

INSIGHTS INTO THE SaeR/S-MEDIATED PATHOGENESIS OF  
*STAPHYLOCOCCUS AUREUS*

by

Tyler Kenji Nygaard

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

Doctor of Philosophy

in

Immunology and Infectious Diseases

MONTANA STATE UNIVERSITY  
Bozeman, Montana

January 2011

©COPYRIGHT

by

Tyler Kenji Nygaard

2011

All Rights Reserved

APPROVAL

of a dissertation submitted by

Tyler Kenji Nygaard

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency and is ready for submission to the Division of Graduate Education.

Dr. Jovanka M. Voyich-Kane

Approved for the Department of Immunology and Infectious Disease

Dr. Mark Quinn

Approved for the Division of Graduate Education

Dr. Carl A. Fox

## STATEMENT OF PERMISSION TO USE

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this dissertation is allowable only for scholarly purposes, consistent with “fair use” as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this dissertation should be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted “the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part.”

Tyler Kenji Nygaard

January 2011

## ACKNOWLEDGEMENTS

I wish to express my utmost gratitude and appreciation to my mentor and advisor Dr. Jovanka M. Voyich-Kane for her exceptional support and guidance. I am greatly indebted to my graduate committee members Dr. Richard Bessen, Dr. Michele Hardy, Dr. Ben Lei, and Dr. Marisa Pedulla for taking time to provide critical direction and advice throughout my graduate education. In particular I would like to thank Dr. Lei, this thesis would not have been possible without his confidence and support prior to joining the Immunology and Infectious Disease graduate program. I would like to express my thanks to all current and past members of the Voyich-Kane lab including, but not limited to, Kyler Pallister, Shannon Griffith, Mark DeWald, Robert Watkins, Danyelle Long, Elizabeth Erickson, Susan Meyer and Cassie Cooper for contributing to an outstanding research environment. I am also grateful for the support of our collaborating investigators Dr. Alex Horswill, Dr. Victor Torres, Dr. Michael Otto, Dr. Eric Wilson, and Dr. Gunnar Kaufmann. I would also like to give a special thanks to my family and friends for their continued support and understanding.

## TABLE OF CONTENTS

1. INTRODUCTION.....	1
Background.....	1
Emergence of USA300.....	1
Bacterial Two-component Sensory Systems.....	2
The SaeR/S Two-component System.....	3
The Role of <i>hla</i> during <i>S. aureus</i> Pathogenesis.....	4
Literature Cited.....	6
Contribution of Authors and Co-Authors.....	11
Manuscript Information Page.....	12
2. COMMUNITY-ASSOCIATED METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS SKIN INFECTIONS: ADVANCES TOWARD IDENTIFYING THE KEY VIRULENCE FACTORS.....	13
Abstract.....	13
Introduction.....	13
The Emergence of Community-associated Methicillin-resistant <i>Staphylococcus aureus</i> .....	13
The USA300 Genome.....	14
General Virulence Elements of USA300.....	14
Arginine Catabolic Mobile Element.....	15
Pore-forming Leukocidins.....	15
Regulation of Virulence.....	16
Conclusion.....	17
Literature cited.....	17
Contribution of Authors and Co-Authors.....	19
Manuscript Information Page.....	20
3. SaeR BINDS A CONSENSUS SEQUENCE WITHIN VIRULENCE GENE PROMOTERS TO ADVANCE USA300 PATHOGENESIS.....	21
Abstract.....	21
Introduction.....	21
Materials and Methods.....	22
Bacterial strains and culture.....	22
Murine Models of Infection.....	22
Oligonucleotide Microarray and TaqMan Real-time Reverse-transcriptase Polymerase Chain Reaction Analysis.....	22
5'-RACE.....	22
Purification of SaeR and EMSAs.....	22
Results.....	22
Importance of SaeR/S in USA300 Pathogenesis.....	22

