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SaeR Binds a Consensus Sequence within Virulence Gene Promoters to Advance USA300 Pathogenesis

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Abstract

This investigation examines the role of the SaeR/S two-component system in USA300, a prominent circulating clone of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA). Using a *saeR/S* isogenic deletion mutant of USA300 (USA300Δ*saeR/S*) in murine models of sepsis and soft-tissue infection revealed this sensory system is critical to USA300 pathogenesis during both superficial and invasive infection. Oligonucleotide microarray and real time RT-PCR identified numerous extracellular virulence genes down-regulated in USA300Δ *saeR/S*. Unexpectedly, an up-regulation of *mecA* and *mecR1* corresponded to increased methicillin-resistance in USA300Δ *saeR/S*. 5'-RACE analysis defined transcript start sites for *sbi*, *efb*, *mecA*, *lukS-PV*, *hly*, *SAUSA300_1975*, and *hla*, to underscore a conserved consensus sequence within promoter regions of genes under strong SaeR/S transcriptional regulation. EMSA experiments illustrated direct binding of SaeR^{His} to promoter regions containing the conserved consensus sequence. Collectively, this investigation demonstrates SaeR/S directly interacts with virulence gene promoters to significantly influence USA300 pathogenesis.

Keywords

Staphylococcus aureus; pathogenesis; virulence; two-component systems; *saeR/S*; USA300; promoter regions; gene expression; *hla*; *mecA*

Introduction

Staphylococcus aureus is a prominent bacterial pathogen responsible for a wide range of disease in humans (1). Within the last decade there has been an emergence of virulent methicillin-resistant *S. aureus* (MRSA) distinct from previously characterized hospital-associated MRSA (HA-MRSA) (2). Termed community-associated MRSA (CA-MRSA) due to apparent origins from outside the healthcare setting, these strains are alarming in their ability to cause life-threatening infections in healthy individuals as well as produce diseases not generally associated with *S. aureus* pathogenesis (3–6). Infection caused by MRSA is a serious health concern even in developed countries; invasive MRSA disease caused over 18,000 deaths during 2005 in the United States alone (7). The recent identification of at least two separate *S.*

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aureus lineages resistant to vancomycin (8), the antibiotic of choice to treat MRSA infection, emphasizes the necessity to further understand fundamental mechanisms of *S. aureus* pathogenesis to promote novel treatment strategies against this bacteria.

The *S. aureus* genome encodes 16 putative two-component gene-regulatory systems (TCSs) identified by sequence homology that collectively monitor environment signals and adjust gene transcription in response (9–11). The SaeR/S TCS is thought to be critical for *S. aureus* pathogenesis by transcriptionally up-regulating numerous virulence genes in response to host specific signals (12–14), yet how this sensory system exerts influence on *S. aureus* transcription has remained uncertain. We have generated an isogenic *saeR/S* deletion mutant in USA300 (USA300Δ *saeR/S*) to examine the role of this TCS during disease caused by a major circulating strain of clinically relevant CA-MRSA. Collectively this investigation demonstrates SaeR/S is critical to USA300 pathogenesis by up-regulating virulence gene transcription through direct interactions with a conserved consensus sequence within gene promoters, a major step towards clearly defining the SaeR/S mediated transcriptome in *S. aureus*.

Materials and methods

Bacterial strains and culture

Staphylococcus aureus strains were cultured and harvested as described previously (14–17) at mid-exponential (ME) and early-stationary (ES) growth defined by OD₆₀₀ = 0.75 or OD₆₀₀ = 2.0, respectively. *Escherichia coli* BL21 and Top10 cells (Invitrogen) were grown in Luria Bertani broth supplemented with 100 mg/L ampicillin (LBA) (EMD chemicals). *S. aureus* pulse-field gel electrophoresis type USA300 strain LAC (18) was used to generate USA300Δ *saeR/S* as described previously (14;¹⁶;19) (Fig 1). USA400 strain MW2 and USA400Δ *saeR/S* were generated in prior investigations (14).

Murine Models of Infection

Murine models of infection were performed as previously described (14–16). 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 CD1 Swiss and Crl;SKH1-hrBR hairless mice were purchased from Charles River Laboratories (Wilmington, MA). Survival statistics were performed using a Logrank test (GraphPad Prism version 4.0).

Oligonucleotide microarray and TaqMan real-time RT-PCR analysis

Oligonucleotide microarray analysis and TaqMan real-time RT-PCR was performed as previously described (14;¹⁵;17). For oligonucleotide microarrays, protocol for Affymetrix prokaryotic target preparation was followed (www.affymetrix.com/support/downloads) using the Standard format hybridized to GeneChip *S. aureus* Genome arrays (Affymetrix). Each experiment was repeated in triplicate. Real-time RT-PCR was performed using primers in Table 1 on at least two separate experiments with triplicate analysis of each sample.

5'-RACE

Rapid-amplification of 5'-complementary DNA ends (5'-RACE) analysis was performed using previously defined protocols (20) with SuperScript III Reverse Transcriptase (Invitrogen Life Technologies), terminal transferase (New England Biolabs), and gene specific primers (Table 1). 5'-RACE products were visualized using 1.5% DNA agarose gel electrophoresis, excised, purified using a QIAquick PCR purification kit (Qiagen), and cloned into pCR2.1 Topo (Invitrogen). Subsequent plasmids were purified using a QIAprep spin miniprep kit (Qiagen) and sequenced with a BigDye terminator v3.1 kit (Applied Biosystems). Sequence alignment of 5'-RACE products against the USA300 genome (NCBI accession # NC_007793) was

performed using nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov>). Multiple sequence alignment was performed using CLUSTALW (<http://align.genome.jp/>) and a consensus sequence logo derived using weblogo (<http://weblogo.berkeley.edu/>). Virtual Footprint Bacterial Regulon Analyzer (<http://prodoric.tu-bs.de/vfp/>) and nucleotide BLAST were used to examine intergenic regions within the USA300 genome.

