, and a SodA-SodM hybrid (Clements et al., 1999; Valderas and Hart, 2001). Wild-type *S. aureus* is more resistant to superoxide stress compared to isogenic *sodA*, or *sodM*, or *sodAsodM*-deficient strains (Karavolos et al., 2003). In a mouse subcutaneous model of infection, significantly higher wild-type *S. aureus* colony forming units were recovered from the site of infection compared to single or double *sod* mutant strains suggesting a role for superoxide dismutase in virulence (Karavolos et al., 2003). The expression of *sodA* and *sodM* is increased under oxidative stress and regulated by the transcriptional regulator SarA (Ballal and Manna, 2009). Interestingly, *S. aureus* uses multiple regulatory systems to respond to oxidative stress. KatA, the only catalase encoded by *S. aureus* that degrades hydrogen peroxide to water and oxygen, is co-regulated by the ferric uptake repressor (Fur) and the peroxide response regulator (Horsburgh et al., 2001b). As expected, *kata* deficient *S. aureus* is more sensitive to killing by hydrogen peroxide compared to wild-type *S. aureus* (Horsburgh et al., 2001a). Furthermore, the alkyl hydroperoxide reductase (AhpC) increases *S. aureus* resistance to the organic-hydroperoxide cumene hydroperoxide and its absence increases *S. aureus* resistance to hydrogen peroxide by a compensatory increase in *kata* expression (Cosgrove et al., 2007). However, the significance of KatA in surviving neutrophil ROS production is unclear since studies have shown no significant differences in neutrophil killing of wild-type *S. aureus* compared to an isogenic *kata* deficient strain (Cosgrove et al., 2007).

Superoxide dismutase and catalase protect *S. aureus* from ROS through enzymatic degradation. *S. aureus* also produces virulence factors that inhibit ROS killing by different mechanisms. The iconic yellow-golden pigment of *S. aureus* is a result of the antioxidant carotenoid named staphyloxanthin and synthesized by genes encoding *crtM* and *crtN* (Wieland et al., 1994; Pelz et al., 2005). The production of staphyloxanthin increases *S. aureus* resistance to killing by hydrogen peroxide and singlet oxygen (Liu et al., 2005). In

addition, staphyloxanthin increases *S. aureus* survival during exposure to neutrophil ROS; in contrast, staphyloxanthin does not confer protection during exposure to neutrophils from patients with chronic granulomatous disease or neutrophils pretreated with diphenyleneiodonium which have deficiencies in ROS production (Liu et al., 2005). Following exposure to neutrophil ROS, surviving bacteria undergo repair of oxidized proteins to maintain homeostasis. *S. aureus* encodes four methionine sulfoxide reductases that play a role in reducing oxidized methionine residues (Singh, 2003; Schöneich, 2005; Singh et al., 2015). Deletion of the methionine sulfoxide reductases *msrA1* and *msrB* increases *S. aureus* susceptibility to exogenous hydrogen peroxide and hypochlorous acid. Furthermore, a *msrA1* and *msrB* double mutant strain of *S. aureus* is more susceptible to neutrophil killing compared to wild-type (Pang et al., 2014). Finally, the SaeR/S TCS also plays a role in regulating virulence factors that decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis (Guerra et al., 2016). The SaeR/S-regulated virulence factors that decrease neutrophil ROS remain unknown and are an active area of research, but are independent of superoxide dismutase and catalase activity since their expression is not regulated by SaeR/S (Rogasch et al., 2006; Voyich et al., 2009; Nygaard et al., 2010; Sun et al., 2010).