## TABLE OF CONTENTS - CONTINUED

Influence of SaeR/S on Transcription of Virulence Factors in vitro.....	26
Identification of Transcript Start Sites of Virulence Genes Influenced by SaeR/S.....	29
Identification of a Conserved Consensus Sequence within Promoter Regions of Genes Influenced by SaeR/S.....	29
Binding of Putative Promoter Regions of USA300 Virulence Factors by SaeR.....	29
Discussion.....	29
Literature cited.....	33
Contribution of Authors and Co-Authors.....	35
Manuscript Information Page.....	36
4. SaeR/S MEDIATED EXPRESSION OF <i>hla</i> PLAYS A PROMINENT ROLE DURING USA300 PATHOGENESIS BY INDUCING T CELL PORE FORMATION AND RAPID PROLIFERATION.....	37
Abstract.....	37
Introduction.....	38
Materials and Methods.....	40
Bacterial Strains and Culture.....	40
Murine Models of Infection.....	41
Transcription and Expression Analysis.....	41
<i>S. aureus</i> Survival in Human Blood and Cytokine Transcription Analysis.....	42
Flow Cytometry.....	43
Results.....	44
<i>In vitro</i> Transcription of <i>hla</i> is dependent upon <i>saeR/S</i> .....	44
SaeR/S-dependent Transcriptional Regulation of <i>hla</i> Promotes Virulence of USA300 during Murine Models of Soft-tissue Infection.....	45
SaeR/S-mediated PMN Plasma Membrane Damage does not Require <i>hla</i> .....	46
Increased Cytokine Production Observed for USA300 $\Delta$ <i>hla</i> Relative to USA300 during Human Blood Infection.....	47
Hla causes Human T Cell Plasma Membrane Damage.....	48
Rapid Human T Cell Proliferation is Promoted by <i>hla</i> .....	49
Discussion.....	50
Literature cited.....	53
5. PROSPECTIVE STUDIES AND CONCLUSIONS.....	64
Defining the SaeR/S Activation Signal.....	64
Investigating the Rapid <i>hla</i> -dependent Proliferation of Human T cells.....	65
Defining the Primary Effector of SaeR/S-mediated PMN Plasma Membrane Damage.....	65
The Role of other SaeR/S Regulated Virulence Genes during USA300 Pathogenesis.....	66
Conclusions.....	67
Literature cited.....	69

LIST OF TABLES

Table	Page
2.1 Primer sequences .....	24

## LIST OF FIGURES

Figure	Page
2.1 Mutiple LukS and LukF subunits are encoded by the USA300 genome.....	16
3.1 Generation of an isogenic <i>saeR/S</i> deletion mutant in USA300 .....	23
3.2 SaeR/S significantly influences the virulence of USA300 during infection.....	26
3.3 Oligonucleotide microarray analysis. ....	27
3.4 Confirmation of oligonucleotide microarray analysis .....	28
3.5 Sequence homology shared within the promoter regions of genes differentially regulated by <i>saeR/S</i> .....	30
3.6 Recombinant SaeR <sup>His</sup> and binding of putative promoter regions of USA300 virulence genes .....	31
4.1 SaeR/S strongly regulates <i>hla</i> during in vitro growth.....	58
4.2 Virulence of USA300 $\Delta$ <i>hla</i> significantly attenuated during murine models of soft-tissue infection .....	59
4.3 The influence of <i>hla</i> during USA300 infection of human blood .....	60
4.4 Hla expression modulates pro-inflammatory cytokine transcript abundance during USA300 infection of human blood .....	61
4.5 Hla causes T cell plasma membrane damage .....	62
4.6 Proliferation of T cells promoted by Hla .....	63
5.1 Human $\alpha$ -defensin induces <i>saeR/S</i> mediated transcription of <i>hla</i> .....	71
5.2 Anti-AIP treatment inhibits USA300 pathogenesis during invasive infection .....	71
5.3 Identification of the <i>nuc</i> transcript start site .....	72

LIST OF FIGURES - CONTINUED

Figure		Page
5.4	SaeR directly binds to the <i>nuc</i> promoter.....	72
5.5	The ArlR/S two component system is important for both superficial and invasive infections caused by USA300.....	73

## ABSTRACT

*Staphylococcus aureus* is an ubiquitous and versatile Gram-positive bacterium capable of producing a wide range of diseases in humans. Within the last ten years there has been a surge of infections caused by hyper-virulent strains of community-associated methicillin-resistant *S. aureus* (CA-MRSA) distinct from previously characterized healthcare-associated MRSA (HA-MRSA). In particular, pulsed-field gel electrophoresis (PFGE) type USA300 has emerged as the prominent circulating strain of CA-MRSA in the United States. How *S. aureus* recognizes environmental signals during infection and responds to promote bacterial survival is clearly an important aspect of pathogenesis but remains incompletely defined. It is believed *S. aureus* recognizes extracellular signals and subsequently alters gene transcription in response via the collective influence of 16 bacterial two-component gene-regulatory systems. This investigation examines the role of one of these two-component systems, *saeR/S*, in USA300 pathogenesis. An isogenic deletion mutant of *saeR/S* generated in USA300 (USA300 $\Delta$ *saeR/S*) demonstrated this two-component system is critical to USA300 infection by upregulating transcription of numerous virulence genes. 5' RACE analysis identified transcript start sites for genes strongly regulated by *saeR/S*. Sequence alignment upstream from transcript start sites revealed a conserved partially palindromic 17 bp sequence specific to promoter regions under strong *saeR/S* regulation and EMSAs confirmed direct binding of recombinant SaeR<sup>His</sup> to this conserved sequence. In particular, strong *saeR/S* dependent transcription of the pore-forming *hla* toxin and robust SaeR<sup>His</sup> binding to the *hla* promoter prompted further studies examining the importance of this virulence gene during USA300 pathogenesis. An isogenic deletion mutant of *hla* in USA300 (USA300 $\Delta$ *hla*) was generated and used to demonstrate *saeR/S*-dependent transcription of *hla* is critical to USA300 pathogenesis during murine models of soft-tissue infection. Though *hla* did not influence *saeR/S*-mediated plasma membrane damage on polymorphonuclear leukocytes, this toxin induced pore formation on human T cells and promoted rapid human T cell proliferation. Collectively this study demonstrates *saeR/S*-dependent transcription of virulence factors is critical to USA300 pathogenesis. In particular, the *saeR/S*-mediated regulation of the potent pore-forming *hla* toxin is a primary effector of USA300 pathogenesis that causes human T cell plasma membrane damage and induces rapid human T cell proliferation.

