

## Book Chapter

# Aspartic Acid Residue 51 of SaeR is Essential for *Staphylococcus aureus* Virulence

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## Abstract

*Staphylococcus aureus* is a common Gram-positive bacteria that is a major cause of human morbidity and mortality. The SaeR/S two-component sensory system of *S. aureus* is important for virulence gene transcription and pathogenesis. However, the influence of SaeR phosphorylation on virulence gene transcription is not clear. To determine the importance of potential SaeR phosphorylation sites for *S. aureus* virulence, we generated genomic alanine substitutions at conserved aspartic acid residues in the receiver domain of the SaeR response regulator in clinically significant *S. aureus* pulsed-field gel electrophoresis (PFGE) type USA300. Transcriptional analysis demonstrated a dramatic reduction in the transcript abundance of

various toxins, adhesins, and immunomodulatory proteins for SaeR with an aspartic acid to alanine substitution at residue 51. These findings corresponded to a significant decrease in cytotoxicity against human erythrocytes and polymorphonuclear leukocytes, the ability to block human myeloperoxidase activity, and pathogenesis during murine soft-tissue infection. Analysis of SaeR sequences from over 8,000 draft *S. aureus* genomes revealed that aspartic acid residue 51 is 100% conserved. Collectively, these results demonstrate that aspartic acid residue 51 of SaeR is essential for *S. aureus* virulence and underscore a conserved target for novel antimicrobial strategies that treat infection caused by this pathogen.

## Keywords

Staphylococcus Aureus.; saeR/S; Virulence; Pathogenesis; Two-Component System; Neutrophil, Hemolysis; MRSA; Transcription; Toxin

## Introduction

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium that can cause a wide range of disease in both humans and animals [1]. The diverse pathogenesis of *S. aureus* can be attributed to the expression of an extensive array virulence factors that are often redundant in function [2]. Expression of these virulence genes *in vivo* is thought to be primarily dictated by the concerted influence of two-component sensory systems that recognize environmental signals and alter gene expression accordingly. The *S. aureus* genome encodes 16 two-component systems that have been identified by sequence analysis [3]. Of these, the SaeR/S two-component system has been shown to be an important mediator of *S. aureus* virulence by transcriptionally upregulating numerous adhesins, toxins, and immunomodulatory proteins that interact directly with host components to advance pathogenesis [4-8]. The mechanisms used by this two-component system to recognize host-specific cues and alter gene transcription in response are not completely understood.

Typical two-component systems are minimally composed of a transmembrane histidine kinase sensor and cognate intracellular response regulator [9]. Recognition of environmental stimulus activates the histidine kinase sensor, inducing autophosphorylation at a histidine residue on the intracellular domain. This phosphate group is then transferred to an aspartic acid residue within the receiver domain of the cognate response regulator. In general, the phosphorylated response regulator then binds to promoter regions within the bacterial genome to mediate gene transcription and promote survival. However, some response regulators such as RcsB from *Escherichia coli* [10] and SreR from *Xanthomonas campestris* (Wang et al., 2014) can regulate gene transcription without being phosphorylated. For the SaeR/S two-component system, the need to artificially phosphorylate recombinant SaeR for the *in vitro* DNA binding activity of this response regulator has not been consistent between studies [7,12]. It has also been suggested that different levels of SaeR phosphorylation correspond to the upregulation of distinct groups of SaeR/S regulated genes [13].

To clarify the importance of potential SaeR phosphorylation sites for mediating *S. aureus* pathogenesis, we have generated individual single amino acid substitutions at conserved aspartic acid residues of SaeR in the genome of *S. aureus* PFGE-type USA300. In this report, we assess the significance of these residues for mediating the virulence of a clinically-relevant MRSA strain using both *in vitro* and *in vivo* models of infection. Results from this study underscore the importance of SaeR aspartic acid residue 51 for virulence gene transcription and demonstrate that the substitution of this single amino acid in the USA300 genome can attenuate pathogenesis of this clinically significant MRSA strain.

## Materials and Methods

### Bacteria Strains and Culture Conditions

*S. aureus* PFGE-type USA300 strain LAC has been described previously [14] and the USA300 isogenic deletion mutant of *saeR/S* (USA300 $\Delta$ *saeR/S*) was generated in previous studies [7]. Bacteria were cultured in an Excella E24 rotary incubator (New

Brunswick) at 250 rpm and 37°C. Unless noted otherwise, overnight bacteria cultures grown in 20 mL of tryptic soy broth (TSB; EMD Millipore) were used to start subcultures in 20 mL TSB containing 0.5% glucose (1:100 dilution). Optical density at 600 nm (OD<sub>600</sub>) was measured using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) and colony forming units (CFUs) were determined by plating diluted samples on tryptic soy agar (TSA; EMD Millipore).

## DNA Sequence Alignment

DNA sequence alignment of SaeR homologues was performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) with protein sequences obtained from the NCBI protein data base using the following accession numbers: ABD22784 for USA300, ATX72322 for USA400, EOR90509 for USA100, WP\_076742615.1 for MT-0541, AAW53763 for *Staphylococcus epidermidis* RP62A, Q8CQ17 for *Staphylococcus epidermidis* 12228, WP\_049307325 for *Staphylococcus capitis*, WP\_029378577 for *Staphylococcus xylosus*, PCQ20359 for *Klebsiella pneumoniae*, NP\_346930 for *Clostridium acetobutylicum*, ACJ50526 for *Escherichia coli*, and NP\_388082 for *Bacillus subtilis*.

