

Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli

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Edited by Richard P. Novick, New York University School of Medicine, New York, New York, and approved March 27, 2014 (received for review November 26, 2013)

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 histidine kinase SaeS recognizes specific host stimuli is unknown. After mutagenizing the predicted extracellular loop of SaeS, we discovered one methionine residue (M31) was essential for the ability of *S. aureus* to transcribe *sae* target genes, including *hla*, *lukAB/lukGH*, and *hlgA*. This single M31A mutation also significantly reduced cytotoxicity in human neutrophils to levels observed in cells following interaction with Δ *saeS*. Another important discovery was that mutation of two aromatic anchor residues (W32A and F33A) disrupted the normal basal signaling of SaeS in the absence of inducing signals, yet both mutant kinases had appropriate activation of effector genes following exposure to neutrophils. Although the transcriptional profile of aromatic mutation W32A was consistent with that of WT in response to human α -defensin 1, mutant kinase F33A did not properly transcribe the γ -toxin genes in response to this stimulus. Taken together, our results provide molecular evidence for how SaeS recognizes host signals and triggers activation of select virulence factors to facilitate evasion of innate immunity. These findings have important implications for signal transduction in prokaryotes and eukaryotes due to conservation of aromatic anchor residues across both of these domains and the important role they play in sensor protein structure and function.

host-pathogen interactions | membrane proteins

The Gram-positive bacterial pathogen, *Staphylococcus aureus* is capable of causing a remarkable range of acute and chronic infections in almost every tissue of the human body (1). The highly adaptable nature of *S. aureus*, including the rapid development of antibiotic resistance and large tissue tropism, has resulted in it being one of the leading causes of infection in the United States (2). This versatility is made possible by the large array of virulence factors encoded in the *S. aureus* genome and on mobile genetic elements that are regulated by a complex, interactive network of regulatory systems including two-component signal transduction systems (TCSs) (3–5).

Following its discovery almost 20 y ago, the *sae* TCS was recognized as a major regulator of secreted toxins and exoenzymes and was named the *S. aureus* exoprotein expression (*sae*) system (6–9). More recently, the system has been shown to be essential for virulence in multiple animal models of infection and to play a critical role in evasion of the innate immune response (10–12). SaeR, the response regulator, and SaeS, the sensor kinase, comprise the traditional machinery of the TCS (13). SaeR is a member of the OmpR family of response regulators and binds a direct DNA repeat sequence upon phosphorylation by

SaeS to activate transcription of genes in the *sae* regulon (11, 14). SaeS, a member of the HisKA family of histidine protein kinases, is predicted to contain a sensing domain composed of only two transmembrane segments connected by a short extracellular loop (15). Work on the antimicrobial peptide sensing TCS ApsXRS in *Staphylococcus epidermidis* suggests the nine amino acid extracellular (EC) loop of the sensor kinase ApsS may be sufficient to detect the presence of specific host antimicrobial peptides. Antisera raised against the extracellular loop rendered the bacteria unable to transcribe ApsR target genes in response to human β -defensin 3, demonstrating the importance of the sensor kinase residues exposed to the external environment regardless of the size of the extracellular domain (16). The topology of ApsS and SaeS is similar, resulting in both being classified as intramembrane-sensing kinases. This class of histidine kinases has traditionally been thought only capable of sensing perturbations to the plasma membrane or receiving signals through auxiliary proteins (17). Indeed, the *sae* system encodes two additional genes, *saeP* and *saeQ*, immediately upstream of *saeRS* and autoinduces their transcription on activation of the *sae* system (13). SaeP and SaeQ, a lipoprotein and integral membrane protein, respectively, have recently been shown to form a protein complex with SaeS, which would seem to support the classification of SaeS as an intramembrane-sensing kinase requiring the presence of the *sae* auxiliary proteins to sense extracellular sig-

Significance

Staphylococcus aureus is a ubiquitous pathogen that causes disease in a variety of tissues. Our studies provide insight into how this pathogen uses the SaeS sensor kinase to recognize innate immunity signals and induce a transcriptional response tailored for its environment. Our results demonstrate that the specificity of these responses is determined by individual amino acids predicted to be in an extracellular domain. These amino acids include aromatic anchors and a methionine residue essential for activation of target gene transcription and virulence. Our findings provide a putative mechanism for the ability of bacterial sensory systems to integrate and diversify the responses to host stimuli. They underscore the exquisite nature of bacterial signaling and the complexity of host-pathogen interactions.

Author contributions: C.E.F., O.W.Z., A.R.H., and J.M.V. designed research; C.E.F., O.W.Z., D.D.M., and K.B.P. performed research; C.E.F. and C.L.M. contributed new reagents/analytic tools; C.E.F., O.W.Z., D.D.M., K.B.P., and J.M.V. analyzed data; C.E.F. and J.M.V. wrote the paper; A.R.H. directed and oversaw studies at the University of Iowa; and J.M.V. directed and oversaw the entire project.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1322125111/-DCSupplemental.

nals (18). However, formation of the protein complex results in repression of the *sae* system and not activation. In addition, *saeP* is not required for the *sae* system to activate (15). Therefore, how SaeS senses host signals known to activate the *sae* system is unclear.

In this study, we investigate the mechanism by which the histidine kinase SaeS is able to specifically detect α -defensin 1 (HNP-1) and human polymorphonuclear leukocytes (PMNs). We mutagenized every residue in the predicted extracellular loop of SaeS and evaluated activation of the mutant kinases in response to each signal by analyzing expression of *sae* target genes. In addition, we evaluated PMN survival when exposed to *S. aureus* USA300 strain LAC expressing each mutant kinase. Our results demonstrate specific residues in the predicted extracellular loop of SaeS are essential to downstream activation of SaeR-regulated genes. Moreover, this study identifies the importance of individual residues for sensing specific host stimuli by demonstrating differential activation of *sae* targets by the mutant kinases in response to the different stimuli.