## INTRODUCTION

### Background

*Staphylococcus aureus* (*S. aureus*) is a common Gram-positive bacterium commensal within the anterior nares of approximately 32% of the population (1). Substantial diversity is exhibited by this organism as approximately 22% of the *S. aureus* genome can differ between strains (2). Though generally presenting as a minor skin abscess, *S. aureus* infection can rapidly progress to more serious and life-threatening disease such as necrotizing fasciitis, necrotizing pneumonia, septicemia, toxic shock syndrome, and infective endocarditis. Invasive *S. aureus* infection is often fatal in the absence of effective antibiotic therapy, indicated by mortality rates for *S. aureus* bacteremia exceeding 80% in 1941 (3). Emerging strains of methicillin-resistant *S. aureus* (MRSA) have become a major cause of human morbidity and mortality throughout the world, demonstrated by a recent survey estimating 18,650 deaths associated with invasive MRSA infection during 2005 in the United States alone (4). The recent identification of at least two separate *S. aureus* lineages resistant to vancomycin (5-7), 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 Emergence of USA300

Within the last ten years there has been a surge of infections caused by hyper-virulent strains of community-associated methicillin-resistant *S. aureus* (CA-MRSA) distinct from

previously characterized healthcare-associated MRSA (HA-MRSA) (8-11). The emergence of a hyper-virulent CA-MRSA clone identified as USA300 by pulsed-field gel electrophoresis (PFGE) is largely responsible for the observed increase in *S. aureus* infections in the United States (4;12;13). USA300 has replaced the dominant strain of CA-MRSA during the turn of the century, PFGE type USA400, and is now the single leading cause of soft-tissue infections presented at emergency departments across the United States (14;15). USA300 has raised concern because of its ability to establish severe life-threatening infections in young, healthy individuals and for causing disease not previously associated with *S. aureus* infection (16-18). Recent surveys indicate USA300 has become resident within many healthcare settings (4;19;20) and exhibits increased antibiotic resistance once established within the hospital environment (21). The mechanisms responsible for the enhanced virulence observed for USA300 relative to other *S. aureus* strains are not entirely clear and are a major focus of this investigation.

### Bacterial Two-component Sensory Systems

The USA300 genome encodes a diverse assortment of virulence factors that promote survival and the ability to express sets of these factors in response to specific environmental cues is likely essential for pathogenesis (13;16;22-24). It is believed bacterial recognition of environmental signals and subsequent alterations in gene transcription are directed by two-component gene-regulatory systems (25-28). Bacterial two-component systems minimally consist of a transmembrane histidine kinase sensor and an intercellular response regulator (29;30). Upon recognizing a specific environmental signal, the transmembrane histidine kinase sensor becomes autophosphorylated at a histidine residue on the intracellular domain.

The activated histidine kinase sensor then transfers this phosphate group to the aspartate residue of the intercellular response regulator. Following activation, the intercellular response regulator of most two-component systems then binds to the bacterial genome to alter gene transcription. However, response regulators have also been shown to possess enzymatic activity, to directly bind RNA, as well as interact with other intercellular proteins to modify gene expression post-transcriptionally (30). In *S. aureus*, it is thought the concerted influence of 16 two-component systems identified by sequence homology direct gene expression in response to various environmental settings encountered during the course of disease (25;28). This investigation characterizes one of these two-component gene-regulatory systems, the *Staphylococcus aureus* exoprotein two-component signal transduction system (SaeR/S), as a major component of *S. aureus* pathogenesis *in vivo*.