## Generation of USA300 Genomic Mutations

Allelic exchange with plasmid pKOR1 was used to impart genomic mutations in USA300 as previously described [15]. All primers used to generate and sequence USA300 mutants are listed in Supplemental Table 1. Primers containing *attB* sites were used to amplify staphylococcal peroxidase inhibitor (*spn*) and *saeR* from the USA300 genome for BP Clonase II (ThermoFisher Scientific) mediated insertion into pKOR1. Spn-check-fwd and *rvs* primers were used to verify the loss of *spn*. Site-directed mutagenesis was performed on pKOR1-*saeR* using *saeR*-D46A, *saeR*-D51A, or *saeR*-D61A primers and PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Inc.) following the manufacture's protocol and as previously performed [16]. The *saePQRS* operon in generated *saeR* point

mutants was PCR amplified using saePQRS\_fwd and rvs primers and sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit) using saeR-seq and saePQRS-seq primers. PCR amplification was performed using saeR-EcoRI-fwd and saeR-XhoI-rvs and cloned into pEPSA5 as previously described [17] to generate the complementary plasmid that expresses wt SaeR, pEPSA5-saeR.

## Whole genome Sequencing and saeR Sequence Analysis

Wild-type USA300 and its SaeR mutants were subject to whole genome sequencing. Briefly, genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI) following by treatment with 20 µg/ml lysostaphin. The DNA library was sequenced on an Illumina NextSeq platform (Illumina, San Diego, CA) with 2 x 150 bp paired-end reads. The reads were mapped against the published NC\_007793 (*S. aureus* USA300\_FPR3757) genome using BWA [18] and Samtools [19], and the SNPs and InDels were examined using freebayes (<https://github.com/ekg/freebayes>). SNPs and InDels were further annotated using snpEff [20].

Blast analysis of the USA300 saeR (locus\_tag SAUSA300\_0691) sequence against more than 8,000 *S. aureus* draft genomes downloaded from the NCBI FTP site (<ftp.ncbi.nih.gov>, dated as June 1, 2018) were extracted, translated into amino acids and aligned using Geneious 11.1.

## Relative Quantitative Real Time RT-PCR

*S. aureus* transcription analysis using relative quantitative real time RT-PCR was performed as previously described [7,8,21]. Briefly, subcultured strains were harvested at mid-exponential (ME; OD<sub>600</sub> = 1.5) or early stationary (ES; OD<sub>600</sub> = 3.0) growth phases, mechanically disrupted using a FastPrep FP120 cell disrupter (ThermoFisher Scientific), and RNA purified with an RNEasy Kit (Qiagen). TaqMan real-time RT-PCR was performed using previously published primer and probe sets [7].

## Hemolysis Assays

Heparinized venous blood from healthy donors was collected in accordance with the protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written informed consent to participate in the study. We adopted protocol described by others [22] to quantify hemolysis of human blood by *S. aureus* extracellular proteins. Briefly, freshly drawn human blood was washed three times with 10 times the volume of sterile DPBS then resuspended at a final dilution of 1:200 with sterile DPBS. Sterile-filtered (0.22  $\mu$ m) *S. aureus* supernatants from 6-hour subcultures grown in TSB were combined with washed diluted blood in individual wells of a 96 well plate on ice at a ratio of 1:1. TSB alone and TSB + 0.5% Triton X-100 were also used as negative and positive controls for hemolysis. Samples were then placed in a SpectraMax 190 microplate reader (Molecular Devices) heated to 37°C and absorbance at 630 nm was measured after 6 minutes of incubation. Percent hemolysis was determined using the following

formula:  $\% \text{Hemolysis} = (\text{Absorbance}_{\text{Experimental}} - \text{Absorbance}_{\text{TSB control}}) / (\text{Absorbance}_{\text{Triton X}} - \text{Absorbance}_{\text{TSB control}}) \times 100$ .

## Human Myeloperoxidase Activity Assays

To obtain extracellular proteins from USA300 strains, supernatant from *S. aureus* sub-cultured for 5 hours was sterile-filtered (0.22  $\mu$ m) and stored at -80 °C. For human myeloperoxidase (MPO) activity assays, 0.5  $\mu$ g of recombinant human MPO (R&D Systems) in 50  $\mu$ L of DPBS was mixed with 50  $\mu$ L of sterile-filtered *S. aureus* supernatant for 30 minutes at room temperature. The MPO-supernatant solution was then exposed to 150  $\mu$ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences) and incubated at 37 °C. The oxidation of TMB catalyzed by human MPO was quantified by measuring the OD<sub>650</sub> every minute for 30 minutes using a SpectraMax Paradigm microplate reader (Molecular Devices). The MPO inhibitor sodium azide was used at a concentration of 1 mM.