Results

Determining the Topology of SaeS. The exact topology of SaeS in the cytoplasmic membrane has not been reported. To address this, we used the substituted cysteine accessibility method (SCAM) (19), which is a technique that allows membrane protein topology to be determined with single amino acid resolution and has recently been adapted for use in *S. aureus* (20). SCAM requires protein constructs that contain only one cysteine residue, which is labeled with a maleimide ring conjugated to biotin (MPB). By engineering individual cysteines throughout a protein, the topology can be determined based on the accessibility of each cysteine residue to labeling. SaeS contains three native cysteines, which needed to be removed to perform SCAM topology analysis. Residues C50 and C56 are near the N terminus of SaeS (depicted green in Fig. 1A), whereas C226 is near the C terminus of the 351 amino acid protein (Fig. S1B). The WT *saeS* gene from USA300 strain LAC (21) was cloned into pEPSA5 (22) with a C-terminal T7-tag for detection by Western blot. Site-directed mutagenesis was used to mutate each cysteine residue to

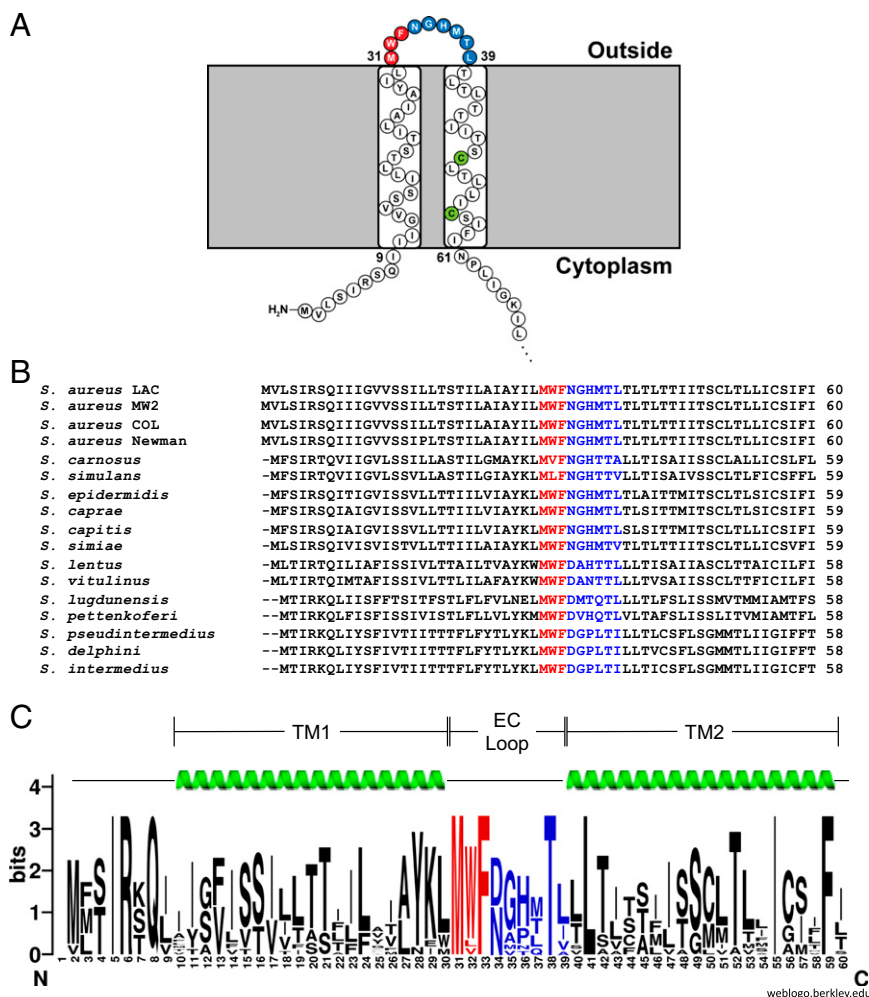


Fig. 1. Residues in the EC loop of SaeS are conserved across staphylococcal strains and species. (A) Cartoon representation of the predicted topology of LAC's SaeS sensing domain from TOPCONS. (B) A sequence alignment of the sensing domain of SaeS from all staphylococcal species and strains of *S. aureus* 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.

a serine to cause minimal structural perturbation, eventually producing constructs with each native cysteine present as the only cysteine in the protein (Table S1). These constructs were used for preliminary SCAM studies in a LAC $\Delta saePQRS$ strain to ensure SaeP, SaeQ, and chromosomally encoded SaeS did not interfere with labeling (*SI Materials and Methods*). All mutant SaeS constructs expressed at levels similar to WT (Fig. S1A, anti-T7). The inability of C50 and C56 to react with the MPB labeling reagent indicates these residues are likely embedded in the cytoplasmic membrane (Fig. S1A, Strep-HRP), whereas the labeling pattern of residue C226 is consistent with a cytoplasmic location (Fig. S1). Interestingly, when either transmembrane cysteine was mutated to a serine either alone or together, the resulting protein ran at a slightly higher molecular weight than WT, as determined by immunoblotting (Fig. S1A). Denaturing and reducing agents did not alter the mobility of SaeS in the gel. Because the altered migration rate indicates a structural change we are unable to fully explain, these constructs were not used for further SCAM analysis.

As an alternative method to obtain the topology for SaeS and validate our SCAM assignments, we used the SaeS amino acid sequence from LAC for TOPCONS analysis, a consensus topology prediction program (23). A small sensing domain composed of two transmembrane passes connected by a nine amino acid extracellular loop was predicted (depicted in red and blue in Fig. 1A). Next, alignment and Weblogo (24) analysis of the SaeS sensing domain from all 14 staphylococcal species currently identified as having the *saeS* gene demonstrated that residues across the entire SaeS sequence are conserved, particularly in the predicted EC loop (Fig. 1B and C).

Mutagenesis of the Predicted Extracellular Loop of SaeS Identified Residues Important for Kinase Activation. To determine the biological relevance of conserved residues in the sensing domain, we performed an alanine scan of the extracellular loop of SaeS and evaluated activation of the *sae* system by the mutant kinases. To this end, we first constructed a markerless $\Delta saeS$ mutant. This strain grew as well as WT LAC but was unable to produce any P_{hla} -dependent fluorescence or red blood cell (RBC) lysis after 24 h of growth (Fig. 2 and Fig. S2). This observation is consistent with previous work showing *sae* regulates transcription of *hla* (11, 25). Both WT LAC and $\Delta saeS$ containing the *hla* reporter were transformed with the empty complementation vector pEPSA5 (WT v and $\Delta saeS$ v, respectively), and complementation of the $\Delta saeS$ mutant with WT SaeS restored both P_{hla} fluorescence and RBC lysis (Fig. 2C and D). Using the SaeS complementing plasmid as a template, each of the nine extracellular residues were mutated to alanines and transformed into the $\Delta saeS$ strain containing the integrated P_{hla} -sGFP reporter (*SI Materials and Methods* and Table S1). These strains were evaluated for growth, expression of the mutant kinases, fluorescence from the *hla* reporter, and RBC lysis activity (Fig. 2). None of the mutations in the EC loop caused any changes in SaeS SDS/PAGE migration like those observed for the transmembrane cysteine mutants (Fig. 2B and Fig. S1A). All mutant protein constructs were expressed; however, N34A demonstrated reduced protein expression at later time points (Fig. 2B). This result may explain the slight decrease observed in RBC lysis for this kinase at 24 h (Fig. 2D).