Purification of SaeR and EMSAs

Expression of recombinant SaeR (SaeR^{His}) was achieved using *saeR* specific primers (Table 1) and a Champion pET Directional TOPO Expression Kit (Invitrogen). *E. coli* BL21 cells transformed with pET100D vector containing *saeR* were grown at 37°C with agitation in 4 L LBA to an OD₆₀₀ = 0.8, induced with 0.5 mM IPTG, and grown for 6 more hours. Cells were harvested by centrifugation (4,000 × g and 4°C for 10 minutes), resuspended in 20 mM Tris, pH 8, (Tris-HCl) then lysed via sonication on ice for 15 minutes. Lysed cells were centrifuged (15,000 × g and 4°C for 10 minutes), samples resuspended in 60 mL 8 M Urea, 0.5 M NaCl, Tris-HCl, sonicated on ice for 15 min, and purified using NI-NTA affinity (Qiagen) and DEAE anion exchange chromatography (GE Healthcare) with a BioLogic LP Chromatography System (Bio-Rad). Purified SaeR^{His} was dialyzed against 3 L Tris-HCl and concentrated using a Centricon Plus-20 centrifugal filter (Millipore). Concentration of SaeR^{His} was determined using a micro BSA protein assay kit (Pierce). Electrophoretic mobility shift assays (EMSAs) were performed as previously described (21) using primers and probes in Table 1. SaeR^{His} was combined with indicated quantity of promoter sequence and 500 ng salmon sperm DNA when indicated in 20 µL binding buffer (100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 5% v/v glycerol, 10 ng/mL BSA) and incubated at room temperature for 15 minutes. Samples were run on a 10% native gel and visualized using a Kodak Image Station 2000mm with excitation at 465 nm and a 535 nm filter or stained with SYBR green (Sigma-Aldrich) and viewed using a UVP BioDoc-It imaging system.

Results

SaeR/S is important for USA300 pathogenesis

As USA300 is a major cause of skin infections, we examined the role of the SaeR/S TCS in this strain using a murine model of soft-tissue infection (Fig. 2). In contrast to mice inoculated with USA300, mice inoculated with USA300Δ*saeR/S* displayed a significant reduction in the incidence and severity of infection. The majority of soft-tissue infections caused by USA300 exhibited dermonecrotic lesions at the dose administered (Fig. 2A and 2B), similar to previous findings (16;²²;23). Surprisingly, nearly half the mice inoculated with USA300Δ*saeR/S* did not display any signs of infection and no dermonecrosis was observed (Fig. 2A and B). Moreover, the size of skin infections generated by USA300Δ*saeR/S* were decreased relative to USA300 (Fig. 2C). This experiment was repeated to show bacterial loads of mice infected with USA300Δ*saeR/S* were the same as USA300 at day 2 post infection but lower after day 4 (Fig. 2D).

A murine sepsis model was used to examine the influence of SaeR/S during invasive infection caused by USA300 (Fig 2E). In accordance with prior research (16), USA300 invasive infection was typically lethal within 24 hours at the dose administered. Conversely, mortality was delayed and considerably reduced in mice infected with USA300Δ*saeR/S*. These findings demonstrate SaeR/S significantly influences the virulence of USA300 during both soft-tissue and invasive infection.

SaeR/S influences transcription of virulence factors in vitro

Gene transcription profiles of USA300Δ*saeR/S* relative to USA300 were defined during in vitro growth using oligonucleotide microarray analysis (Fig 3). Approximately 1.9% of genes

in USA300 Δ *saeR/S* exhibited a greater than two-fold change in transcription relative to USA300, including numerous hemolysins, proteases, host immunomodulatory proteins, and adhesion factors. A transcriptional up-regulation of the *mecA* (penicillin-binding protein 2') and *mecR1* genes that impart methicillin resistance was observed in USA300 Δ *saeR/S*, findings not reported for isogenic *saeR/S* deletion mutants in other *S. aureus* strains (12;¹⁴;24). However, USA300 Δ *saeR/S* is primarily characterized by a down-regulation of transcripts encoding a variety of secreted virulence genes relative to USA300. These include different hemolysins and leukocidins, such as α -toxin (*hla*), β -hemolysin (*hly*), and two-component leukotoxins (*hlgB-hlgC*, *lukE-lukD*, and SAUSA300_1974-SAUSA300_1975). A down-regulation was also observed for known immunomodulatory genes, namely *sbi*, *efb*, and *chs* (encoding CHIPS), secreted into the extracellular environment to subvert the host immune response (25–27). The transcriptional regulator *tcaR*, a member of the *tcaR-tcaA-tcaB* operon, was down-regulated in USA300 Δ *saeR/S*. In addition to associations with teicoplanin resistance and cell wall biosynthesis pathways (28), *tcaR* is thought to be a repressor of the *ica* locus (29) and directly regulate *sasF* and *sarS* transcription (30). Other transcripts down-regulated in USA300 Δ *saeR/S* included various host-binding proteins (*fmbA*, *fmbB*, *empbp*, *coa*, and SAUSA300_1052), several lipase precursors, and the majority of the *spl* operon containing 6 distinct serine proteases.