## Human PMN Plasma Membrane Integrity Assays

Human polymorphonuclear leukocytes (neutrophils or PMNs) were isolated under endotoxin-free conditions (< 25.0 pg/ml) using freshly drawn heparinized venous blood from healthy donors with written informed consent as previously described [21,23]. Cell viability and purity of preparations were assessed using a FACSCalibur Flow cytometer (BD Biosciences) and only preparations containing  $\geq 98\%$  viable PMNs were used. Assays intoxicating PMNs with extracellular *S. aureus* proteins were performed as previously described [17,24]. Briefly, supernatant from *S. aureus* subcultured for 5 hours in TSB was sterile-filtered (0.22  $\mu$ m) and diluted by 1:10 with TSB. To intoxicate PMNs, 20  $\mu$ l of diluted *S. aureus* supernatant was combined with 100  $\mu$ l Roswell Park Memorial Institute (RPMI) 1640 Medium (Corning Cellgro) with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Corning Cellgro) containing  $1 \times 10^6$  purified human PMNs in a serum coated well of a 96 well plate. Samples were incubated at 37°C for 90 minutes then stained with propidium iodide (PI; ThermoFisher Scientific) following the manufactures protocol and then analyzed with a FACSCalibur Flow cytometer. Triton-X 100 (0.5%) was used as a positive control for causing PMN plasma membrane permeability. The following formula was used to determine % propidium iodide<sup>+</sup>:  $\% \text{Propidium Iodide}^+ = (\text{Mean PI signal}_{\text{Experimental}} - \text{Mean PI signal}_{\text{untreated}}) / (\text{Mean PI signal}_{\text{Triton-X}} - \text{Mean PI signal}_{\text{untreated}})$ .

Assays measuring human PMN plasma membrane permeability following the phagocytosis of live *S. aureus* were performed as previously described [8,17,24]. Briefly, subcultured *S. aureus* was harvested at ME growth by centrifugation (5,000  $\times$  g, 5 min, 4°C), washed with DPBS, then opsonized with 50% normal human serum for 15 minutes at 37°C. Opsonized bacteria were washed with DPBS and then  $2 \times 10^7$  CFU in 100  $\mu$ l of DPBS was combined with 100  $\mu$ l of RPMI/H containing  $1 \times 10^6$  purified human PMNs in a serum coated well of 96 well plate. Phagocytosis was synchronized by centrifugation (524  $\times$  g, 8°C, 8 minutes) in an Allegra X-15R centrifuge (Beckman Coulter) and samples were incubated at 37°C for 90 minutes. Following

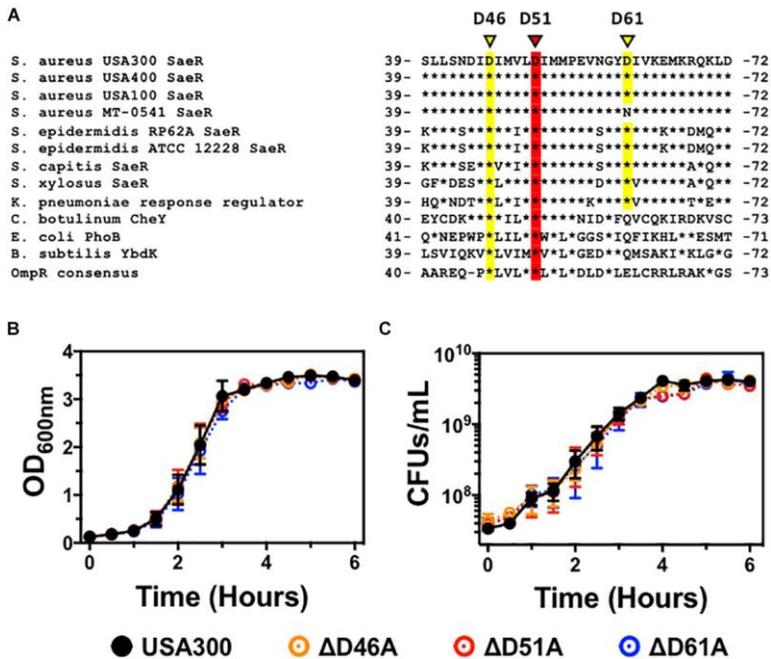
incubation, human PMNs were stained with PI and analyzed using flow cytometry as described above.

## Murine Model of Soft-Tissue Infection

All animal studies conformed to National Institute of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University-Bozeman. Female BALB/C mice (8-10 weeks old) with an average weight of 22 g were purchased from Animal Resource Facility at Montana State University (Bozeman, MT). The murine model of soft-tissue infection was performed as previously described [7,25,26]. Briefly, 12-week-old BALB/C mice were shaved and hair completely removed with Nair™ treatment. Two days later, shaved mice (5 per group) were subcutaneously inoculated with  $1 \times 10^7$  CFUs of *S. aureus* in 100 uL DPBS. Mice were weighed and abscess size measured at indicated times post-inoculation. The area of soft-tissue infections was determined using the following formula as previously published [25]:  $\text{Area} = \pi (\text{Length}/2) \times \text{Width}/2$ .

## Results

**The generation of point mutations in the USA300 genome that confer aspartic acid to alanine substitutions within the receiver domain of SaeR.** Analysis of the three-dimensional crystal structure of the two-component response regulator PhoB of *Escherichia coli* [27] indicates that the homologous *S. aureus* response regulator SaeR is phosphorylated on a conserved aspartic acid at residue 51 (Figure 1A). Aspartic acid residue 46 is also highly conserved in the OmpR family of proteins, suggesting this residue might be important for SaeR function. Analysis of the SaeR sequences extracted from over 8,000 draft *S. aureus* genomes revealed that aspartic acid residue 51 is 100% conserved. The biological significance of this conservation is heightened by the identification of 50 unique SaeR protein sequences identified from the draft genomes (Supplemental Figure 1).



**Figure 1: The generation of USA300 genomic point mutations in specific aspartic acid residues of *saeR*.**

**A)** DNA sequence alignment of *saeR* homologues from different bacteria highlighting the putative phosphorylation site at conserved aspartic acid residue 51 (red) as well as proximal aspartic acid residues 46 and 61 (yellow). *In vitro* growth as measured by **B)** Absorbance at 600 nm and **C)** colony forming units (CFU) per mL of USA300, USA300 with an aspartic acid to alanine point mutation at SaeR residue 46 ( $\Delta$ D46A), at residue 51 ( $\Delta$ D51A), or at residue 61 ( $\Delta$ D61A). Data in panels **B** and **C** are presented as the mean  $\pm$  SEM of three independent experiments.