#### The SaeR/S Two-component System

A strong upregulation of *saeR/S* following human polymorphonuclear leukocyte (PMN) phagocytosis instigated studies examining the importance of this two-component system during infections caused by *S. aureus* (31). Preliminary studies with the prototypic CA-MRSA strain USA400 demonstrated this two-component system is critical to pathogenesis during murine models of invasive infection and survival in human blood (32). Transcriptional analysis indicated *saeR/S* mediates USA400 pathogenesis by altering transcription of many genes implicated in *S. aureus* virulence. Using an isogenic deletion mutant of *saeR/S* in USA300, this investigation demonstrates this two component system is critical to USA300 pathogenesis during both superficial and invasive infections by directly upregulating the transcription of numerous virulence factors that directly interact with host

proteins to damage host cells, manipulate the host immune response, and improve *S. aureus* adherence to host tissue. In particular, this study illustrates the *saeR/S* two component system has a major influence on the transcriptional regulation of  $\alpha$ -toxin (*hla*), a prominent *S. aureus* virulence factor.

### The Role of *hla* during *S. aureus* Pathogenesis

The *S. aureus* genome encodes an assortment of pore forming toxins with specificity towards different host cell types (24;33-35). These include *hla*,  $\delta$ -toxin, phenol-soluble modulins,  $\gamma$ -hemolysin, the Panton-Valentine Leukocidin, the LukD/E leukocidin, and the recently characterized LukA/B leukocidin. Expression of these toxins can vary dramatically between *S. aureus* isolates. For example, *hla* is highly conserved between *S. aureus* strains while the Panton-Valentine leukocidin is common only among CA-MRSA isolates and rarely expressed by HA-MRSA (35;36). Though SaeR/S influenced the transcription of several of these toxins, including the Panton-Valentine leukocidin, LukA/B, and  $\gamma$ -hemolysin, this two-components system had the most robust regulatory effect on *hla* transcription (37).

The *hla* gene encodes a potent hexameric toxin capable of producing pores in a variety of cell types including rabbit erythrocytes, human lymphocytes, human keratinocytes, and human endothelial cells (38-42). USA300 expresses Hla at higher levels during human infection than *in vitro* growth (43) and recent evidence suggests an overall increased expression of *hla* in USA300 is largely responsible for the virulence of this strain during animal models of infection (44-48). This toxin is highly conserved among *S. aureus* isolates and recent work has demonstrated the efficacy of immunization strategies targeting Hla (45;46;49). However, the primary *in vivo* regulator of *hla* and the mechanisms by which this

toxin promotes *S. aureus* virulence have remained unclear. Strong transcriptional influence of SaeR/S on *hla* transcription and robust binding of recombinant SaeR<sup>His</sup> to the *hla* promoter prompted studies exploring the importance of *hla* on SaeR/S-mediated *S. aureus* pathogenesis (37). This research illustrates SaeR/S strongly mediates transcriptional regulation of *hla* and this toxin plays a significant role during murine soft-tissue infections caused by USA300. Though Hla was not required for SaeR/S mediated PMN plasma membrane damage, deletion of *hla* from USA300 during infection of human blood resulted in an exaggerated pro-inflammatory cytokine transcription profile. Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) treated with USA300 supernatants demonstrated Hla-dependent human T cell plasma membrane damage. Further, Hla promoted a rapid expansion of human T cells following exposure to USA300 supernatants. Collectively, this investigation demonstrates Hla is a primary effector of the SaeR/S two component system and acts by manipulating the T cell response to advance USA300 pathogenesis.

Literature Cited

- (1) Kuehnert MJ, Kruszon-Moran D, Hill HA, et al. Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001-2002. *J Infect Dis* 2006 Jan 15;193(2):172-9.
- (2) Fitzgerald JR, Sturdevant DE, Mackie SM, Gill SR, Musser JM. Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci U S A* 2001 Jul 17;98(15):8821-6.
- (3) Skinner D and Keefer CS. Significance of bacteremia caused by *Staphylococcus aureus*. *Arch Inter Med* 1941;68:851-75.
- (4) Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 2007 Oct 17;298(15):1763-71.
- (5) Graber CJ, Wong MK, Carleton HA, Perdreau-Remington F, Haller BL, Chambers HF. Intermediate vancomycin susceptibility in a community-associated MRSA clone. *Emerg Infect Dis* 2007 Mar;13(3):491-3.
- (6) Howe RA, Monk A, Wootton M, Walsh TR, Enright MC. Vancomycin susceptibility within methicillin-resistant *Staphylococcus aureus* lineages. *Emerg Infect Dis* 2004 May;10(5):855-7.
- (7) Sakoulas G, Moellering RC, Jr. Increasing antibiotic resistance among methicillin-resistant *Staphylococcus aureus* strains. *Clin Infect Dis* 2008 Jun 1;46 Suppl 5:S360-S367.
- (8) Naimi TS, LeDell KH, Como-Sabetti K, et al. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA* 2003 Dec 10;290(22):2976-84.
- (9) Okuma K, Iwakawa K, Turnidge JD, et al. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J Clin Microbiol* 2002 Nov;40(11):4289-94.
- (10) Vandenesch F, Naimi T, Enright MC, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* 2003 Aug;9(8):978-84.