TaqMan real-time RT-PCR was performed to support oligonucleotide microarray analysis and to determine the influence of SaeR/S on transcription of two genes not included on the oligonucleotide microarray (Fig 4), Pantone-Valentine Leukocidin (PVL) and novel arginine catabolic mobile element (ACME) of USA300 (18). TaqMan real time RT-PCR confirmed the isogenic deletion of *saeR* and *saeS* (Fig 4A), down-regulation of *sbi*, *efb*, *hla*, *hly*, SAUSA300_1975 (Fig 4B), and up-regulation of *mecA* (Fig. 4C) in USA300 Δ *saeR/S*. Complementation with a plasmid encoding *saeR* and *saeS* (USA300 Δ *saeR/S* + Comp) partially restored gene transcription in USA300 Δ *saeR/S*, reinforcing real-time RT-PCR and microarray results. Transcriptional regulation of *lukS-PV* (PVL component) was down-regulated in USA300 Δ *saeR/S*, though USA300 Δ *saeR/S* + Comp did not exhibit restored transcription of *lukS-PV* (Fig 4B). Transcription of *arcA* (ACME component) was similar between USA300 and USA300 Δ *saeR/S*, indicating SaeR/S is not involved with regulating ACME (Fig 4C). The up-regulation of *mecA* and *mecR1* in USA300 Δ *saeR/S* relative to USA300 was coupled with significantly improved growth in sub-inhibitory concentrations of oxacillin (Fig 4D and E). Improved growth (Fig 4F and G) and a subtle increase of *mecA* transcription during growth in oxacillin (Fig 4H) was also observed for USA400 Δ *saeR/S* relative to USA400, further supporting results demonstrating a transcriptional up-regulation of methicillin-resistance genes in the absence of SaeR/S.

Identification of transcript start sites of virulence genes influenced by SaeR/S

Sequence homology of SaeR/S with other bacterial TCSs suggests the DNA-binding response regulator SaeR controls *S. aureus* virulence through direct interactions with virulence gene promoters (13). To define a consensus SaeR recognition sequence specific to virulence gene promoters influenced by SaeR/S, 5'-RACE was used to first identify various transcript start sites (TSSs) in USA300 and USA300 Δ *saeR/S* (Fig 5). Genes shown to be positively regulated by SaeR/S produced more robust 5'-RACE products in USA300 relative to USA300 Δ *saeR/S* (Fig 5B). Alternatively, transcription of *agrB* was not influenced by SaeR/S (Fig 4C) and 5'-RACE products for *agrB* were indistinguishable between USA300 wt and the USA300 Δ *saeR/S* (Fig 5A). 5'-RACE products for *mecA*, a transcript up-regulated in USA300 Δ *saeR/S* relative to USA300, were also identical between USA300 and USA300 Δ *saeR/S* (Fig 5A). These results indicate the transcriptional down-regulation of *sbi*, *efb*, SAUSA300_1975, *lukS-PV*, *hla*, and *hly* in USA300 Δ *saeR/S* likely accounts for the diminished 5'-RACE products for these genes in this strain.

5'-RACE products were excised, cloned, sequenced and aligned against the USA300 genome to define the TSS for these genes (Fig 5C and D). For *hla* and *agrB*, the TSS was found to be 330 and 18 bp upstream, respectively, from the start codon in accordance with prior research (31;32). Previously undefined TSSs of *sbi*, *efb*, *SAUSA300_1975*, *hly*, *lukS-PV*, and *mecA* were also determined. Sequences of 5'-RACE products for *lukS-PV*, *agrB*, and *mecA* isolated from USA300Δ*saeR/S* corresponded to those obtained from USA300. The 5'-RACE product for *sbi* from USA300Δ*saeR/S* mapped to the intergenic region immediately upstream from 50S ribosomal protein L13 and is presumed to be a product of nonspecific primer binding.

Identification of a conserved consensus sequence within promoter regions of genes influenced by SaeR/S

Multiple sequence alignment performed on intergenic sequences immediately upstream from the defined TSS of genes positively regulated by SaeR/S revealed two conserved domains corresponding to the -10 and -35 promoter boxes (Fig 5C). In contrast, intergenic sequences immediately upstream from the TSS of *agrB* and *mecA* did not share the extensive homology observed for genes up-regulated by SaeR/S (Fig 5D). A consensus sequence 17 bp in length was derived from the conserved domain immediately upstream from the -35 promoter box (Fig 5E). This putative SaeR recognition sequence (SRS) has a relatively low overall G/C content and is partially palindromic. In silico examination of intergenic regions within the USA300 genome identified the SRS within putative promoter regions of numerous genes under SaeR/S regulation including *coa*, *saeP*, *splA*, *SAUSA300_1052*, *SAUSA300_0215*, and *SAUSA300_0407* (Fig 3, Fig 5F). Investigators elsewhere have demonstrated a *sae* deletion mutant of strain COL exhibits a reduced expression of Ear and increased expression of Plc (24). Interestingly, the SRS was found within the putative promoter region of *ear* while the reverse complement of the SRS was located within the putative promoters of *plc* and the phosphate starvation-inducible protein *phoH* (Fig 5H). Subsequent real time RT-PCR confirmed a down-regulation of *ear* in USA300Δ*saeR/S* (Fig 4B). The SRS was also found immediately upstream from -35 promoter region of *rot* defined by primer extension analysis (33) and between the -35 and -10 promoter regions of the *arlR/S* TCS also defined by primer extension analysis (34) (Fig 5G).