Human neutrophils extrude DNA decorated with antimicrobial proteins termed neutrophil extracellular traps (NETs) to ensnare and kill bacteria as a terminal cell fate pathway that has been named NETosis and is dependent on ROS (Brinkmann et al., 2004; Fuchs et al., 2007; Galluzzi et al., 2012). Neutrophil exposure to *S. aureus* is a potent inducer of NETs (Pilschek et al., 2010). However, *S. aureus* escapes NETs by secreting nuclease (*nuc*), an SaeR/S-regulated factor (Berends et al., 2010; Olson et al., 2013). Furthermore, the nuclease products from DNA degradation 2'-deoxyadenosine-3'-monophosphate and 2'-deoxyadenosine-5'-monophosphate are converted by the *S. aureus* exoprotein adenosine synthase (*adsA*) into 2'-deoxyadenosine (dAdo), which induces caspase-3-mediated apoptosis in macrophages (Thammavongsa et al., 2013). A murine intravenous infection model showed macrophages are unable to diffuse into kidney abscesses containing neutrophils and wild-type *S. aureus*; however, infection with a *nuc* or *adsA* deficient *S. aureus* strain allowed macrophages to efficiently infiltrate the neutrophil abscess (Thammavongsa et al., 2013).

S. AUREUS MODULATION OF NEUTROPHIL FATE

Neutrophils undergo an apoptotic differentiation program in response to bacterial pathogens to limit host damage caused by a prolonged inflammatory response (Kobayashi et al., 2003). For excellent reviews on the mechanisms of neutrophil apoptosis and how bacterial pathogens modulate neutrophil fate, we recommend (Kennedy and DeLeo, 2009; Rigby and DeLeo, 2012; Kobayashi et al., 2017). *S. aureus* produces pore-forming

toxins that lyse neutrophils, other leukocytes, and red blood cells and this lytic activity promotes an intense inflammatory response. While these toxins clearly play a role in lysing neutrophils and other immune cells, their role if any beyond neutrophil cytolysis is unknown. Notable exceptions include Pantone-Valentine Leukocidin (PVL) and LukGH which have been shown to prime neutrophils at sublytic concentrations and promote neutrophil extracellular trap formation, respectively (Graves et al., 2012; Malachowa et al., 2013). In addition, neutrophils exposed to *S. aureus* have been shown to undergo cell death with intact phagosomes undermining the role of cytolytic toxins in *S. aureus* escape from neutrophils (Kobayashi et al., 2010). For reviews on the lytic properties of these toxins and their targets, please refer to (DuMont and Torres, 2014; Reyes-Robles and Torres, 2016). Herein, we will highlight recent studies suggesting *S. aureus* modulates neutrophil fate to disrupt proper neutrophil clearance following phagocytosis of *S. aureus* independent of lytic activity.

Following phagocytosis, neutrophils are ingested by macrophages in a process called efferocytosis to remove spent neutrophils and limit inflammation (Martin et al., 2014). *S. aureus* inhibits macrophage efferocytosis of *S. aureus* laden neutrophils by upregulating the "don't eat me" signal CD47 (Greenlee-Wacker et al., 2014). Interestingly, macrophage production of cytokines that modulate neutrophil fate was altered and inflammasome activation was reduced in response to neutrophils harboring *S. aureus* compared to *S. aureus* alone. *In vitro*, human neutrophils containing *S. aureus* do not undergo the classical apoptotic death pathway as they fail to activate caspase-3, as well as caspase-2, -8, and -9. Instead, neutrophil death in this *in vitro* model in response to *S. aureus* is dependent on receptor-interacting protein 1 (RIP-1) which is a hallmark of programmed cell death or necroptosis. Studies by Zurek et al. showed that the SaeR/S TCS plays a role in modulating neutrophil fate by inhibiting IL-8 production and NF- κ B activation (Greenlee-Wacker et al., 2014; Zurek et al., 2015). Programmed neutrophil death in response to wild-type *S. aureus* was accelerated compared to exposure to a *saeR/S* deletion mutant.