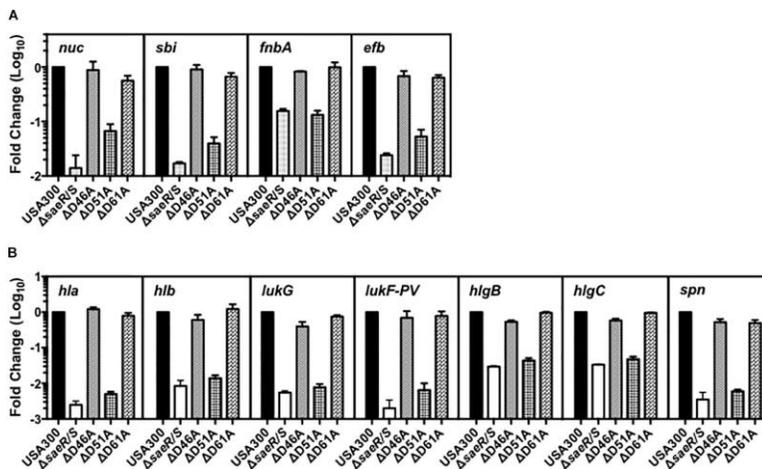
To determine if these aspartic acid residues in the receiver domain of SaeR are important for *S. aureus* pathogenesis, we used allelic recombination to induce substitutions of specific nucleic acids within the *S. aureus* genome as previously described by others [15,28,29]. Using this technique, we generated USA300 strains with an aspartic acid to alanine substitution at the conserved SaeR residue 51 (USA300 $\Delta$ *saeR*-D51A) or the proximal conserved residue 46 (USA300 $\Delta$ *saeR*-D46A). In addition, we generated an aspartic acid to alanine substitution at the less maintained residue 61 of SaeR (USA300 $\Delta$ *saeR*-D61A) to serve as a control for this study.

Primary DNA sequencing analysis and additional whole genome sequencing analysis verified the targeted amino acid substitutions that were generated in the three mutants and showed that there were no additional SNPs or InDels when compared to the wild-type strain. As with previously generated isogenic deletion mutants of *saeR/S* in *S. aureus* [7,8], no differences during *in vitro* growth could be detected in these SaeR point mutants relative to the parental USA300 strain (Figures 1B and 1C).

**Substitution of SaeR aspartic acid residue 51 with alanine substantially decreases the transcript abundance of USA300 virulence genes.** The SaeR/S two-component system plays an essential role during pathogenesis by upregulating *S. aureus* virulence gene transcription [5-8]. It is thought that this process requires the activation of SaeR via phosphorylation at an aspartic acid residue within the receiver domain of this response regulator. Indeed, others have indicated that aspartic acid residue 51 of SaeR is necessary for the transcription of  $\alpha$ -hemolysin (Hla) using an artificial plasmid overexpression system in *S. aureus* strain Newman lacking wild-type *saeR/S* [13]. However, the transcription of *saeR* that is under a positive feedback loop by the SaeR/S two-component system was actually enhanced in the absence of aspartic acid 51 while no difference in SaeR/S regulated coagulase A transcription could be demonstrated [13]. This study suggested that different levels of SaeR phosphorylation corresponds to the up-regulation of specific sets of virulence genes, prompting us to also examine the influence of the highly conserved SaeR aspartic acid residue 46 and partially conserved aspartic acid residue 61 on SaeR/S-mediated virulence gene transcription.

To resolve the importance of these SaeR aspartic acid residues for virulence gene expression in clinically relevant MRSA, we examined the transcript abundance of numerous adhesins, toxins, and immunomodulatory proteins in the USA300 aspartic acid point mutant strains using relative quantitative real time RT-PCR (Figure 2). Compared to USA300, we observed substantial decreases in the abundance of transcripts encoding various adhesins, toxins, and immunomodulatory proteins in a USA300

isogenic deletion mutant of *saeR/S* (USA300 $\Delta$ *saeR/S*) relative to the USA300 wt (Figures 2A and 2B). The virulence gene transcription profile for USA300 $\Delta$ *saeR*-D51A was almost identical to that of USA300 $\Delta$ *saeR/S*. In contrast, the transcript abundance of virulence genes in USA300 $\Delta$ *saeR*-D46A and USA300 $\Delta$ *saeR*-D61A was comparable to the USA300 parental wt (Figures 2A and 2B). Reintroduction of wild-type SaeR to USA300 $\Delta$ *saeR*-D51A rescued defects in gene transcription (Supplemental Figure 2), supporting results indicating aspartic acid residue 51 of SaeR is required for the upregulation of virulence gene expression by this response regulator.