SaeR binds putative promoter regions of USA300 virulence factors

EMSAs were used to demonstrate recombinant SaeR^{His} binds numerous virulence gene promoter regions in a concentration dependent manner (Fig. 6). Purified SaeR^{His} (Fig 6A) specifically bound the -35 region of the *hla* and *sbi* promoters defined by 5'-RACE in a concentration dependent manner while no binding was detected for the -35 region of the *agrB* promoter (Fig 6B and C). The *hla* promoter probe sequence contains two SRSs, the *sbi* promoter probe contains one SRS, while the *agrB* promoter probe has no SRS, paralleling the relative affinity of these probes for SaeR^{His}. Dissection of the *hla* promoter using overlapping probe sequences suggests binding of SaeR^{His} is most robust to the sequence containing both SRSs immediately upstream from the -35 promoter region (Fig. 6D). EMSAs indicate strong binding of SaeR^{His} to the *arlR*, *saeP*, and *splA* promoter regions (Fig 6E). SaeR^{His} binding was also observed for the *efb*, *hly*, *rot*, *lukS-PV*, and *SAUSA300_1975* promoter regions while no binding was detected for the *mecA* promoter region (Fig 6E). These findings establish direct SaeR-DNA interaction specific to virulence gene promoters containing the SRS.

Discussion

Cumulative evidence indicates *S. aureus* virulence is determined by the coordinated expression of specific virulence gene ensembles that further bacterial survival and dissemination (2;¹⁵; ^{16,35}-38). *S. aureus* sensory mechanisms are thought to be primarily composed of 16 TCSs that dictate gene expression in response to specific environmental signals (9-11). TCSs offer

intriguing drug targets; inhibition of these sensory systems may effectively “blind” *S. aureus* and result in the reduced expression of numerous virulence genes (39). Indeed, preliminary investigations by others demonstrates targeted inhibition of the *agr* quorum-sensing TCS attenuates soft-tissue infections caused by *S. aureus* (40;41). The SaeR/S TCS is thought to be critical during *S. aureus* pathogenesis by up-regulating expression of numerous secreted virulence genes during infection. As approximately 22% of the *S. aureus* genome can differ between isolates (42) and virulence appears to be largely dictated by differences in expression of common genes between strains (36), we verified the importance of SaeR/S in clinically relevant USA300.

Results demonstrating SaeR/S significantly influences virulence during soft-tissue infections caused by USA300 were unexpected given previous findings showing this TCS is dispensable during soft-tissue infections caused by CA-MRSA strain USA400 (14). Compared to USA400, the SaeR/S mediated transcriptome in USA300 is almost exclusively composed of extracellular virulence genes with SaeR/S exerting a stronger regulatory influence on many of these genes. For example, *hla* transcription was reduced by over 33-fold in USA300 Δ *saeR/S* compared to a 4-fold reduction of *hla* transcription in USA400 Δ *saeR/S* (14). Others have shown in vitro transcription of α -toxin (*hla*), *agr*, *saeR/S*, and *sarA* is higher in USA300 relative to USA400 (37). Thus it is likely differences in genomic content and extent of USA300 SaeR/S transcriptional regulation on common virulence gene expression that accounts for the significant influence of this TCS on virulence and pathogen survival during USA300 soft-tissue infections.

The USA300 SaeR/S mediated transcriptome is primarily composed of secreted virulence genes that interact directly with host components including numerous hemolysins and leukocidins, immunomodulatory genes, cell surface fibronectin binding proteins, lipases, nucleases and proteases similar to findings in other *S. aureus* strains (14;²⁴;43). We also report an up-regulation of *mecA* and *mecR1* in USA300 Δ *saeR/S* that corresponds to improved in vitro growth in the presence of subinhibitory concentrations of oxacillin, findings supported by the improved growth of USA400 Δ *saeR/S* relative to USA400 in the presence of oxacillin. Direct binding could not be detected between SaeR^{His} and the *mecA* promoter region defined by 5'-RACE analysis, suggesting an indirect up-regulation of *mecA* in USA300 Δ *saeR/S* by SaeR/S. The down-regulation of the transcriptional regulator *tcaR* in USA300 Δ *saeR/S*, involved with cell wall biosynthesis and implicated in methicillin resistance (28), may account for the differential regulation of *mecA* and *mecR1*.

We identified a conserved palindromic consensus sequence 17 bp in length specific to promoters of genes regulated by SaeR/S. EMSAs revealed SaeR^{His} directly bound various virulence gene promoters containing the conserved consensus sequence while no binding was detected for promoter regions lacking this sequence motif. Corresponding to published findings in other SaeR/S deficient *S. aureus* strains (12;¹⁴;24), a dramatic down-regulation of *saeP* was observed in USA300 Δ *saeR/S*. Not surprisingly, the SRS was identified within the previously defined P1 primary start site for the SaeR/S transcript (44). EMSAs demonstrated direct binding of SaeR^{His} to this promoter region, supporting a positive feedback loop of SaeR/S transcription suggested by others (12). The promoter region of *lukS-PV* was defined using 5'-RACE and weak binding of SaeR^{His} to this promoter sequence reinforces recent work indicating SaeR/S positively regulates *luk-PV* transcription (45). The SRS was also located between the -35 and -10 promoter regions of the *arlR/S* operon (34) and strong binding of SaeR^{His} to this promoter region was observed. The ArlR/S TCS is thought to negatively regulate transcription of numerous secreted virulence genes including *hla*, *hly*, and *coa*, implicating a negative-feedback loop on secreted virulence gene expression (34). Additionally, the SRS specifically mapped to the region immediately upstream from the -35 promoter box of the repressor of secreted virulence genes, *rot* (46), defined by others using primer extension analysis (33). Recent work

on strain COL has demonstrated the repression of *hla* expression by *rot* requires *saeR/S* and that *rot* downregulates the expression of *SaeR/S* (47). Weak binding of *SaeR^{His}* to the *rot* promoter supports the hypothesis made by others (47) that *SaeR/S* mediated transcription of *rot* acts as a negative feedback loop to curtail over expression of *SaeR/S* and secreted virulence genes positively regulated by *SaeR/S*. We were unable to identify a differential regulation of the *rot* or *arlR* transcription in USA300Δ*saeR/S* during in vitro growth. However, because *SaeR/S* is thought to be activated by host tissue-specific signals it follows that changes in transcription of virulence gene regulators controlled by *SaeR/S* may only be resolved during growth in vivo. Taken together, these findings indicate a complex regulatory network of multiple feedback loops influences the *SaeR/S* mediated transcriptional regulation of secreted virulence genes in USA300.