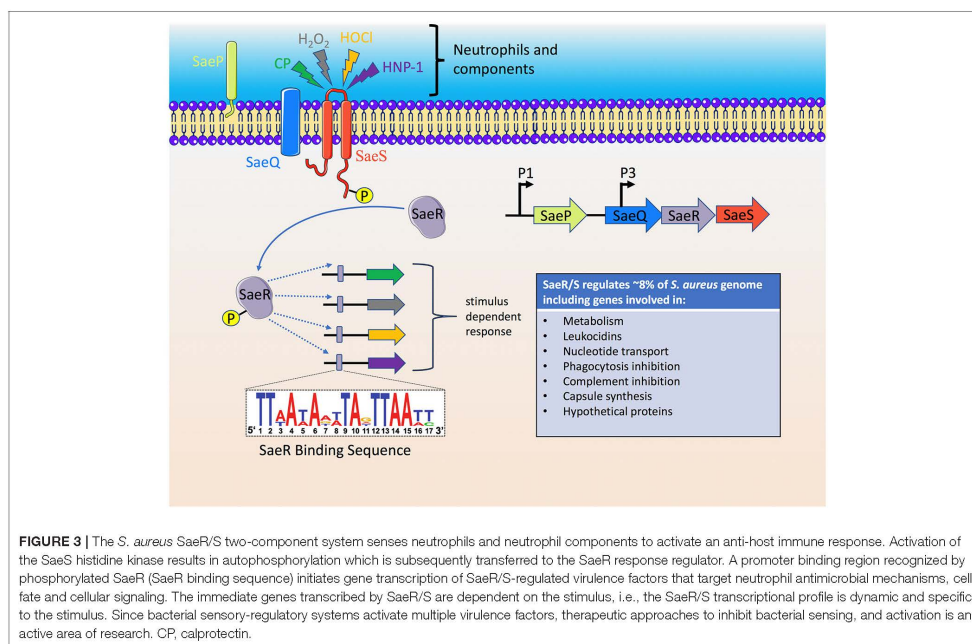
In addition, ROS production is implicated in programmed neutrophil death leading to NET formation (NETosis) and as discussed above *S. aureus* inhibits neutrophil ROS production (Fuchs et al., 2007; Akong-Moore et al., 2012). Interestingly, Pilschek et al. described a NET forming mechanism that did not require neutrophil lysis and is ROS independent. Instead, DNA-containing vesicles budding from the nuclear membrane are released while maintaining neutrophil plasma membrane integrity (Pilschek et al., 2010). Thus, these anuclear neutrophils may still retain phagocytic and chemotactic activity, as previously observed (Malawista et al., 1989). Further studies are needed to determine if inhibition of neutrophil ROS production by *S. aureus* modulates NETosis and to better elucidate ROS independent and dependent NET formation and NETosis. However, it is clear that *S. aureus* alters the neutrophil death program through a variety of mechanisms that impact not only cellular signaling by the neutrophil but clearance via efferocytosis.

THE OTHER SIDE OF THE EQUATION: THE ABILITY OF *S. AUREUS* TO SENSE NEUTROPHILS

As reviewed above, the neutrophil has many mechanisms to sense and respond to *S. aureus* and likewise individual *S. aureus* factors have evolved to disrupt every step of the neutrophil response to the invading pathogen. However, the ability of *S. aureus* to sense the host has been underestimated. The *S. aureus* genome consists of 16 two-component systems (TCS) that sense environmental stimuli and regulate gene expression accordingly (Kawada-Matsuo et al., 2011). Of these 16 TCSs, SaeR/S is recognized as a major contributor to *S. aureus* pathogenesis and neutrophil evasion (Voyich et al., 2009; **Figure 3**). Geiger et al. was the first to demonstrate that the TCS SaeR/S contained an upstream promoter element that could be activated by human neutrophil products H_2O_2 and alpha-defensin (Geiger et al., 2008). SaeR/S target genes are differentially regulated in response to whole blood, neutrophils and neutrophil components (H_2O_2 , alpha-defensin, and calprotectin; Voyich et al., 2005; Palazzolo-Ballance et al., 2007; Malachowa et al., 2011; Flack et al., 2014; Zurek et al., 2014; Cho et al., 2015). The nine amino acid extracellular loop of SaeS plays an important role in sensing different neutrophil-derived stimuli (Flack et al., 2014; Liu et al., 2015). Highly conserved residues M31, W32, and F33 on SaeS are essential to the appropriate activation of