- (11) Zetola N, Francis JS, Nuernberger EL, Bishai WR. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging threat. *Lancet Infect Dis* 2005 May;5(5):275-86.
- (12) Chambers HF. The changing epidemiology of *Staphylococcus aureus*? *Emerg Infect Dis* 2001 Mar;7(2):178-82.
- (13) Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol* 2008 Jun 26.
- (14) Johnson JK, Khoie T, Shurland S, Kreisel K, Stine OC, Roghmann MC. Skin and soft tissue infections caused by methicillin-resistant *Staphylococcus aureus* USA300 clone. *Emerg Infect Dis* 2007 Aug;13(8):1195-200.
- (15) Moran GJ, Krishnadasan A, Gorwitz RJ, et al. Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med* 2006 Aug 17;355(7):666-74.
- (16) Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* 2008 Jun 1;46 Suppl 5:S350-S359.
- (17) Miller LG, Perdreau-Remington F, Rieg G, et al. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N Engl J Med* 2005 Apr 7;352(14):1445-53.
- (18) Sifri CD, Park J, Helm GA, Stemper ME, Shukla SK. Fatal brain abscess due to community-associated methicillin-resistant *Staphylococcus aureus* strain USA300. *Clin Infect Dis* 2007 Nov 1;45(9):e113-e117.
- (19) Klevens RM, Morrison MA, Fridkin SK, et al. Community-associated methicillin-resistant *Staphylococcus aureus* and healthcare risk factors. *Emerg Infect Dis* 2006 Dec;12(12):1991-3.
- (20) Seybold U, Kourbatova EV, Johnson JG, et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. *Clin Infect Dis* 2006 Mar 1;42(5):647-56.
- (21) Davis SL, Rybak MJ, Amjad M, Kaatz GW, McKinnon PS. Characteristics of patients with healthcare-associated infection due to SCCmec type IV methicillin-resistant *Staphylococcus aureus*. *Infect Control Hosp Epidemiol* 2006 Oct;27(10):1025-31.
- (22) Diep BA, Gill SR, Chang RF, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 2006 Mar 4;367(9512):731-9.

- (23) Miller JF, Mekalanos JJ, Falkow S. Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* 1989 Feb 17;243(4893):916-22.
- (24) Nygaard TK, Deleo FR, Voyich JM. Community-associated methicillin-resistant *Staphylococcus aureus* skin infections: advances toward identifying the key virulence factors. *Curr Opin Infect Dis* 2008 Apr;21(2):147-52.
- (25) Cheung AL, Bayer AS, Zhang G, Gresham H, Xiong YQ. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 2004 Jan 15;40(1):1-9.
- (26) Groisman EA, Mouslim C. Sensing by bacterial regulatory systems in host and non-host environments. *Nat Rev Microbiol* 2006 Sep;4(9):705-9.
- (27) Laub MT, Goulian M. Specificity in two-component signal transduction pathways. *Annu Rev Genet* 2007;41:121-45.
- (28) Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* 2003 Jun;48(6):1429-49.
- (29) Krell T, Lacal J, Busch A, Silva-Jimenez H, Guazzaroni ME, Ramos JL. Bacterial sensor kinases: diversity in the recognition of environmental signals. *Annu Rev Microbiol* 2010 Oct 13;64:539-59.
- (30) Galperin MY. Diversity of structure and function of response regulator output domains. *Curr Opin Microbiol* 2010 Apr;13(2):150-9.
- (31) Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* 2005 Sep 15;175(6):3907-19.
- (32) Voyich JM, Vuong C, Dewald M, et al. The SaeR/S Gene Regulatory System Is Essential for Innate Immune Evasion by *Staphylococcus aureus*. *J Infect Dis* 2009 Apr 17.
- (33) Graves SF, Kobayashi SD, Deleo FR. Community-associated methicillin-resistant *Staphylococcus aureus* immune evasion and virulence. *J Mol Med* 2010 Feb;88(2):109-14.
- (34) Kobayashi SD, Deleo FR. An update on community-associated MRSA virulence. *Curr Opin Pharmacol* 2009 Oct;9(5):545-51.
- (35) Otto M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol* 2010 Oct 13;64:143-62.