This investigation is the first to examine the role of *SaeR/S* in USA300 pathogenesis and demonstrates this TCS is important during both invasive and superficial disease caused by this clinically relevant strain. Our results define the TSSs for *sbi*, *efb*, *SAUSA300_1975*, *hly*, *lukS-PV*, and *mecA* and are congruent with previously established TSSs for *hla* and *agrB* (31;32). A conserved partially palindromic SRS was identified within virulence gene promoters transcriptionally regulated by *SaeR/S* and EMSAs established direct binding of *SaeR^{His}* to promoter regions containing the conserved consensus sequence. Collectively these findings demonstrate *SaeR/S* plays a major role during USA300 pathogenesis of both superficial and invasive infections by directly binding the SRS within virulence gene promoters to up-regulate transcription of a precise exoprotein array that furthers *S. aureus* survival and dissemination.

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Fig 1. Generation of an isogenic *saeR/S* deletion mutant in USA300

A) Construction of USA300 Δ *saeR/S* via allelic gene replacement. B) PCR verification of mutant genotype as described previously (14) and C) in vitro growth of USA300, USA300 Δ *saeR/S*, and USA300 Δ *saeR/S* supplemented with a complement plasmid encoding *saeR/S* (USA300 Δ *saeR/S*+ Comp).

Fig 2. SaeR/S significantly influences the virulence of USA300 during infection

A) Soft-tissue infections in mice produced by USA300 relative to USA300 Δ saeR/S at day 4 post-infection. B) Percentage of mice exhibiting infection and dermonecrosis following subcutaneous injection of USA300, USA300 Δ saeR/S, or a mock PBS control with * $P < 0.001$ and ** $P < 0.0001$ as determined by T-test. C) Volume of abscess produced by USA300, USA300 Δ saeR/S, or a mock PBS control with * $P < 0.01$ as determined by T-test. D) In a separate experiment CFUs per abscess were enumerated for three mice from each group at days 2, 4, and 7 post-infection. E) Survival during invasive infection by USA300 wt, USA300 Δ saeR/S, or mock PBS control. * $P < 0.001$ as determined by logrank test. Unless otherwise stated, murine models used 15 mice per experimental group and 10 mice per PBS control group.

Mid-exponential		Early Stationary		Locus Tag	SRS	Gene Annotation
Fold Increase	P Value	Fold Increase	P Value			
8.09	1.6E-04	18.69	3.9E-05	synthetic construct		spectinomycin 3'-adenylyltransferase
4.14	7.3E-06	2.24	2.3E-04	SAUSA300_0032		penicillin-binding protein 2
		3.45	3.7E-02	SAUSA300_1098		<i>pyrE</i>
2.44	1.6E-04	1.87	6.7E-03	SAUSA300_0035		hypothetical protein
		3.16	2.2E-02	SAUSA300_1096		<i>carB</i>
2.37	3.0E-04	1.77	4.5E-03	SAUSA300_0033		methicillin-resistance MecR1 regulatory protein
		2.80	3.5E-03	SAUSA300_2572		<i>aur</i>
		2.44	4.0E-02	SAUSA300_1093		<i>pyrB</i>
		2.34	4.6E-02	SAUSA300_2482		hypothetical protein
Fold Decrease	P Value	Fold Decrease	P Value	Locus Tag	SRS	Gene Annotation
		-2.02	9.8E-03	SAUSA300_1380		hypothetical protein
		-2.06	1.0E-02	SAUSA300_2303		<i>tcaR</i>
		-2.10	1.0E-02	SAUSA300_1053		formyl peptide receptor-like 1 inhibitory protein
		-2.17	2.3E-02	SAUSA300_1769	+	<i>lukE</i>
		-2.32	1.4E-02	SAUSA300_0223	+	hypothetical protein
		-2.46	8.5E-03	SAUSA300_1330	~	<i>ltaA</i>
		-2.51	2.0E-03	SAUSA300_1768	+	<i>lukD</i>
-1.19	9.7E-03	-2.31	5.3E-04	SAUSA300_0774		<i>empbp</i>
		-4.76	3.9E-03	SAUSA300_0370		putative staphylococcal enterotoxin
-2.54	5.1E-03	-2.67	4.4E-04	SAUSA300_0320		triacylglycerol lipase precursor
-3.08	2.2E-03	-2.72	4.5E-04	SAUSA300_2603		<i>lip</i>
-4.95	8.8E-04	-1.26	4.8E-02	SAUSA300_0773		putative staphylocoagulase
-2.67	1.1E-02	-3.69	4.5E-04	USA300HOU_1091		similar to permease
-2.25	3.7E-02	-4.46	5.2E-03	SAUSA300_1919		hypothetical protein
-3.51	2.2E-03	-4.81	5.7E-05	SAUSA300_0028		putative transposase
-1.21	3.2E-02	-7.41	4.2E-04	SAUSA300_2367		<i>hlgB</i>
		-10.10	7.1E-03	SAUSA300_1753	~	<i>spIF</i>
-5.52	7.5E-03	-5.10	4.4E-03	SAUSA300_2441		<i>fnbA</i>
-1.16	1.5E-02	-9.52	3.1E-03	SAUSA300_1754	~	<i>spIE</i>
-11.95	1.2E-03			SAUSA300_0408	+	hypothetical protein
		-12.17	2.0E-03	SAUSA300_1756	~	<i>spIC</i>
-9.62	2.2E-05	-1.90	1.9E-02	SAUSA300_0224	+	staphylocoagulase precursor
-1.52	7.9E-03	-10.75	1.2E-03	SAUSA300_1917	+	map pseudogene
		-13.77	3.4E-03	SAUSA300_1757	~	<i>spIB</i>
		-16.84	9.0E-05	SAUSA300_1758	+	<i>spIA</i>
		-17.99	8.4E-04	SAUSA300_2366		<i>hlgC</i>
-6.54	9.6E-03	-12.99	6.1E-03	SAUSA300_0409	+	hypothetical protein
-13.61	2.5E-03	-6.54	2.2E-02	SAUSA300_2440		<i>fnbB</i>
-11.19	1.1E-03	-10.22	3.0E-03	SAUSA300_0776		<i>nuc</i>
-13.33	5.3E-03	-11.33	5.1E-03	SAUSA300_1920		<i>chs</i>
-2.49	5.4E-03	-22.22	2.0E-03	SAUSA300_1974	~	leukocidin/hemolysin toxin family protein
-2.82	3.3E-02	-24.39	3.0E-03	SAUSA300_1918	+	truncated beta hemolysin
-5.13	7.3E-03	-25.58	4.1E-03	SAUSA300_1975	+	Aerolysin/leukocidin family protein
-33.78	3.1E-04	-1.73	8.4E-03	SAUSA300_0407	+	superantigen-like protein
-10.95	1.3E-06	-28.17	1.7E-02	SAUSA300_0692	~	<i>saeQ</i>
-35.59	6.1E-04	-3.72	1.4E-02	SAUSA300_0215	+	hypothetical protein
-9.80	6.5E-04	-33.78	1.5E-03	SAUSA300_1058	++	<i>hla</i>
-36.63	1.7E-03	-10.20	6.8E-04	SAUSA300_1052	+	fibrinogen-binding protein
-17.09	1.7E-04	-37.31	1.9E-03	SAUSA300_1056	~	hypothetical protein
-22.22	3.3E-04	-36.36	9.1E-03	SAUSA300_1055	+	<i>efb</i>
-28.82	1.6E-06	-34.72	1.6E-02	SAUSA300_2384	+	<i>sbl</i>
-22.52	1.0E-04	-46.51	2.9E-02	SAUSA300_0693	+	<i>saeP</i>
-119.90	2.3E-07	-72.46	5.0E-02	SAUSA300_0690	~	<i>saeS</i>
-177.62	1.8E-04	-72.46	4.5E-02	SAUSA300_0691	~	<i>saeR</i>