The most striking observation was the complete inability of single mutations M31A, W32A, and F33A (depicted red in Fig. 1) to complement the *saeS* deletion (Fig. 2C and D). These constructs did not cause any growth defects (Fig. 2A), expression

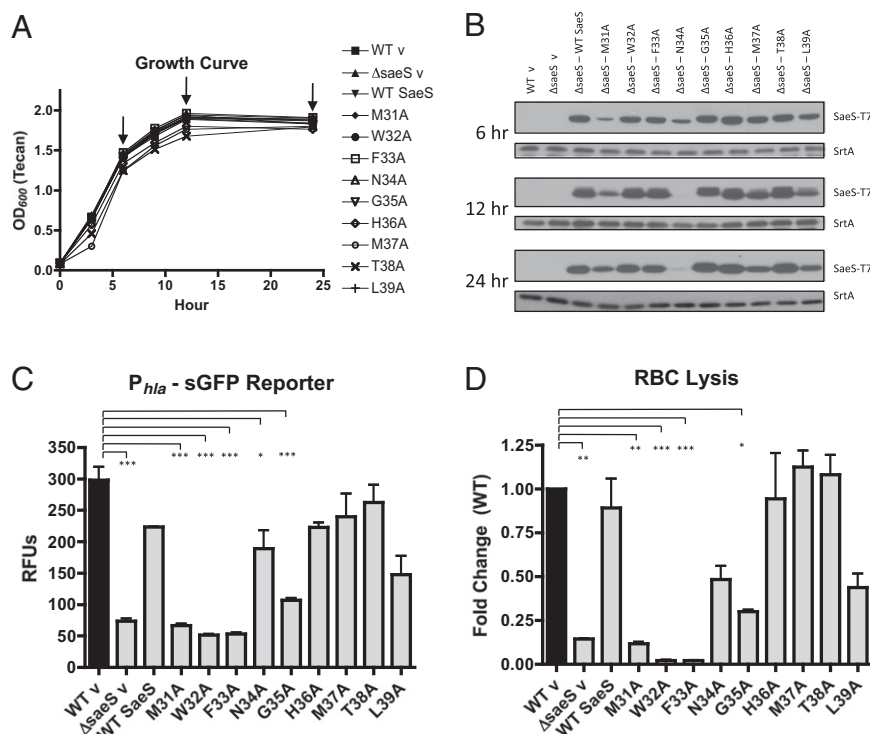


Fig. 2. In vitro analysis of SaeS EC loop mutations. (A) Growth of LAC and $\Delta saeS$ expressing WT and EC loop mutant copies of SaeS. (B) T7 immunoblot of cell pellets from strains depicted in A to evaluate kinase expression levels during in vitro growth at 6, 12, and 24 h (indicated by arrows). Sortase A was used as a loading control. (C) Fluorescence from a P_{hla} -sGFP reporter at 24 h, expressed as relative fluorescence units (RFUs, fluorescence divided by OD_{600}). (D) Lysis of RBCs following incubation with bacterial supernatants harvested after 24 h. Error bars are SEM of two biological replicates examined in triplicate. Asterisks denote significance as evaluated by a one-way ANOVA with Tukey's posttest based on *P* values as follows: *0.01–0.05, **0.001–0.01, and ***<0.001.

of *saeR* was not disrupted, and *saeS* was transcribed in a manner similar to the other plasmid based sensor kinases (Fig. S3). Even with its reduced protein expression, the N34A mutation was able to complement Δ *saeS* (Fig. S3), suggesting concentration is likely not the issue with the inability of M31A, W32A, and F33A mutant kinases to signal. Although M31 and F33 are completely conserved among staphylococcal species, two substitutions can be found for W32. *Staphylococcus simulans* and *Staphylococcus canosus* encode a leucine and a valine, respectively, maintaining the hydrophobicity of the position without the aromatic residue (Fig. 1B). Although the effect that these specific mutations have on *S. aureus* gene expression is unknown and will require further study, we identified the importance of the alanine substitutions in the SaeS EC loop in vitro and next evaluated their significance in an ex vivo model.

Supernatants from M31A, W32A, and F33A Mutant Strains Are Unable to Cause PMN Plasma Membrane Permeability. SaeRS regulates transcription of multiple toxins, including γ -toxin (*hlgA*, *hlgB*, *hlgC*), LukSF-PVL, and LukAB/LukGH, which have been shown to contribute to PMN lysis (26–31). Additionally, transcription of the *saePQRS* operon and SaeR target genes are activated in response to PMN phagocytosis and PMN components (32–34). Collectively, these data suggest sensing and responding to PMNs is a central function of the *sae* system. To evaluate the effect of SaeS EC mutations on the cytolytic capacity of bacterial supernatants, we exposed PMNs to spent media from strains containing the SaeS EC point mutations for varied periods of time. PMN membrane permeability was assessed using propidium iodide (PI) (Fig. 3). Consistent with previous observations, the WT

LAC spent media caused ~85% of PMNs to be PI positive within 15 min, with similar results after 1 h (Fig. 3) (35). Almost no cytotoxicity was observed for Δ *saeS*, and complementation with WT SaeS was able to return cytotoxicity to WT levels. Although all other EC loop mutations retained cytotoxicity, the single point mutations M31A, W32A, and F33A were unable to cause any membrane damage, similar to the RBC lysis assay (Fig. 2D), suggesting they are important for SaeS-mediated expression of cytolytic toxins.

Activation Profile of Specific SaeS EC Mutants Is Signal Dependent.

Differential transcription of *sae* regulated genes has been shown to be dependent on specific host stimuli including HNP-1 and PMNs, implying another level of target gene regulation via specific stimuli (34). To investigate the role of the SaeS EC residues in the transcriptional response previously identified (34), we exposed mutant strains that were unable to activate the *hla* reporter or cause PMN cytotoxicity (M31A, W32A, and F33A) to a subinhibitory concentration of HNP-1 or human PMNs and measured transcript levels of *sae* target gene *hlgA* by real-time quantitative PCR (Fig. 4). To ensure the reduced protein expression of M31A (Fig. 2B) did not influence target gene transcription, we included N34A as a copy level control that also demonstrated reduced SaeS protein expression (Fig. 2B). Additionally, we included point mutation H36A that did not appear to alter activation of the *hla* reporter or PMN cytotoxicity. As expected, α -defensin induced an increase in *hlgA* transcription compared with media controls in a *saeS*-dependent manner (Fig. 4A), and mutation of residue H36 had no impact on *hlgA* expression (Fig. 4A). Consistent with previous experiments