- (36) Boyle-Vavra S, Daum RS. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab Invest* 2007 Jan;87(1):3-9.
- (37) Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 2010 Jan 15;201(2):241-54.
- (38) Grimminger F, Rose F, Sibelius U, et al. Human endothelial cell activation and mediator release in response to the bacterial exotoxins *Escherichia coli* hemolysin and staphylococcal alpha-toxin. *J Immunol* 1997 Aug 15;159(4):1909-16.
- (39) Hildebrand A, Pohl M, Bhakdi S. *Staphylococcus aureus* alpha-toxin. Dual mechanism of binding to target cells. *J Biol Chem* 1991 Sep 15;266(26):17195-200.
- (40) Jonas D, Walev I, Berger T, Liebetrau M, Palmer M, Bhakdi S. Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect Immun* 1994 Apr;62(4):1304-12.
- (41) Valeva A, Walev I, Pinkernell M, et al. Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. *Proc Natl Acad Sci U S A* 1997 Oct 14;94(21):11607-11.
- (42) Walev I, Martin E, Jonas D, et al. Staphylococcal alpha-toxin kills human keratinocytes by permeabilizing the plasma membrane for monovalent ions. *Infect Immun* 1993 Dec;61(12):4972-9.
- (43) Loughman JA, Fritz SA, Storch GA, Hunstad DA. Virulence Gene Expression in Human Community-Acquired *Staphylococcus aureus* Infection. *J Infect Dis* 2009 Feb 1;199(3):294-301.
- (44) Bubeck WJ, Bae T, Otto M, Deleo FR, Schneewind O. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat Med* 2007 Dec;13(12):1405-6.
- (45) Bubeck WJ, Schneewind O. Vaccine protection against *Staphylococcus aureus* pneumonia. *J Exp Med* 2008 Feb 18;205(2):287-94.
- (46) Kennedy AD, Bubeck WJ, Gardner DJ, et al. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. *J Infect Dis* 2010 Oct 1;202(7):1050-8.
- (47) Li M, Cheung GY, Hu J, et al. Comparative Analysis of Virulence and Toxin Expression of Global Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strains. *J Infect Dis* 2010 Nov 4.

- (48) Montgomery CP, Boyle-Vavra S, Adem PV, et al. Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. *J Infect Dis* 2008 Aug 15;198(4):561-70.
- (49) Ragle BE, Bubeck WJ. Anti-alpha-hemolysin monoclonal antibodies mediate protection against *Staphylococcus aureus* pneumonia. *Infect Immun* 2009 Jul;77(7):2712-8.

CONTRIBUTION OF AUTHORS AND CO-AUTHORS

Chapter 2: COMMUNITY-ASSOCIATED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* SKIN INFECTIONS: ADVANCES TOWARD IDENTIFYING THE KEY VIRULENCE FACTORS

Author: Tyler K. Nygaard

Contributions: Collected/analyzed references and wrote majority of manuscript.

Co-author: Dr. Frank R. DeLeo

Contributions: Wrote last paragraph of section titled "Pore-forming Leukocidins", generated Figure 1, contributed to editing of final manuscript.

Co-author: Dr. Jovanka M. Voyich

Contributions: Wrote manuscript abstract, edited and commented on the manuscript at all stages.

MANUSCRIPT INFORMATION PAGE

COMMUNITY-ASSOCIATED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* SKIN INFECTIONS: ADVANCES TOWARD IDENTIFYING THE KEY VIRULENCE FACTORS

Tyler K. Nygaard, Frank R. DeLeo, and Jovanka M. Voyich

Journal Name: Current Opinions in Infectious Disease

Status of Manuscript:

- Prepared for submission to a peer-reviewed journal
- Officially submitted to a peer-reviewed journal
- Accepted by a peer-reviewed journal
- Published in a peer-reviewed journal

Publisher: Wolters Kluwer Health, Lippincott Williams & Wilkins Publishing

Issue in which manuscript appears in: April 2008, Volume 21, Issue 2: 147-152.

# Community-associated methicillin-resistant *Staphylococcus aureus* skin infections: advances toward identifying the key virulence factors

Tyler K. Nygaard<sup>a</sup>, Frank R. DeLeo<sup>b</sup> and Jovanka M. Voyich<sup>a</sup>

<sup>a</sup>Department of Veterinary Molecular Biology, Montana State University, Bozeman and <sup>b</sup>Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, NIH, Hamilton, Montana, USA

Correspondence to Jovanka M. Voyich, PhD, Veterinary Molecular Biology Department, Molecular Biosciences Building, 960 Technology Blvd., Bozeman, MT 59718, USA  
Tel: +1 406 994 7184; e-mail: jovanka@montana.edu

**Current Opinion in Infectious Diseases** 2008, 21:147–152

## Purpose of review

In recent years there has been an increase in the incidence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections in healthy individuals, the cause of which is largely unknown. CA-MRSA primarily causes skin and soft-tissue infections but certain strains are also associated with unusually severe pathology. The purpose of this review is to provide a critical analysis of our current knowledge of virulence factors contributing to skin and soft-tissue infections caused by CA-MRSA.