sae-target genes. Residue M31 is essential to the activation of *sae* regulon and plays an important role in sensing neutrophil-derived stimuli including HNP-1. Strains with mutations in aromatic residues W32 and F33 have disrupted normal basal signaling of SaeS in the absence of inducing signals, yet both mutant kinases have appropriate activation of effector genes following exposure to neutrophil-derived stimuli (Flack et al., 2014). This posits that response regulators turn-on only subsets of genes based on the host stimulus. Such observations support a unique hypothesis that emerging strains of bacteria are not more virulent because they harbor new virulence factors but are better able to sense and respond to their human hosts.

SaeR/S is the best studied for its role in sensing the neutrophil but additional studies are needed to understand just how *S. aureus* senses the neutrophil *in vivo*. Certainly other regulatory systems play a role either directly or potentially by responding to the activation of SaeR/S, however, these studies are currently limited. Considering HNP-1 is recognized early following phagocytosis, the recognition of HNP-1 is likely very important for *S. aureus* adaptation to the neutrophil environment. Interestingly, the recognition of HNP-1 by SaeS has not been demonstrated to influence the genes in *S. aureus* directly correlated with combating the antimicrobial capacity of antimicrobial peptides [e.g., *dlt* and *mprF* are regulated by the GraRS two-component system (Yang et al., 2012)] but instead



appears to ready *S. aureus* for combating neutrophil reactive oxygen species and for producing cytolytic toxins. Additionally, the GraRS system recognizes cationic peptides including LL-37, and polymyxin B but does not recognize HNP-1 implying a complex interplay of sensory systems to combat the neutrophil (Yang et al., 2012). Another interesting two-component gene regulatory system is WalK/R. Studies have demonstrated this system is essential for *S. aureus* viability (Dubrac and Msadek, 2004; Dubrac et al., 2007). However, studies using a strain of *S. aureus* with a constitutively active WalR response regulator demonstrated constitutive expression caused up-regulation of virulence genes that are known to be SaeR-regulated including *coa*, *hla*, *hlgA*, *hlgB*, *hlgC*, and *sbi* (Delauné et al., 2012). Although the exact mechanism of communication between WalR and SaeR/S is unknown data suggest WalR influences the SaeR/S system indirectly. This is inferred since deletion of *saeR/S* in a strain that constitutively expressed WalR showed the impact of WalR on the virulence factors during growth *in vitro* was through SaeR/S (Delauné et al., 2012). However, in mouse models of infection constitutive expression of WalR in *S. aureus* caused increased neutrophil recruitment and enhanced bacterial clearance compared to wild-type *S. aureus*. Constitutive expression of WalR decreasing virulence *in vivo* was independent of SaeR/S and linked to WalR dependent peptidoglycan release promoting an inflammatory environment that recruited neutrophils (Delauné et al.). Currently the stimulus recognized by WalK is unknown. Understanding what stimulus(i) activates this system may reveal how WalK/R and SaeR/S regulation of virulence are linked. Additionally, the fatty acid kinase YrfB, that lacks any traditional DNA-binding domain, has been shown to influence gene expression of well-defined SaeR/S-dependent genes (Bose et al., 2014; Krute et al., 2017). However, when a *yrfB* mutant strain was exposed to the known SaeS-activation stimulus HNP-1, this kinase no longer impacted virulence genes regulated by SaeR/S (Krute et al., 2017). These data further emphasize the importance of defining virulence regulation in physiologically relevant environments and highlight the complexity of virulence gene regulation in *S. aureus*.