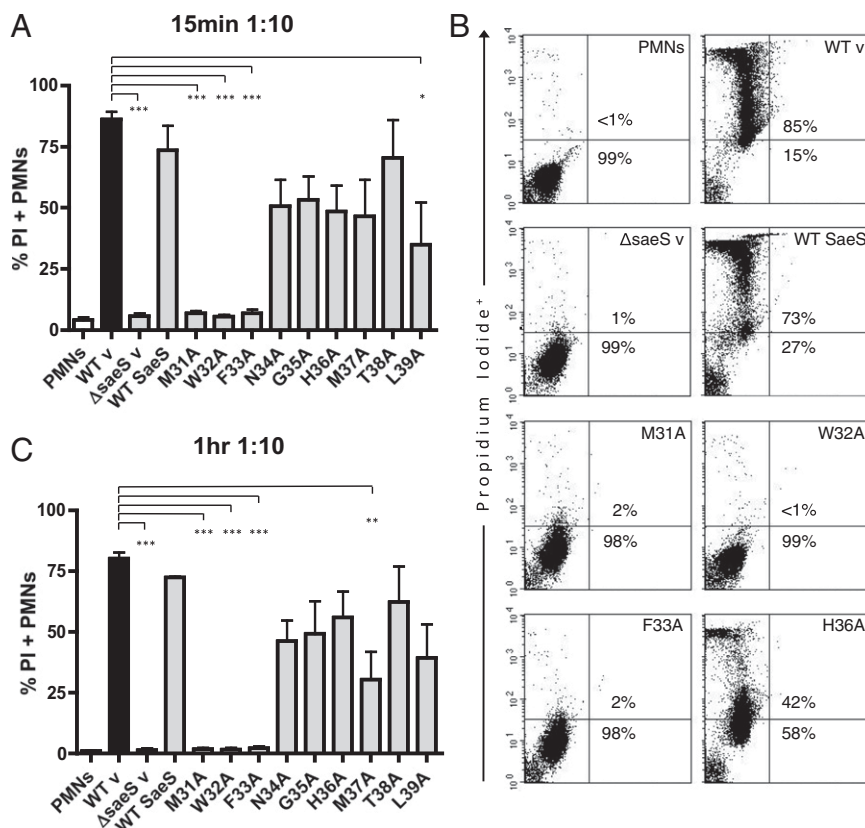


Fig. 3. Supernatants from M31A, W32A, and F33A mutant strains are unable to cause PMN plasma membrane permeability. (A) PMN plasma membrane permeability at 15 min after incubation with bacterial supernatants. (B) Representative dot plots of PMNs stained with PI following 1-h incubation with spent supernatants. (C) PMN plasma membrane permeability at 1 h after incubation with bacterial supernatants. 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.

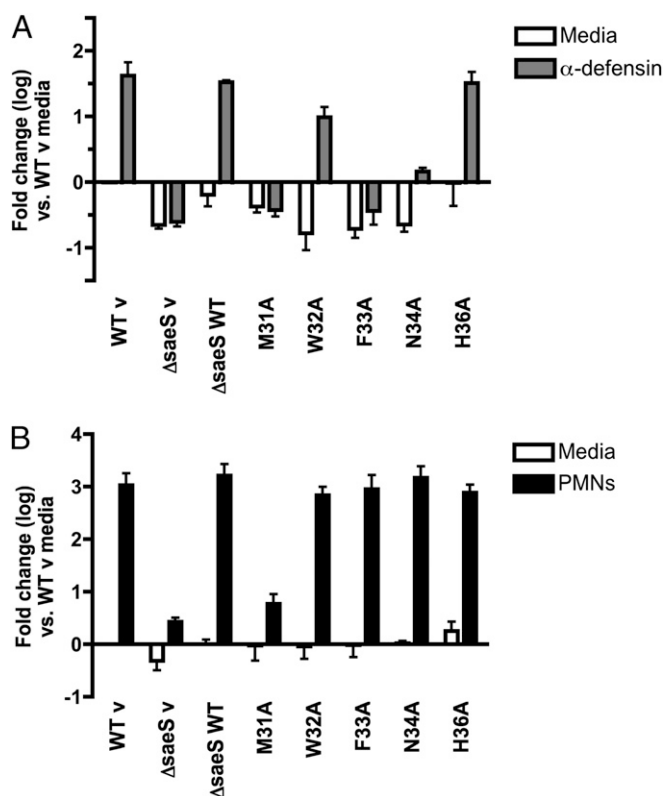


Fig. 4. Expression of *sae* target gene *hlgA* in mutant kinase strains following exposure to human α -defensin 1 and human PMNs. (A) Relative gene expression of *hlgA* in SaeS EC mutants was analyzed following a 30-min exposure to a subinhibitory concentration of human α -defensin 1 (0.48 μ M). All transcript levels are normalized to *gyrB* and calibrated to transcript abundance in WT strains in media only. Data are shown as mean fold change of two biological replicates analyzed in triplicate. (B) Transcription of *hlgA* was measured 30 min after PMN phagocytosis (10:1 MOI). Data are reported as mean fold change of two to four biological replicates analyzed in duplicate.

(Figs. 2D and 3), mutants M31A and F33A were unable to activate; however, W32A was able to induce a 1.2 log increase in transcription of *hlgA* in response to α -defensin. The N34A mutant elicited a weak response to α -defensin, suggesting it may play a role in sensing α -defensin or alternatively weak expression resulted in an inability to fully activate *sae* target gene expression.

Transcription of *hlgA* was also analyzed following a 30-min exposure to human PMNs. PMNs induced a robust increase in *hlgA* expression in the WT strain, whereas M31A transcription mirrored $\Delta saeS$ (Fig. 4B). Unlike α -defensin, N34A and both aromatic mutations (W32A and F33A) were able to activate transcription of *hlgA* to WT levels in response to human PMNs. F33A and N34A had similar activation profiles following exposure to α -defensin and PMNs, confirming that reduced SaeS protein expression was not the reason the M31A mutant was unable to activate *sae* target gene transcription following either stimulus. Taken together, these data suggest that the M31 residue is essential for activation of the *sae* system. In addition, it appears that single residues in the EC domain control the ability of SaeS to integrate and diversify responses to host stimuli.