Fig 3. Oligonucleotide microarray analysis

Mean fold-change in USA300 *saeR/S* gene transcription relative to USA300. Experiments were performed in triplicate. The presence of the SaeR recognition sequence (SRS) immediately preceding a gene is indicated by a +, two SRSs are indicated by ++, and a ~ indicates the presence of the SRS within the putative operon promoter of that gene.

Fig 4. Confirmation of Oligonucleotide microarray analysis

Relative quantitative real time RT-PCR examining expression of A) *saeR* and *saeS*; B) *sbi*, *efb*, *hla*, *hly*, *lukS*, *SAUSA300_1975* (1975) and *ear*; and C) *agrB*, *mecA*, and *arcA*. TaqMan real time RT-PCR experiments were performed twice at two different growth phases (ME: $\text{OD}_{600} = 0.75$ and ES: $\text{OD}_{600} = 2.0$) with triplicate measurement of each sample. D) OD_{600} and E) CFUs/mL during growth in TSB containing 10 $\mu\text{g/mL}$ oxacillin for USA300, USA300 Δ *saeR/S*, and USA300 Δ *saeR/S* supplemented with a complement plasmid encoding *saeR/S* (USA300 Δ *saeR/S*+ Comp). F) OD_{600} and G) CFUs/mL during growth of USA400 and USA400 Δ *saeR/S* in TSB containing 2.5 $\mu\text{g/mL}$ oxacillin. H) Real Time RT-PCR for *mecA* in USA400 and USA400 Δ *saeR/S* at mid-exponential (ME) growth or in the presence of 2.5 $\mu\text{g/mL}$ oxacillin at 3 hrs. Experiments from D, E, F, and G were performed at least in triplicate with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ relative to the parental strain as determined by T-test.

Fig 5. Genes differentially regulated by *SaeR/S* share sequence homology within their promoter regions
5'-RACE products using gene-specific primers for A) *sbi*, *efb*, *mecA*, and *agrB*; B) *SAUSA300_1975*, *lukS-PV*, *hla*, and *hly*. C) 5'-RACE products were sequenced to define transcript start sites (TSSs) and upstream alignment underscoring nucleic acids shared by all sequences (red) and highly conserved between sequences (yellow). Putative -35 and -10 promoter regions are indicated and the SRSs are boxed. D) Promoter regions of *agrB* and *mecA* do not share strong homology with the consensus sequence upstream of genes transcriptionally regulated by *SaeR/S*. E) A consensus sequence was generated (weblogo.berkeley.edu) from the conserved 17 bp sequences immediately upstream of the putative -35 promoter box of *SaeR/S* regulated genes. F) In silico analysis identified the SRS within intergenic regions upstream from the start codon of other genes influenced by *SaeR/S*. G) -35 promoter region of *rot* defined by primer extension analysis (33) and between the -35 and -10 promoter regions of the *arlR/S* TCS (34). H) The reverse complement of the SRS was identified upstream of the *plc* and *phoH* start codons.