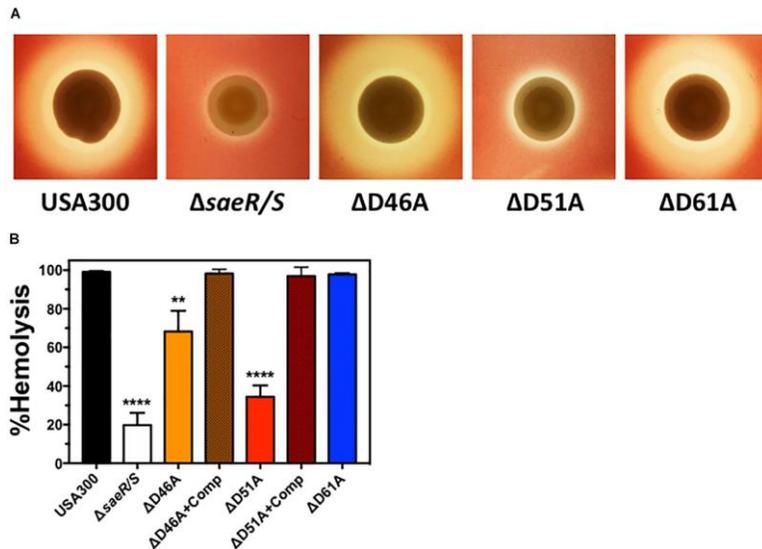
**Figure 2: Aspartic acid residue 51 of SaeR is important for the transcription of numerous virulence genes in USA300.**

Taqman® RT-PCR analysis of USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300 $\Delta$ *saeR/S*), and USA300 genomic point mutants that confer aspartic acid to alanine substitutions at SaeR residue 46 ( $\Delta$ D46A), 51 ( $\Delta$ D51A), or 61 ( $\Delta$ D61A) during growth *in vitro*. Transcriptional analysis was performed at **A** mid-exponential growth for nuclease (*nuc*), the second binder of IgG (*sbi*), fibronectin-binding protein A (*fnbA*), and the extracellular fibrinogen-binding protein (*efb*) or at **B** early-stationary growth for  $\alpha$ -hemolysin (*hla*),  $\beta$ -hemolysin (*hlb*), leukocidin subunit G (*lukG*), the Panton-Valentine leukocidin subunit F (*lukF-PV*),  $\gamma$ -hemolysin component B (*hlgB*),  $\gamma$ -hemolysin component C (*hlgC*), and the staphylococcal peroxidase inhibitor (*spn*). All panels show the mean  $\pm$  SEM of at least two separate experiments and are presented as fold change relative to USA300 wt.

**Aspartic acid residue 51 of SaeR is essential for the SaeR/S mediated hemolysis of human erythrocytes by USA300.** *S. aureus* expresses numerous hemolysins under direct transcriptional regulation by the SaeR/S two-component system [7,8] that lyse mammalian erythrocytes including Hla, Hlb, and HlgA/B [30,31]. Real time RT-PCR analysis indicated aspartic acid residue 51 of SaeR is essential for the transcriptional upregulation of these hemolysins (Figure 2). To determine the importance of this putative SaeR phosphorylation site for hemolysis caused by *S. aureus*, we first qualitatively assessed the ability of USA300, USA300 $\Delta$ saeR/S, USA300 $\Delta$ saeR-D46A, USA300 $\Delta$ saeR-D51A, and USA300 $\Delta$ saeR-D61A to lyse human red blood cells during growth on agar (Figure 3A). The zone of hemolysis generated by USA300 $\Delta$ saeR/S on agar was decreased relative to USA300 (Figure 3A), corresponding to observations in this study (Figure 2) and in previously published reports [7,8] that demonstrate a decrease in transcript abundance of various *S. aureus* hemolysins when this two-component system is absent. A reduced hemolysis of human erythrocytes during growth on agar of USA300 $\Delta$ saeR-D51A paralleled that of USA300 $\Delta$ saeR/S, supporting the notion that aspartic acid residue 51 is essential for SaeR/S activity. In contrast, the zone of hemolysis for USA300 $\Delta$ saeR-D46A and USA300 $\Delta$ saeR-D61A was indistinguishable from that caused by USA300.

To quantify the hemolysis caused by aspartic acid point mutants of SaeR, we measured the turbidity of human erythrocytes in solution after being combined with extracellular proteins produced by these strains. Corresponding to hemolysis during growth on agar, significantly less human red blood cells were lysed by filtered supernatants from USA300 $\Delta$ saeR/S or USA300 $\Delta$ saeR-D51A relative to the USA300 parental strain (Figure 3B). In addition, extracellular proteins produced by USA300 $\Delta$ saeR-D46A generated significantly less hemolysis than USA300. In support of these findings, the reintroduction of wild-type SaeR to USA300 $\Delta$ saeR-D46A or USA300 $\Delta$ saeR-D51A rescued the hemolytic activity of supernatants from these strains to levels observed for USA300. Taken together, these findings show that aspartic acid residue 51 of SaeR is necessary for causing the hemolysis of human erythrocytes that is

facilitated by the SaeR/S two-component system. In addition, SaeR aspartic acid residue 46 appears to play a significant but less important role than residue 51 mediating hemolysis caused by USA300.