To further examine this hypothesis, we analyzed the expression profile of a large number of *sae* target genes for point mutations M31A, W32A, F33A, and H36A in response to both α -defensin and PMNs using the QuantiGene 2.0 assay (34). Changes in gene expression in response to different host stimuli

were determined by comparing levels in *S. aureus* SaeS EC point mutants treated with the described stimulus to those in the same strain exposed to media only (Fig. 5 and Tables S2 and S3). Due to autoregulation of the *sae* system from the P1 promoter, transcript levels of *saeR* and *saeS* increase moderately on activation of the system (13, 15). An increase in expression of *saeR* was observed in all strains capable of activating *sae*-dependent gene expression (WT v, $\Delta saeS$ -WT SaeS, W32A, F33A, and H36A; Tables S2 and S3). Because complementation was plasmid based with a nonnative promoter, up-regulation of *saeS* in response to stimuli was only observed in the WT strain. Consistent with previous reports, both stimuli caused an increase in expression of several genes containing the SaeR binding domain upstream of their coding sequence in the WT, and $\Delta saeS$ complemented with WT SaeS and H36A strains but not in $\Delta saeS$ (Fig. 5) (11, 34). The M31A mutation was unable to activate expression of genes in the *sae* regulon and had profiles similar to $\Delta saeS$ in the presence of either stimulus. W32A and F33A had a larger fold increase in expression of *sbi*, *saeP*, *saeR*, and *hla* than WT in response to both stimuli. Intriguingly, F33A was only able to weakly transcribe *hlgA* and *hlgC* in response to α -defensin, yet both genes had high levels of induction following PMN exposure, similar to the quantitative RT-PCR (qRT-PCR) results (Fig. 4). Genes that do not contain a SaeR binding domain upstream of their coding sequence (*agrA*, *sarA*, *dltA*, and *mecA*) were not induced after either stimulus in any of the strains, demonstrating specificity of the SaeS-mediated responses. These data demonstrate the essential nature of residue M31 for SaeS sensing and signaling and suggest other residues in the extracellular loop may refine the pathogen's response to external stimuli including α -defensin.

To evaluate the effect the SaeS EC mutants have on overall gene expression, the QuantiGene 2.0 data for each point mutation in the presence or absence of either host stimulus were normalized to the WT v media control. The expression profile of *hlgA* was consistent with qRT-PCR analysis showing all strains had a large increase in transcription, with the exception of $\Delta saeS$ and M31A, following exposure to PMNs (Fig. 6A). Expression of *lukA* in M31A, W32A, and F33A was slightly reduced compared with that of WT in the absence of any signal, but contact with PMNs restored expression in W32A and F33A (Fig. 6B). *Hla* is expressed at a very high level in USA300 strains (36), making it difficult to measure induced expression. Consistent with this, little change was observed in *hla* transcript levels in WT or H36A following incubation with PMNs (Fig. 6C). Expression of *hla* dropped substantially in $\Delta saeS$, M31A, W32A, and F33A mutants in the absence of any stimulus (supported by Fig. 2), yet PMNs were able to induce overall transcript levels similar to WT in W32A and F33A. As a control, the expression profile of *dltA* was assessed to ensure the SaeS point mutations were not causing any changes in gene expression unrelated to the *sae* system (Fig. 6D) (37). Results for α -defensin-induced gene expression followed the same trends as the PMN results with the exception of *hlgA* in F33A, which had a robust response to PMNs and a diminished response to α -defensin (Fig. S4). These data demonstrate that, although the aromatic point mutations W32A and F33A are able to activate *sae*-dependent gene expression in a signal-specific manner, basal expression of several *sae*-regulated toxins is reduced in these mutants.

SaeS Mutant M31A Is Unable to Induce Cytotoxicity in Human PMNs Following Phagocytosis. To investigate whether our transcriptional findings translate to a pathogenesis phenotype, we infected human neutrophils with the SaeS EC mutant strains and assessed PMN cytotoxicity by evaluating membrane permeability and lactate dehydrogenase (LDH) release (Fig. 7). In contrast to the >90% cytotoxicity caused by WT, the M31A mutant was only able to induce membrane permeability in ~50% of PMNs and was indistinguishable from the attenuated $\Delta saeS$ strain. Consistent

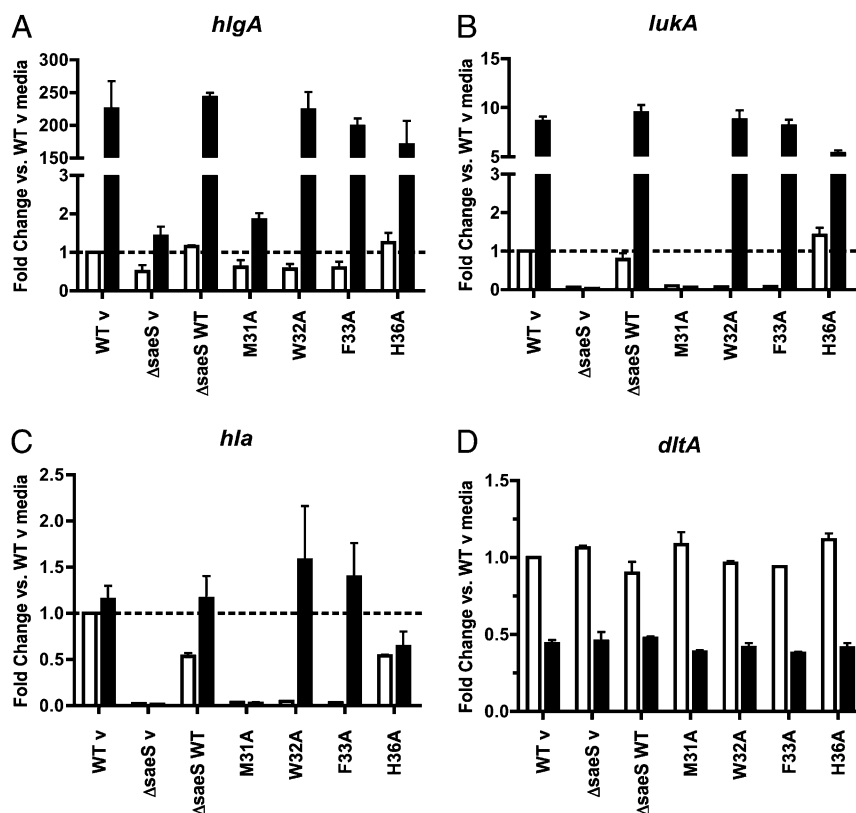


Fig. 6. SaeS mutant kinase strains have lower basal gene expression of *sae*-regulated toxins. QuantiGene 2.0 assay results from PMN induced gene expression in *S. aureus* strains were normalized to *gyrB*, calibrated to WT v without stimulus (media control), and displayed as mean fold changes. Error bars are SEM of two biological replicates analyzed in duplicate. White bars, media control; black bars, PMNs; dashed lines, calibrator (WT v without stimulus). (A) *hlgA*, (B) *luka*, (C) *hla*, and (D) *dltA* (control, gene without SaeR binding domain).

second extracellular loop of the human vasopressin receptor V_{1a} found four aromatic residues to be important for ligand binding and receptor activation (43). Together these findings demonstrate the global importance of aromatic amino acids in the transmembrane and extracellular domains of sensing proteins in a wide range of organisms and hint at potential mechanisms for the altered transcriptional profile of the *sae* regulon in *S. aureus* SaeS mutants. It is also conceivable that aromatic residues W32 and F33 may directly interact with inducing agents, including α -defensin or other unidentified signals in PMNs. Another possibility is that mutation of W32 or F33 may alter protein-protein interactions within the SaeS homodimer or the SaePQS complex, resulting in the altered gene expression we observed. The recent finding that auxiliary proteins SaeP and SaeQ stimulate the phosphatase activity of the bifunctional kinase to modulate expression of the *sae* regulon supports this hypothesis (18). More studies are needed to investigate these possibilities and to examine the effect of other point mutations on the refinement of the pathogen response to stimuli.