Fig 6. Recombinant SaeR^{His} binds putative promoter regions of USA300 virulence genes
 A) SDS-PAGE verifying expression and purification of SaeR^{His}. B) EMSAs demonstrating SaeR^{His} (0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16 μM) binding to 50 bp oligonucleotides encoding -35 promoter region (0.25 pmol) of *hla*, *agrB*, and *sbi* in the presence of 500 ng salmon sperm DNA. C) Binding of SaeR^{His} (6 μM) to *hla*, *agrB*, and *sbi* promoters (0.25 pmol) in the presence of 2.5, 10, or 25 pmol of indicated cold promoter and 500 ng of salmon sperm DNA. D) Interaction of SaeR^{His} (0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16 μM) with four overlapping probe sequences (2.5 pmol) of the *hla* promoter. E) EMSAs examining binding of SaeR^{His} (0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16 μM) to 1 pmol of promoter regions (~100 bp) for *arlR*, *saeP*, *rot*, *splA*, *mecA*, *lukS*-PV, *efb*, *hly*, and SAUSA300_1975.

Table 1

Primer and probe Sequences

Real Time RT-PCR Primers	Sequence
<i>gyrB</i> fwd	5' - CAAATGATCACAGCTTTGGTACAG -3'
<i>gyrB</i> rvs	5' - CGGCATCAGTCATAATGACGAT -3'
<i>gyrB</i> probe	5' - AATCGGTGGCGACTTTGATCTAGCGAAAG -3'
<i>saeS</i> fwd	5' - CGTACATTCAGAGTAGAAAACTCTCGTAATAC -3'
<i>saeS</i> rvs	5' - GTTGCGCGAGTTCATTAGCTATATAT -3'
<i>saeS</i> probe	5' - AGCCTAATCCAGAACCACCCGTT -3'
<i>saeR</i> fwd	5' - CTGCCAAAACACAAGAACATGATAC -3'
<i>saeR</i> rvs	5' - ATTTACGCCTTAACCTTAGGTGCAGAT -3'
<i>saeR</i> probe	5' - ATTTACGCCTTAACCTTAGGTGCAGAT -3'
<i>sbi</i> fwd	5' - ATACATCAAAACATTACGCGAACAC -3'
<i>sbi</i> rvs	5' - CTGGGTCTTGCTGTCTTTAAGTG -3'
<i>sbi</i> probe	5' - CAGGTAGCTTTATGGTTGCTACAAAAAT -3'
<i>efb</i> fwd	5' - CTACAATTGCGTCAACAGCAGAT -3'
<i>efb</i> rvs	5' - ACCATCATTGTACTCTACGATATTGTGA -3'
<i>efb</i> probe	5' - CGAGCGAAGGATACGGTCCAAGAGAAA -3'
<i>hla</i> fwd	5' - CAACAACACTATTGCTAGGTTCCATATT -3'
<i>hla</i> rvs	5' - CCTGTTTTTACTGTAGTATTGCTTCCA -3'
<i>hla</i> probe	5' - ATGAATCCTGTCGCTAATGCCGAGA -3'
<i>SAUSA300_1975</i> fwd	5' - GCGTCATCATTATCATGTGCAA -3'
<i>SAUSA300_1975</i> rvs	5' - TCTTTCTTATTTTGGTCTTGAGAGTCTTT -3'
<i>SAUSA300_1975</i> probe	5' - CAGCAACGACTCAAGCAAATTCAGCTCA -3'
<i>lukS</i> fwd	5' - GAGGTGGCCTTTCCAATACAAT -3'
<i>lukS</i> rvs	5' - CCTCCTGTTGATGGACCACTATTA -3'
<i>lukS</i> probe	5' - TGGTCTCAAAACAAATGACCCCAATGTAGA -3'
<i>arcA</i> fwd	5' - CAACAGGTAATGGCGACGTT -3'
<i>arcA</i> rvs	5' - CAACCCCTGGTCGAATACATAA -3'
<i>arcA</i> probe	5' - TGATGGTGCACGTGAACAATGGAATG -3'
<i>mecA</i> fwd	5' - ACTGATTAACCCAGTACAGATCCTTTC -3'
<i>mecA</i> rvs	5' - TCCAAACTTTGTTTTTCGTGTCTTT -3'
<i>MecA</i> probe	5' - ATCTATAGCGCATTAGAAAA -3'
<i>agrB</i> fwd	5' - GCCCATTCCTGTGCGACTTA -3'
<i>agrB</i> rvs	5' - GGGCAAATGGCTCTTTGATG -3'
<i>agrB</i> probe	5' - AACCTTTTCATTATCACACTT -3'
<i>ear</i> fwd	5' - ACCGATCAATAGCGATAGTGTAATAA -3'
<i>ear</i> rvs	5' - ATGCATCATTGCGTCAAATCT -3'
<i>ear</i> probe	5' - TGATTGTCAAGGAAAAATCCCAATGGAGA -3'
5' RACE primers	Sequence