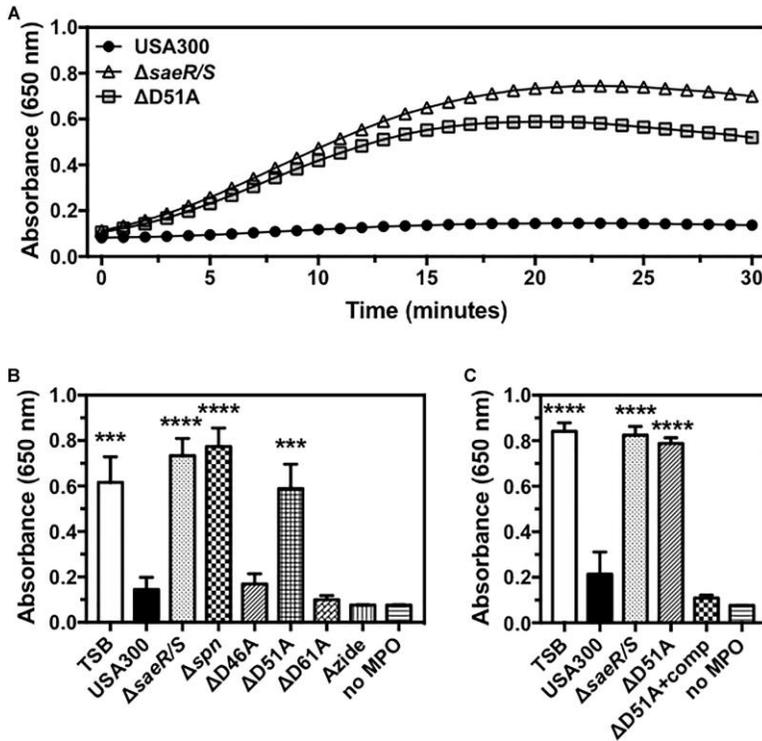


**Figure 3: Aspartic acid residues 46 and 51 of SaeR are important for the hemolysis of human erythrocytes by USA300.**

A) Zone of hemolysis caused by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300 $\Delta$ saeR/S), or USA300 expressing SaeR with aspartic acid to alanine substitutions at residue 46 ( $\Delta$ D46A), 51 ( $\Delta$ D51A), or 61 ( $\Delta$ D61A) during growth on agar containing 2.5% human red blood cells. B) Percent hemolysis of human red blood cells in solution exposed to 6 hour supernatants from USA300, USA300 $\Delta$ saeR/S,  $\Delta$ D46A,  $\Delta$ D51A,  $\Delta$ D61A or  $\Delta$ D46A and  $\Delta$ D51A complemented with a plasmid expressing wt SaeR ( $\Delta$ D46A+Comp or  $\Delta$ D51A+comp, respectively). All panels represent at least 3 separate experiments. Panels B and C show the mean  $\pm$  SEM with \*\*  $P \leq 0.01$  and \*\*\*\*  $P \leq 0.0001$  relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

**Inhibition of human MPO activity by USA300 extracellular proteins requires aspartic acid residue 51 of SaeR.** Recently published findings demonstrate that *S. aureus* prevents the generation of reactive oxygen species by human polymorphonuclear leukocytes (neutrophils or PMNs) via SPIN, a protein under strong SaeR/S regulation that binds directly to the active site of human MPO and inhibits peroxidase activity of

this enzyme [32,33]. To determine if the putative phosphorylation site at aspartic acid residue 51 of SaeR is required for the inhibition of MPO activity by USA300, we assessed the activity of human MPO in the presence of extracellular proteins produced by USA300, an isogenic deletion mutant of SPIN in USA300 (USA300 $\Delta$ *spn*), USA300 $\Delta$ *saeR/S*, USA300 $\Delta$ *saeR*-D46A, USA300 $\Delta$ *saeR*-D51A, and USA300 $\Delta$ *saeR*-D61A (Figure 4). Congruent with previously published findings [32], a strong inhibition of human MPO activity was observed in the presence of the MPO inhibitor azide or extracellular proteins produced by USA300 but not extracellular proteins produced by USA300 $\Delta$ *saeR/S* or by USA300 $\Delta$ *spn* (Figures 4A and 4B). As with USA300 $\Delta$ *saeR/S* and USA300 $\Delta$ *spn*, extracellular proteins produced by USA300 $\Delta$ *saeR*-D51A did not inhibit human MPO activity (Figures 4A and 4B). Reintroduction of wild-type SaeR to USA300 $\Delta$ *saeR*-D51A rescued the inhibition of human MPO activity by extracellular proteins produced from this strain (Figure 4C). In contrast, supernatant from USA300 $\Delta$ *saeR*-D46A and USA300 $\Delta$ *saeR*-D61A blocked the activity of human MPO.

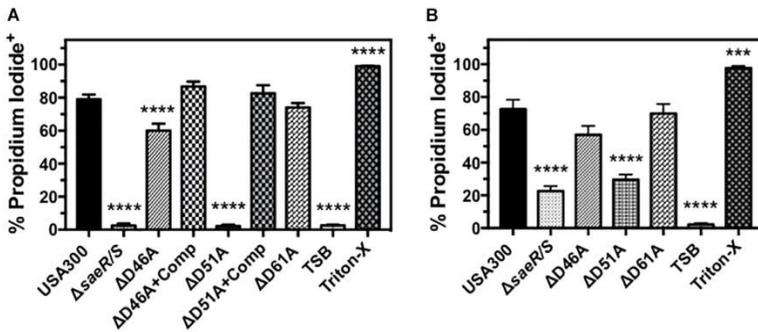


**Figure 4: Aspartic acid residue 51 of SaeR is essential for the inhibition of human myeloperoxidase activity by USA300.**

(A) Human myeloperoxidase (MPO) activity in the presence of extracellular proteins produced by USA300, an isogenic deletion mutant of *saeR/S* in USA300 ( $\Delta$ saeR/S), or an aspartic acid to alanine substitution at residue 51 of SaeR in USA300 ( $\Delta$ D51A) as measured by absorbance at 650 nm following exposure to 3,3',5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide. (B) The activity of human MPO in the presence of extracellular proteins produced by USA300,  $\Delta$ saeR/S, an isogenic deletion mutant of SPIN in USA300 ( $\Delta$ spn),  $\Delta$ D51A, or USA300 with an aspartic acid to alanine substitution at residue 46 ( $\Delta$ D46A) or 61 ( $\Delta$ D61A) of SaeR. Peroxidase activity in the presence of the MPO inhibitor sodium azide (Azide) or samples lacking MPO (no MPO) were included as negative controls. (C) Experiments in panel (B) were repeated using a D51A point mutant of *saeR* complemented with a plasmid expressing wt SaeR ( $\Delta$ D51A+comp). All panels show the mean  $\pm$  SEM of at least three independent experiments. For panels (B) and (C), absorbance is shown at 20 minutes with \*\*\* P < 0.001 and \*\*\*\* P < 0.0001 relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparisons test.