## Recent findings

Isolates classified as pulsed-field gel electrophoresis type USA300 have emerged as the predominant CA-MRSA genotype and in most geographic areas account for 97% or more of CA-MRSA infections. Recent key studies, such as those reporting the complete genome sequence of USA300, and the discovery of cytolytic peptides that contribute significantly to CA-MRSA virulence, lead the way for future investigations.

## Summary

Although we have only a cursory understanding of the molecular mechanisms of CA-MRSA virulence, studies using clinically relevant CA-MRSA isolates are beginning to identify virulence determinants specific to this pathogen. Identifying CA-MRSA virulence determinants and the concerted regulation of these factors will foster development of vaccines and therapeutics designed to control CA-MRSA skin infections.

## Keywords

CA-MRSA, skin infection, virulence

Curr Opin Infect Dis 21:147–152  
© 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins  
0951-7375

## Introduction

*Staphylococcus aureus* is one of the most common causes of skin and soft-tissue infections worldwide. These infections typically present as minor abscesses but may progress to severe life-threatening disease such as necrotizing fasciitis. Recently there has been a dramatic increase in the number of skin and soft-tissue infections caused by methicillin-resistant *S. aureus* (MRSA) strains originating from outside healthcare settings. The mechanisms that account for the increased prevalence and virulence of these community-associated MRSA (CA-MRSA) isolates are not completely understood. The objective of this review is to provide a critical assessment of our current knowledge of virulence factors contributing to CA-MRSA skin and soft-tissue infections.

## The emergence of community-associated methicillin-resistant *Staphylococcus aureus*

*S. aureus* is a ubiquitous Gram-positive bacteria and a commensal organism of humans, colonizing the anterior nares of approximately 32% of the population [1]. This

versatile pathogen is responsible for a wide variety of disease, including skin infections, bacteremia, endocarditis, sepsis, necrotizing fasciitis, pneumonia, and toxic-shock syndrome. Our capacity to treat *S. aureus* disease has been increasingly challenged by emergence and re-emergence of antibiotic-resistant isolates, especially those that are multidrug resistant. Antibiotic-resistant *S. aureus* has surfaced as a major cause of human mortality, as an estimated 18 650 deaths were associated with invasive MRSA infections during 2005 in the United States alone [2•]. Approximately 14% of these deadly infections were attributed to CA-MRSA [2•].

Resistance to methicillin is provided by the *mecA* gene encoded within the staphylococcal cassette chromosome *mec* (SCC*mec*), which has been horizontally transferred multiple independent times from related staphylococcal species into methicillin-susceptible precursor strains [3]. To date, five primary SCC*mec* types have been identified with differing gene content and organization. Prior to the 1980s, MRSA was primarily associated with healthcare facilities and carried SCC*mec* types I, II, or III. Recently

there has been a global surge in CA-MRSA strains with an epidemiology distinct from hospital-associated MRSA (HA-MRSA) [4,5]. These emerging CA-MRSA strains have acquired SCC*mec* types IV or V, which distinguish them from earlier HA-MRSA strains. It is apparent that CA-MRSA has arisen multiple times from integration events of these different SCC*mec* types into methicillin-susceptible *S. aureus* lineages as opposed to having evolved from previously characterized HA-MRSA strains [3,6]. Current reports indicate hypervirulent USA300 is replacing some of the HA-MRSA in the healthcare setting [2<sup>••</sup>,7]. Furthermore, it appears that strains of CA-MRSA exhibit increased antibiotic resistance once established in the hospital environment [8]. Given the influx of USA300 into healthcare settings, the current MRSA differentiation scheme based on community or healthcare origins may soon be obsolete.

Though primarily manifesting as skin and soft-tissue infections, CA-MRSA can also cause a number of life-threatening invasive ailments including several not previously associated with *S. aureus* infection, such as necrotizing pneumonia and necrotizing fasciitis [9–13]. Predisposing risk factors to CA-MRSA skin and soft-tissue infections include trauma, insect bites, injection drug use, and poor hygiene [14]. CA-MRSA has gained notoriety, however, because of its ability to cause infection in otherwise healthy individuals without identifiable risk factors and spread rapidly. Outbreaks of CA-MRSA infections have been reported in athletes, prisoners, military personnel, and children [14,15]. Clearly, CA-MRSA strains have acquired advantages for transmission and virulence relative to HA-MRSA strains. These advantages, however, remain incompletely defined.