Inasmuch as we observed different abilities of the mutant kinases to activate downstream effectors of the *sae* system, we evaluated the virulence of these mutant strains during interaction with human PMNs. Reflective of transcript induction of target genes following exposure to human PMNs, we observed that W32A and F33A kinase mutants had cytolytic capabilities equivalent to the WT strain, whereas following PMN phagocytosis, the M31A mutant strain produced significantly less cytotoxicity. These data combined with the lack of cytolytic activity of supernatants from all three mutant kinases harvested during *in vitro* growth illustrate the importance of not only specific residues in the predicted extracellular domain but the essential

nature of the host stimulus to trigger virulence in *S. aureus*. Thus, it would be interesting to examine the SaeS EC point mutations in other infections models, notably the mouse skin, as the transcriptional profile for the *sae* regulon has already been shown to be modified in this environment compared with PMNs (34).

Although our findings have exciting implications for the importance of aromatic residues in signal transduction and pathogenesis, the significance of the M31A mutation is intriguing. Future studies will elucidate exactly why this methionine is essential to the SaeS sensing domain. Because this kinase appears to be completely unable to activate transcription of *sae* target genes, suggesting it may be in a “locked off” conformation, it would be informative to combine the M31A mutation with the constitutively active mutation L18P found in strain Newman (15, 44, 45). It should be noted that, although we predict M31 to be EC regardless of its exact localization, we identified a residue that is essential for signaling in a major virulence regulatory system in *S. aureus*.

This study advances our understanding of how *S. aureus* can sense and respond to changing environments within the host and provides an explanation for the coordination of multiple signals via specific residues within a single sensing domain. Therefore, our findings support the hypothesis that individual residues in the sensing domain of SaeS are able to dictate the outcome of infections based on the presence of specific host stimuli. These discoveries will be valuable for the development of novel anti-virulence therapeutics, because we identified residues within SaeS that are essential for proper activation of the *sae* system and for *S. aureus* pathogenesis.

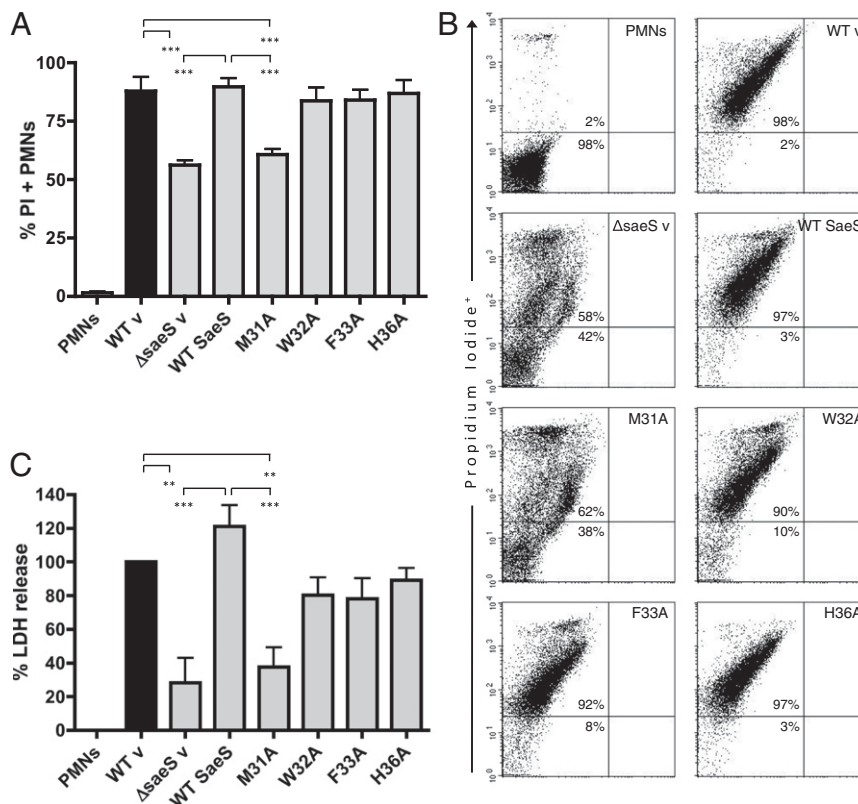


Fig. 7. Residue M31 in the SaeS sensing domain is essential for SaeS-induced PMN plasma membrane permeability and lysis. (A) Compiled results representing three separate experiments investigating PMN plasma membrane permeability 3 h after infection with SaeS EC mutants (10:1 MOI). (B) Representative dot plots of PMNs stained with PI 3 h after infection. (C) LDH assays of matching supernatants from membrane permeability studies in A showing PMN cytotoxicity normalized to WT v. Results are the mean \pm SEM of three PMN donors. Significance was evaluated by a one-way ANOVA with Tukey's posttest. *P* values are represented as **0.001–0.01 and ***<0.001.

Materials and Methods

Bacterial Strains and Growth Conditions, Plasmid and Strain Construction, Substituted Cysteine Accessibility Method (SCAM), *hla* Reporter Construction, SaeS Extracellular Point Mutations, Immunoblotting, and Statistics. Detailed protocols are described in *SI Materials and Methods* and *Table S4*.

Fluorescent *hla* Reporter Assays. Overnight cultures were diluted 1:100 in tryptic soy broth (TSB) containing appropriate antibiotics and incubated in a 37 °C shaker. At each time point, 200 μ L was removed and placed in a black 96-well plate in triplicate. The OD₆₀₀ and fluorescence (480-nm excitation/515-nm emission) was measured in a Tecan Infinite M200 plate reader (Tecan). The 24-h fluorescence readings were divided by the corresponding OD₆₀₀ and plotted as relative fluorescence units (RFUs). At 6, 12, and 24 h, 1-mL samples were pelleted and stored at –20 °C for immunoblot analysis (*SI Materials and Methods*) to evaluate expression of mutant SaeS constructs. At 24 h, supernatants were filter sterilized with 0.22- μ m Spin-X columns (Costar) and stored at –20 °C for future evaluation by the RBC lysis assay, as described below.