THE CHALLENGE OF INVESTIGATING NEUTROPHIL—*S. AUREUS* INTERACTIONS *IN VIVO*

Investigating the neutrophil-*S. aureus* interactions *in vivo* has been challenging. The relevance of using the murine immune system to mimic interactions between *S. aureus* and the human immune system is heavily debated (Buer and Balling, 2003; Kim et al., 2014; Montgomery et al., 2014; Reizner et al., 2014; Tseng et al., 2015). Interestingly, though HNP-1 is recognized as the most prominent trigger of *saeR/S*, murine neutrophils are deficient in its production (Inaba et al., 2010). Not only do they differ in the production of HNP-1 but notable differences are observed in chemokine production, cell trafficking, and susceptibility to secreted toxins (Johnston et al., 1990; Singer and Sansonetti, 2004; Parker and Prince, 2012; Tseng et al., 2015). Often the importance of single *S. aureus* virulence genes is

highlighted in models of infection; however, canonical *S. aureus* toxins such as HlgA, HlgC, and LukF-PVL have extremely limited lytic activity on murine cells (Liu, 2009; Baba-Moussa et al., 2011; Parker and Prince, 2012; Vandenesch et al., 2012). While deficiencies in the murine model are apparent it remains the most prominent and cost effective method to explore relationships *in vivo* between *S. aureus* and the host immune system. To that end, advances are being made toward creating more effective murine models that retain benefits such as availability of working with immune-deficient lines while displaying similar disease characteristics as observed in human hosts (Montgomery et al., 2014; Tseng et al., 2015). Tseng et al. developed a “humanized” murine model wherein NOD/SCID/IL2r^{null} mice are engrafted with human CD34⁺ umbilical cord blood cells leading to the production of human immune cells within mice (Tseng et al., 2015). These mice demonstrated susceptibility to *S. aureus* infections at concentrations similar to what is observed in humans as well as increased susceptibility to LukF-PVL. In future efforts, humanized murine models such as this may be key to understanding how *S. aureus* regulates its pathogenesis in the context of a more relevant host environment and may be critical to advance research into effective therapeutics.

POTENTIAL OF THERAPEUTICS TO AUGMENT NEUTROPHIL FUNCTION AGAINST *S. AUREUS*

Vaccine development to prevent or reduce *S. aureus* infections has not been successful (Fowler and Proctor, 2014). Antibody based vaccines targeting individual virulence factors have not conferred significant protection against *S. aureus*, and in some cases have even increased mortality (Fowler et al., 2013). As discussed above, *S. aureus* produces virulence factors that target specific neutrophil receptors to inhibit neutrophil functions. However, *S. aureus* virulence factors show redundancy in the neutrophil functions they inhibit. For example, multiple virulence factors target the complement pathway and immunoglobulin recognition to inhibit neutrophil phagocytosis and neutralizing one of these virulence factors is not likely to have a significant effect *in vivo*. Thus, vaccine approaches that simultaneously target multiple *S. aureus* virulence factors are needed and encouragingly some are in the pipeline (Torre et al., 2015; Frenck et al., 2016).

Targeting *S. aureus* sensory/regulatory systems has great potential for therapeutic and vaccine development. Theoretically, chemical inhibitors and neutralizing antibodies of sensory/regulatory systems could inhibit *S. aureus* from sensing its environment and producing virulence factors that disrupt host antimicrobial functions. The SaeR/S TCS is an attractive candidate for vaccine development since SaeR/S-regulated factors disrupt key neutrophil functions that include production of ROS, cytokine expression, and cell fate (Watkins et al., 2011, 2013; Nygaard et al., 2013; Zurek et al., 2014; Guerra et al., 2016). Many of the best studied immunomodulatory factors and cytolytic proteins that target neutrophil function are regulated by SaeR/S including Sbi, Efb, HlgABC, Hla, LukG/H (LukA/B),