**Human PMN plasma membrane permeability caused by USA300 requires aspartic acid at residue 51 of SaeR.**

Previous studies have demonstrated that the SaeR/S two-component system upregulates *S. aureus* toxins that target human PMNs [7,8,34-37]. To assess the importance of aspartic acid residues within the receiver domain of SaeR for mediating toxicity against PMNs, we first examined the ability of extracellular proteins produced by D46A, D51A and D61A point mutants of SaeR to cause human PMN plasma membrane permeability as measured by propidium iodide staining (Figure 5A). No plasma membrane permeability was observed for PMNs exposed to extracellular proteins produced by USA300 $\Delta$ saeR-D51A, indicating aspartic acid residue 51 of SaeR is necessary for the toxicity of USA300 against human PMNs. A significant reduction in PMN plasma membrane permeability was also observed for extracellular proteins produced by USA300 $\Delta$ saeR-D46A, though this difference was less than that observed for USA300 $\Delta$ saeR-D51A. In support of these findings, complementation of USA300 $\Delta$ saeR-D46A or USA300 $\Delta$ saeR-D51A with wild-type SaeR rescued the lytic capacity of extracellular proteins produced by these strains against human PMNs. Corresponding to the toxicity of extracellular proteins produced by these strains, significantly less PMN plasma membrane permeability was observed following phagocytosis of live USA300 $\Delta$ saeR/S, USA300 $\Delta$ saeR-D51A, and USA300 $\Delta$ saeR-D46A relative to USA300 (Figure 5B). These results demonstrate that SaeR aspartic acid residue 51 and, to a lesser extent, aspartic acid residue 46 are important for activity of this two-component system.



**Figure 5: Human PMN plasma membrane permeability caused by USA300 is significantly influenced by aspartic acid residues 46 and 51 of SaeR.**

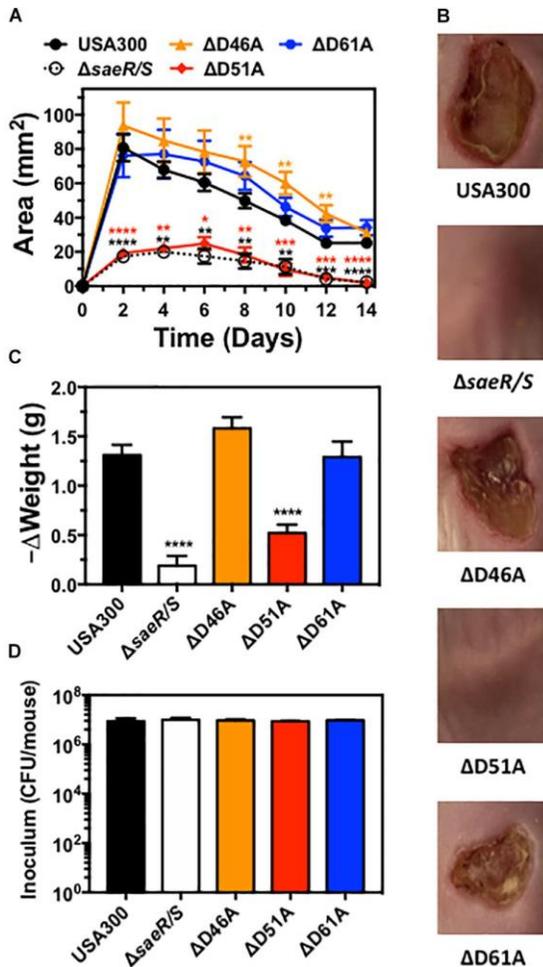
**A)** Flow cytometry was used to assess the percentage of purified human PMNs permeable to propidium iodide 90 minutes after exposure to 5 hour supernatants from USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300 $\Delta$ saeR/S), or USA300 expressing SaeR with aspartic acid to alanine substitutions at residue 46 ( $\Delta$ D46A), 51 ( $\Delta$ D51A), 61 ( $\Delta$ D61A), and  $\Delta$ D46A or  $\Delta$ D51A complemented with plasmids expressing wt SaeR ( $\Delta$ D46A+Comp or  $\Delta$ D51A+comp, respectively). **B)** The percentage of propidium iodide positive human PMNs 90 minutes after the phagocytosis of live *S. aureus* at a ratio of 20 colony forming units per PMN. For these experiments, PMNs were also treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X). All panels show the mean  $\pm$  SEM of at least 5 independent experiments. \*\*\*  $P \leq 0.001$  and \*\*\*\*  $P \leq 0.0001$  relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

### Aspartic acid residue 51 of SaeR is essential for the pathogenesis of USA300 during murine soft-tissue infections.

Previously published findings demonstrate the upregulation of virulence gene transcription *in vivo* by the SaeR/S two-component system is essential for the pathogenesis of *S. aureus* during both systemic and localized infection [7,8].