RBC Lysis Assay. RBC lysis activity in bacterial supernatants was determined using the previously described RBC lysis titration assay with slight modifications (46). Culture supernatants from the fluorescent *hla* reporter assay (described above) were twofold serially diluted across a 96-well plate. Rabbit erythrocytes from HemoStat Laboratories were washed in PBS until the supernatant was clear. Washed rabbit RBCs were resuspended in PBS to create a 3% (vol/vol) solution. Seventy microliters of the freshly diluted 3% RBC solution was aliquoted into 96-well plates containing 30 μ L of serially diluted culture supernatant. Plates were incubated statically at room temperature for 1 h. Hemolytic activity was evaluated by measuring the loss of turbidity at 630 nm using the Tecan Infinite M200 plate reader. The resulting curves were fit with a four-parameter logistic fit using Prism (version 4.0c for Mac; GraphPad Software) to determine the midpoint. Midpoints of each fit were normalized to WT, and all measurements were performed in triplicate.

PMN Cytotoxicity Assays. PMNs (or neutrophils) were isolated from healthy human donors following procedures described elsewhere (33). All procedures were performed in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. *S. aureus*-induced cytotoxicity assays were performed as previously described with minor adjustments (47). PMNs (1×10^6) were resuspended in RPMI + 5 mM HEPES and plated in 96-well plates precoated with 20% (vol/vol) human serum diluted in Dulbecco's phosphate buffered saline (DPBS). For assays using spent supernatants, *S. aureus* overnight cultures were diluted 1:100 and grown for 5 h. Spent supernatants were filter sterilized and diluted 1:10 in RPMI. Diluted supernatants were added to plated PMNs, synchronized (400 \times g, 8 min, 4 °C), and then incubated statically at 37 °C with 5% CO₂. Membrane permeability and cytotoxicity caused by whole bacteria were evaluated as described using flow cytometry (11, 47) or the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega) according to the manufacturer's instructions (31, 33).

Analysis of Gene Expression Following Exposure to Host Stimuli. Overnight cultures were diluted 1:100 and grown to an OD₆₀₀ = 1.5. Bacteria were washed in DPBS and then plated in 96-well round bottom plates at 5×10^7 CFUs per well. A subinhibitory concentration of human α -defensin 1 [0.48 μ M, as previously determined (34); Millipore] was added to bacteria, and plates were incubated at 37 °C while shaking (250 rpm) for 30 min. RNA was harvested using RNeasy Mini Kits (Qiagen) as described elsewhere (48). For PMN-induced gene expression, PMNs were plated in a 96-well plate at 2×10^6 cells per well, and bacteria were added at a 10:1 bacteria:PMN ratio. Infections were synchronized (400 \times g, 8 min, 4 °C), and plates were incubated statically at 37 °C. After 30 min, RNA was purified from bacteria as previously described (48).

QuantiGene 2.0 Assays. QuantiGene 2.0 assays (Affymetrix) were performed on RNA harvested from α -defensin and PMN experiments as previously described (33, 34). Briefly, magnetic microsphere beads containing capture probes unique to 15 genes or operons with the SaeR binding sequence (*hlgA*, *hlgB*, *hlgC*, *lukA*, *sbi*, *saeP*, *saeR*, *saeS*, *hla*, *lukD*, *lukF-PVI*, *splA*, *ssl7*, *arlR*, and *rot*) and five genes

without the SaeR binding site (*gyrB*, *sarA*, *agrA*, *mecA*, and *dltA*) were incubated with purified RNA samples (50 ng) and target-specific DNA probes. DNA probes contained biotinylation sites that allow amplification of the fluorescent signal. Following signal amplification, the fluorescence intensity of each capture bead was detected using a Luminescence flow cytometer (BioRad) and reported as the mean fluorescent signal for each gene-specific bead. Two biological replicates of each experiment were examined in duplicate, and gene expression levels were normalized to *gyrB*. Changes in gene expression in response to different host stimuli were determined by comparing fluorescence levels in *S. aureus* SaeS EC point mutants treated with the described stimulus to those of the same strain in media only. To evaluate the effect the SaeS point mutations have on overall gene expression, transcript levels for each point mutation in the presence and absence of stimuli were compared with the WT media control.

Stimulus-dependent gene expression was verified by TaqMan real-time RT-PCR on bacterial RNA harvested from the aforementioned α -defensin and PMN phagocytosis experiments as previously described (11, 31). Relative quantification of *hlgA* was expressed as the log₁₀ change relative to the WT v media control for each stimulus. Two to four biological replicates of each experiment were analyzed in duplicate (PMN) or triplicate (α -defensin).

ACKNOWLEDGMENTS. This work was supported by National Institute of Health (NIH) Grants NIH-RR020185 and NIH-R01 Award A1090046-01, Molecular Biosciences Fellowship P20RR16455-07 (to O.W.Z.), Montana State University Agriculture Experiment Station funds, and an equipment grant from the Murdoch Charitable Trust. Additional support for C.E.F., C.L.M., and A.R.H. was from project 3 of NIH Grant A1083211.