and PVL (Nygaard et al., 2010). Thus, inhibiting SaeS from sensing its environment has the potential to prevent expression of multiple virulence factors in response to host signals. In this line of thinking, targeting *S. aureus* quorum sensing via inhibition of Agr has been proposed as a therapeutic approach to inhibit production of virulence factors (Clatworthy et al., 2007; Park et al., 2007; Cegelski et al., 2008; Kaufmann et al., 2008; Njoroge and Sperandio, 2009; Rutherford and Bassler, 2012). For a review on the *S. aureus* Agr regulatory system please refer to (Painter et al., 2014). In brief, findings have identified synthetic peptides, small molecules, and antibodies that disrupt *S. aureus* Agr-dependent quorum sensing either by blocking the sensor histidine kinase AgrC from recognizing its cognate signal autoinducing peptide (AIP), by inhibiting the AgrA response regulator from binding DNA, or by antibody interference of AIP (Park et al., 2007; Kaufmann et al., 2008; Kirchdoerfer et al., 2011; Tal-Gan et al., 2013; Broderick et al., 2014; Murray et al., 2014; Sully et al., 2014). Using this approach, reductions in mouse dermonecrosis following infection with *S. aureus*, increased bacterial clearance, and reduced hemolytic activity have been reported (Tal-Gan et al., 2013; Sully et al., 2014). In addition, the development of materials with quorum sensing inhibitors to apply during a skin infection is an active area of research and showing promising results (Broderick et al., 2014; Kratochvil et al., 2017). However, studies are needed to fully elucidate the effects of inhibiting sensory/regulatory systems since this can also increase the production of negatively regulated virulence factors and the impact on cross talk between regulatory systems that might result in compensation by one system at the loss of another, is not known.

Modulation of the inflammatory response may be another attractive target for therapeutics that might increase the likelihood of an effective neutrophil response. However, additional research is needed to identify key inflammatory mediators that resolve *S. aureus* infection and to understand those that exacerbate disease. Studies have identified IL-17 as a key determinant of proper host defense during *S. aureus* cutaneous infection (Cho et al., 2010). Murphy et al. identified $\gamma\delta$ T cells as the major source of IL-17 during peritonitis caused by *S. aureus* and identified a subset of memory $\gamma\delta$ T cells that enhanced IL-17 production to protect the host during subsequent *S. aureus* infection (Murphy et al., 2014). Thus, immunotherapies that enhance host bactericidal functions by neutralizing detrimental inflammatory responses, increasing beneficial cytokines at the site of infection, or stimulating the expansion of protective T cell subpopulations in response to *S. aureus* show therapeutic potential. Further research is also needed to understand site-specific cytokine requirements to mount an effective host response to *S. aureus* infection.

Lastly, as discussed above, the use of mouse models has undeniably increased our understanding of *S. aureus*-host interactions. However, due to the specificity of many of the *S. aureus* virulence factors toward human cells, improved models of disease are needed like the “humanized” mouse as discussed above. With improved models and advances in understanding neutrophil-*S. aureus* interactions, perhaps future reviews will be able to document neutrophils as the clear winner in this epic immune battle.

In conclusion, the ability of *S. aureus* to survive a neutrophil encounter is thought to contribute significantly to the virulence of this pathogen. This is exemplified by the observed increase in susceptibility to *S. aureus* infections in individuals suffering from defects that alter normal neutrophil function, such as chronic granulomatous disease, leukocyte adhesion deficiency, and neutropenia (Bodey et al., 1966; Pincus et al., 1976; Dale et al., 1979; Leksstrom-Himes and Gallin, 2000). It is clear that neutrophil antimicrobial activity is essential to eliminate *S. aureus*. However, studies have also demonstrated that increased neutrophil numbers at the site of infection can exacerbate disease (Gresham et al., 2000; McLoughlin et al., 2008) and that modulation of the neutrophil inflammatory response can significantly impact outcome of infection (Watkins et al., 2013; Zurek et al., 2015). Clearly more studies are needed to define the neutrophil responses that resolve a *S. aureus* infection. Finding the right balance of controlling inflammation while maintaining an effective neutrophil antimicrobial response will be key to the design of an effective therapeutic.

AUTHOR CONTRIBUTIONS

FG and JV wrote, edited, prepared manuscript. TB, DP, and ES wrote sections and contributed equally.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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