Transcriptional analysis of USA300 $\Delta$ saeR-D51A in this report (Figure 2) indicates aspartic acid residue 51 of SaeR is required for upregulating *S. aureus* virulence gene transcription mediated by SaeR/S *in vitro*. To determine if potential SaeR phosphorylation sites are important for pathogenesis *in vivo*, we assessed the virulence of USA300, USA300 $\Delta$ saeR/S, USA300 $\Delta$ saeR-D46A, USA300 $\Delta$ saeR-D51A, and USA300 $\Delta$ saeR-D61A during a murine model of soft-tissue infection (Figure 6). In a manner indistinguishable from USA300 $\Delta$ saeR/S, soft-tissue infections caused by USA300 $\Delta$ saeR-D51A were significantly smaller than infections

caused by USA300 (Figure 6A) and did not exhibit the open dermonecrotic lesions characteristic of USA300 pathogenesis (Figure 6B) that is attributed to the high expression of Hla by this strain [38]. In addition, a significant decrease in the weight of mice following infection with USA300 was not observed following inoculation with USA300 $\Delta$ *saeR/S* or USA300 $\Delta$ *saeR*-D51A (Figure 6C). These findings could not be explained by differences in inoculum concentration given to mice for each *S. aureus* strain tested (Figure 6D). As opposed to USA300 $\Delta$ *saeR*-D51A, no differences could be distinguished between soft-tissue infections caused by USA300 $\Delta$ *saeR*-D46A or USA300 $\Delta$ *saeR*-D61A relative to USA300 (Figures 6A, 6B, and 6C). These results demonstrate that USA300 pathogenesis during murine soft-tissue infection is dependent upon aspartic acid residue 51 of SaeR while aspartic acid residues 46 and 61 are not essential for causing disease.



**Figure 6: Aspartic acid residue 51 of SaeR significantly influences the pathogenesis of murine soft-tissue infections caused by USA300.** (A) Area of soft-tissue infection caused by USA300, an isogenic deletion mutant of *saeR/S* in USA300 ( $\Delta$ saeR/S), or USA300 with an aspartic acid to alanine substitution at SaeR residue 46 ( $\Delta$ D46A), 51 ( $\Delta$ D51A) or 61 ( $\Delta$ D61A). (B) Representative images of soft-tissue infections from experiments in panel (A) on day 6 post-inoculation. (C) Change in the weight of mice during experiments in panel (A) between days 0 and 2 post-inoculation. (D) Inoculum of each strain given to mice in panels (A-C). Panels (A), (C), and (D) show the mean  $\pm$  SEM of two independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  and \*\*\*\*  $P \leq 0.0001$  relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

## Discussion

Bacterial pathogens must recognize environmental cues and respond appropriately to cause disease. For *S. aureus*, the SaeR/S two-component system plays an important role during pathogenesis by sensing host-specific signals and up-regulating virulence gene expression in response [39,40]. In this report, we show that the substitution of the putative SaeR phosphorylation site at aspartic acid residue 51 completely ameliorates SaeR/S-mediated virulence. Specifically, we observed a dramatic decrease in the transcript abundance of various hemolysins and leukocidins in USA300 lacking aspartic acid residue 51 of SaeR. These findings correspond to a decrease in the ability of this strain to lyse human erythrocytes and PMNs. SaeR aspartic acid residue 51 was also shown to be important for the transcription of the human MPO inhibitor SPIN and USA300 lacking SaeR aspartic acid residue 51 did not inhibit human MPO activity. Moreover, we show that the virulence of USA300 $\Delta$ saeR-D51A during murine models of soft-tissue infection paralleled the transcript abundance of Hla in this strain, consistent with previous findings demonstrating this toxin is a major *S. aureus* virulence determinant in this model [38]. Collectively, these findings indicate that SaeR residue 51 is essential for *S. aureus* evasion of innate immunity and support other studies that suggest only phosphorylated SaeR has DNA binding activity [12].

We also observed a more subtle decrease in the toxicity of USA300 against human erythrocytes and PMNs in the absence of the conserved aspartic acid residue 46 of SaeR. It is not clear if substitution of this residue simply perturbs the receiver domain structure of SaeR to diminish activity or if aspartic acid residue 46 plays a more direct role in the phosphorylation state of this response regulator. Regardless, a reduction in the lytic capacity of USA300 $\Delta$ saeR-D46A suggests this conserved residue also influences SaeR function and will be further examined in future studies.

This study demonstrates that the substitution of a single amino acid residue in the SaeR response regulator of USA300 renders

this highly pathogenic strain avirulent, highlighting the critical importance of two-component sensory systems for bacterial pathogenesis. Indeed, others have shown that point mutations in the dimerization interface of the histidine kinase sensor AgrC [28] or in the P2 promoter region of the Agr two-component system [29] have a profound effect on the lytic capacity and colonization potential of *S. aureus* while a point mutation in the histidine kinase sensor LiaS of *Streptococcus pyogenes* contributes to the carrier phenotype of this bacterium [41]. Interestingly, no natural mutations of aspartic acid residue 51 could be identified in over 8,000 *S. aureus* genomes, suggesting this putative phosphorylation site is imperative for the fitness of this pathogen. Taken together, these studies indicate that novel therapeutic strategies that inhibit the activation of this highly conserved response regulator would impede the expression of multiple virulence factors and effectively block different aspects of pathogenesis resulting in clearance of *S. aureus* by innate immune mechanisms.

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