- Lowy FD (1998) Staphylococcus aureus infections. *N Engl J Med* 339(8):520–532.
- DeLeo FR, Chambers HF (2009) Reemergence of antibiotic-resistant Staphylococcus aureus in the genomics era. *J Clin Invest* 119(9):2464–2474.
- Cheung AL, Bayer AS, Zhang G, Gresham H, Xiong Y-Q (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. *FEMS Immunol Med Microbiol* 40(1):1–9.
- Bronner S, Monteil H, Prévost G (2004) Regulation of virulence determinants in Staphylococcus aureus: Complexity and applications. *FEMS Microbiol Rev* 28(2): 183–200.
- Beier D, Gross R (2006) Regulation of bacterial virulence by two-component systems. *Curr Opin Microbiol* 9(2):143–152.
- Giraud AT, Raspanti CG, Calzolari A, Nagel R (1994) Characterization of a Tn551-mutant of Staphylococcus aureus defective in the production of several exoproteins. *Can J Microbiol* 40(8):677–681.
- Giraud AT, Rampone H, Calzolari A, Nagel R (1996) Phenotypic characterization and virulence of a sae- agr- mutant of Staphylococcus aureus. *Can J Microbiol* 42(2): 120–123.
- Rampone H, Martínez GL, Giraud AT, Calzolari A, Nagel R (1996) In vivo expression of exoprotein synthesis with a Sae mutant of Staphylococcus aureus. *Can J Vet Res* 60(3):237–240.
- Giraud AT, Calzolari A, Cataldi AA, Bogni C, Nagel R (1999) The sae locus of Staphylococcus aureus encodes a two-component regulatory system. *FEMS Microbiol Lett* 177(1):15–22.
- Liang X, et al. (2006) Inactivation of a two-component signal transduction system, SaeRS, eliminates adherence and attenuates virulence of Staphylococcus aureus. *Infect Immun* 74(8):4655–4665.
- Nygaard TK, et al. (2010) SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 201(2):241–254.
- Watkins RL, Pallister KB, Voyich JM (2011) The SaeRS gene regulatory system induces a pro-inflammatory cytokine response during Staphylococcus aureus infection. *PLoS ONE* 6(5):e19939.
- Novick RP, Jiang D (2003) The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. *Microbiology* 149(Pt 10):2709–2717.
- Sun F, et al. (2010) In the Staphylococcus aureus two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J Bacteriol* 192(8):2111–2127.
- Adhikari RP, Novick RP (2008) Regulatory organization of the staphylococcal sae locus. *Microbiology* 154(Pt 3):949–959.
- Li M, et al. (2007) Gram-positive three-component antimicrobial peptide-sensing system. *Proc Natl Acad Sci USA* 104(22):9469–9474.
- Mascher T (2006) Intramembrane-sensing histidine kinases: A new family of cell envelope stress sensors in Firmicutes bacteria. *FEMS Microbiol Lett* 264(2):133–144.
- Jeong DW, et al. (2012) The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of Staphylococcus aureus. *Mol Microbiol* 86(2):331–348.
- Bogdanov M, Zhang W, Xie J, Dowhan W (2005) Transmembrane protein topology mapping by the substituted cysteine accessibility method (SCAM(TM)): Application to lipid-specific membrane protein topogenesis. *Methods* 36(2):148–171.
- Thoendel M, Horswill AR (2013) Random mutagenesis and topology analysis of the autoinducing peptide biosynthesis proteins in Staphylococcus aureus. *Mol Microbiol* 87(2):318–337.
- Boles BR, Thoendel M, Roth AJ, Horswill AR (2010) Identification of genes involved in polysaccharide-independent Staphylococcus aureus biofilm formation. *PLoS ONE* 5(4): e10146.
- Forsyth RA, et al. (2002) A genome-wide strategy for the identification of essential genes in Staphylococcus aureus. *Mol Microbiol* 43(6):1387–1400.
- Bernsel A, Viklund H, Hennerdal A, Elofsson A (2009) TOPCONS: Consensus prediction of membrane protein topology. *Nucleic Acids Res* 37(Web Server issue, suppl 2): W465–W468.
- Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: A sequence logo generator. *Genome Res* 14(6):1188–1190.
- Cho H, Jeong DW, Li C, Bae T (2012) Organizational requirements of the SaeR binding sites for a functional P1 promoter of the sae operon in Staphylococcus aureus. *J Bacteriol* 194(11):2865–2876.
- Menestrina G, et al. (2003) Ion channels and bacterial infection: The case of β -barrel pore-forming protein toxins of Staphylococcus aureus. *FEBS Lett* 552(1):54–60.
- Prévost G, et al. (1995) Panton-Valentine leukocidin and gamma-hemolysin from Staphylococcus aureus ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect Immun* 63(10):4121–4129.
- Dumont AL, et al. (2011) Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. *Mol Microbiol* 79(3):814–825.
- Ventura CL, et al. (2010) Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. *PLoS ONE* 5(7):e11634.
- Rigby KM, DeLeo FR (2012) Neutrophils in innate host defense against Staphylococcus aureus infections. *Semin Immunopathol* 34(2):237–259.
- Voyich JM, et al. (2009) The SaeRS gene regulatory system is essential for innate immune evasion by Staphylococcus aureus. *J Infect Dis* 199(11):1698–1706.
- Palazzolo-Ballance AM, et al. (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant Staphylococcus aureus. *J Immunol* 180(1):500–509.
- Voyich JM, et al. (2005) Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. *J Immunol* 175(6):3907–3919.
- Zurek OW, et al. (2014) The role of innate immunity in promoting SaeRS-mediated virulence in Staphylococcus aureus. *J Innate Immun* 6(1):21–30.
- Nygaard TK, DeLeo FR, Voyich JM (2008) Community-associated methicillin-resistant Staphylococcus aureus skin infections: advances toward identifying the key virulence factors. *Curr Opin Infect Dis* 21(2):147–152.
- Kennedy AD, et al. (2008) Epidemic community-associated methicillin-resistant Staphylococcus aureus: Recent clonal expansion and diversification. *Proc Natl Acad Sci USA* 105(4):1327–1332.
- Herbert S, et al. (2007) Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PLoS Pathog* 3(7):e102.
- Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C (2008) The virulence regulator Sae of Staphylococcus aureus: Promoter activities and response to phagocytosis-related signals. *J Bacteriol* 190(10):3419–3428.
- Gleason NJ, et al. (2012) Tyrosine replacing tryptophan as an anchor in GWALP peptides. *Biochemistry* 51(10):2044–2053.
- Draheim RR, Bormans AF, Lai RZ, Manson MD (2006) Tuning a bacterial chemoreceptor with protein-membrane interactions. *Biochemistry* 45(49):14655–14664.
- Adase CA, Draheim RR, Manson MD (2012) The residue composition of the aromatic anchor of the second transmembrane helix determines the signaling properties of the aspartate/maltose chemoreceptor Tar of Escherichia coli. *Biochemistry* 51(9): 1925–1932.
- Zhou X, et al. (2007) Yeast screens show aromatic residues at the end of the sixth helix anchor transient receptor potential channel gate. *Proc Natl Acad Sci USA* 104(39): 15555–15559.
- Conner M, et al. (2007) Systematic analysis of the entire second extracellular loop of the V(1a) vasopressin receptor: Key residues, conserved throughout a G-protein-coupled receptor family, identified. *J Biol Chem* 282(24):17405–17412.
- Schäfer D, et al. (2009) A point mutation in the sensor histidine kinase SaeS of Staphylococcus aureus strain Newman alters the response to biocide exposure. *J Bacteriol* 191(23):7306–7314.
- Mainiero M, et al. (2010) Differential target gene activation by the Staphylococcus aureus two-component system saeRS. *J Bacteriol* 192(3):613–623.
- Pang YY, et al. (2010) agr-Dependent interactions of Staphylococcus aureus USA300 with human polymorphonuclear neutrophils. *J Innate Immun* 2(6):546–559.
- Nygaard TK, et al. (2012) Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS ONE* 7(5):e36532.
- Voyich JM, Sturdevant DE, DeLeo FR (2008) Analysis of Staphylococcus aureus gene expression during PMN phagocytosis. *Methods Mol Biol* 431:109–122.