Real Time RT-PCR Primers	Sequence
poly adapter	5' - CACCTGAGCAGAGTGACGAGGACTACGGCTTCAGCTTTTTTTTTTTTTTTTTT -3'
poly outer adapter	5' - CACCTGAGCAGAGTGACG -3'
sbi cDNA	5' - GTAATTTTGAGTTAGCTTCATATGCTGCTG -3'
sbi PCR	5' - GTCTTTAAGTGATTGAGAGAACTACTTCTTG -3'
efb cDNA	5' - TTTGTATTCAAACGAAACTAAGTTGACTGC -3'
efb PCR	5' - AGTACCATCATTGTACTCTACGATATTGTG -3'
agrB cDNA	5' - CCCTAATCGTACTTGCAAAAAATTGAATATG -3'
agrB PCR	5' - ATACGTGGCAAACCTGGTCAATTTTATTATC -3'
mecA cDNA	5' - AGCATAAAAAATATATACCAAACCCGACAAC -3'
mecA PCR	5' - CCCGACAACACTACAACATTAAAAATAAGTG -3'
SAUSA300_1975 cDNA	5' - GTAACATTACGTTTGTCTTTTGTGAGAC -3'
SAUSA300_1975 PCR	5' - CACATGATAATGATGACGCTATTAACAC -3'
lukS cDNA	5' - TTATCGCTACTTGTATCTTCTGTTCTTTTG -3'
lukS PCR	5' - CAATGTTGCAGCTAATAGTCTTTTTTTGAC -3'
hla cDNA	5' - AAGTGACTAAATCACCTGTTTTTACTGTAG -3'
hla PCR	5' - TTAATATGGAACCTAGCAATAGTGTGTTG -3'
hly cDNA	5' - TGGATACAAAACGGTCGATAACATATAAAC -3'
hly PCR	5' - CTAACCTAATCAATCAGTATCATCTTTC -3'
SaeR cloning primers	Sequence
saeR Up	5' - CACCATGGTGTATCAATTAGAAGTC -3'
SaeR Dwn	5' - GATTATGACGTAATGTCTAATTTGTG -3'
EMSAs probes	Sequence
agrB promoter top	5' - ATTTAACAGTTAAGTATTTATTTCCTACAGTTAGGCAATATAATGATAAA -3'
agrB promoter bottom	5' - TTTATCATTATATTGCCTAAGTGTAGGAAATAAATACTTAAGTGTAAAT -3'
sbi promoter top	5' - CACTAGTTAATCTATTAGTTAACATTAGTTAATAATTAGTTAATTTCCAT -3'
sbi promoter bottom	5' - ATGGAAATTAAGTAATTATTAAGTAATGTAACTAATAGATTAAGTAGTG -3'
hla probe-1 top	5' - GTAAATTAATAAATAGAAATAGAGAAAAAGGGTATTAATTATGTTGGAA -3'
hla probe-1 bottom	5' - TTCCAAACATAATTAATACCCTTTTCTCTATTCTATTATTAATTAC -3'
hla probe-2 top	5' - ATAAAAATTAAGTATATTAAGTGTAAATTAATAAATAGAAATAGA -3'
hla probe-2 bottom	5' - TCTATTCTATTATTAATTTACACTAGTTAATATATAGTTAATTTTAT -3'
hla probe-3 top	5' - AAATCAATTAAGTATTAATAAATAAATAAGTATATTAAGTAGTG -3'

Real Time RT-PCR Primers	Sequence
<i>hla</i> probe-3 bottom	5' - CACTAGTTAATATATAGTTAATTTTATTTAATAGTTAATTAATTGATT -3'
<i>hla</i> probe-4 top	5' - AAAAATACAAATATCTTAGAATTAATCAATTAATTAAGTATTAAATAAA -3'
<i>hla</i> probe-4 bottom	5' - TTTATTTAATAGTTAATTAATTGATTAAATTTCTAAGATATTTGTATTTT -3'
6FAM- <i>hla</i> probe-3 top	5' - 6FAM/ AAATCAATTAATTAAGTATTAAATAAAAAATTAAGTATTAACTAGTG -3'
6FAM- <i>agrB</i> pro bottom	5' - 6FAM/ TTTATCATTATATTGCCTAAGTGTAGGAAATAAATCTTAAGTGTAAAT -3'
6FAM- <i>sbi</i> pro bottom	5' - 6FAM/ ATGGAAATTAAGTAAATTAAGTAAATGTTAACTAATAGATTAAGTGTAGTG -3'
EMSA primers	Sequence
<i>arlR</i> EMSA fwd	5' - AATTAACAGAATGCCATTAAGTATTAAATTTTG -3'
<i>arlR</i> EMSA rvs	5' - TCACAAATCAATAACCATGTTTAAATTTTTC -3'
<i>splA</i> pro fwd	5' - GTCAATTCATGTAAAAATAATTAATAAAACGTTAATAAG -3'
<i>splA</i> pro rvs	5' - CTAGATACACATTACTATATACATTTTAAATAAAAAATATTAA -3'
<i>efb</i> pro fwd	5' - ATAGATAGCTATATTTTGTCTATATTATAAAGTGTATTATAG -3'
<i>efb</i> pro rvs	5' - TTACATTTATTAAGTTATCCTATTTTATACAACTTTG -3'
<i>saeP</i> EMSA fwd	5' - CAAATTATCTTCTTCAATATTAGTTAAGCGA -3'
<i>saeP</i> EMSA rvs	5' - GATAAGTTAAGTTTAAATGAAGGCAAATAATATG -3'
<i>mecA</i> pro fwd	5' - TAAAAAAGATGATAACACCTTCTACACCTC -3'
<i>mecA</i> pro rvs	5' - AAGACTACATTTGTAGTATATTACAAATGTAGTATTTATG -3'
<i>hly</i> EMSA fwd	5' - TACTCAAAAAACATTTACTTAAAAATATAAATTCGAT -3'
<i>hly</i> EMSA rvs	5' - TTTTATATAGCTTACAACAAAATAGATGCAAAATTG -3'
<i>rot</i> EMSA fwd	5' - GTTTTATGTACTATTATCTTATTCTAAATATTAAGTCTATTG -3'
<i>rot</i> EMSA rvs	5' - TTGCTTTCTATTCAATTGTCAAAGTTG -3'
<i>lukS-PV</i> EMSA fwd	5' - ATAGTAAAAAACACATTTGTAGATATTTTAAACTC -3'
<i>lukS-PV</i> EMSA rvs	5' - TTACATTTTATATTAAACCTTTTAACTTTAATAAAATTAATAT -3'
<i>1975</i> pro fwd	5' - TTTTAAAGAGGATAAAGTATTTAATAATATTAACAAAATC -3'
<i>1975</i> pro rvs	5' - GTATCGACATGTGAATAATATCACAAAAAC -3'