Strains classified as pulsed-field gel electrophoresis (PFGE) type USA300 (USA300) are particularly adept at causing skin and soft-tissue infections [16,17]. A recent report identified MRSA as the leading cause of infections presenting to emergency departments across the United States, accounting for approximately 59% of skin and soft-tissue infections [17]. USA300 was identified as the causative agent in 97% or more of these infections [17]. Additionally, USA300 has been shown to cause necrotizing fasciitis, a life-threatening deep-tissue infection atypical of *S. aureus* pathogenesis [12]. The ability of USA300 to cause skin and soft-tissue infections is likely attributed to virulence factors encoded within the USA300 genome and the concerted regulation of these factors by sensory two-component systems.

### The USA300 genome

Over 20% of the *S. aureus* genome varies among strains [3] and virulence factors horizontally transferred by mobile genetic elements contribute to the extensive genetic

diversity exhibited by this organism [18,19<sup>••</sup>,20,21]. As with other *S. aureus* strains, many known and putative virulence molecules as well as some antibiotic resistance genes are located within extrachromosomal plasmids and mobile genetic elements of the USA300 genome [19<sup>••</sup>]. For the purposes of this review, reference to the USA300 genome and its elements refers specifically to the first sequenced strain as reported by Diep *et al.* [19<sup>••</sup>]. Antibiotic resistance is attributed in part to plasmids found in USA300 (e.g., penicillin-resistance), while function of one of the plasmids (pUSA01) remains unclear. Mobile genetic elements include a novel *S. aureus* pathogenicity island 5 (SaPI5) that encodes pyrogenic toxins closely resembling superantigens SEQ and SEK, prophage  $\phi$ SA3usa encoding both staphylokinase and a chemotaxis inhibiting protein, and prophage  $\phi$ SA2usa encoding the Panton–Valentine leukocidin (PVL). USA300 typically carries two genetic cassettes similar to those found in *S. epidermidis*: SCC*mec* IV that confers methicillin-resistance and the arginine catabolic mobile element (ACME) encoding an arginine deiminase pathway and a putative oligopeptide permease operon.

### General virulence elements of USA300

The USA300 genome contains numerous genes common to other *S. aureus* isolates that contribute to the survival of this organism. These genes include a bacteriocin operon that may improve the ability of USA300 to outcompete other bacteria, multiple cell surface adhesion proteins, as well as numerous extracellular proteases and lipases [19<sup>••</sup>,21]. Relative to other strains of *S. aureus*, USA300 encodes an abundance of cell surface fibrinogen binding proteins, namely Ebh, FnbA, and FnbB [21]. There is evidence indicating *S. aureus* cell surface fibrinogen-binding proteins are directly involved in the internalization of *S. aureus* by nonphagocytic host cells [22] and their collective contribution to skin and soft-tissue infections may be significant.

SaPI5 encodes two genes with 98% amino acid identity to previously identified *S. aureus* enterotoxins, SEQ and SEK [18]. Recombinant SEK has been shown to be superantigenic [23], but unlike other pyrogenic toxin superantigens, neither SEQ nor SEK appear emetic [18]. The USA300 genome also encodes other molecules annotated as exotoxins previously identified in other strains of *S. aureus* and some may possess superantigenic properties [21]. Superantigens bind outside the peptide binding cleft of major histocompatibility complex class II molecules of antigen-presenting cells and crosslink them to the  $\beta$ -chain variable region of specific T-cell receptors. Cross linking induces T-cell activation independent of peptide presentation in the peptide binding cleft, leading to proliferation of polyclonal T-cell populations and subsequent immune dysfunction that can eventually lead to multiple organ

failure. In essence, superantigens manipulate the adaptive immune response to encourage proliferation of nonspecific T-cells, thereby hindering proliferation of T-cells specific to *S. aureus* peptides [24]. The individual and concerted role of SEQ, SEK, and other exotoxins produced by USA300 has not been directly tested in skin and soft-tissue infection models. Given the extraordinary tissue damage incurred with staphylococcal necrotizing fasciitis, however, future studies may define a role for superantigens in this dramatic disease presentation.

The USA300 genome contains prophage  $\phi$ SA3usa that encodes staphylokinase (SAK) and a chemotaxis inhibitor protein (CHIP) that hinder the host immune response. SAK has been shown to bind  $\alpha$ -defensins and inactivate their bactericidal properties [24,25]. SAK also binds and activates plasminogen to produce plasmin, a potent protease that cleaves C3b and IgG bound to the *S. aureus* cell surface thereby hindering polymorphonuclear leukocyte (PMN) phagocytosis. Alternatively, CHIP acts to impede PMN chemotaxis by associating with the formyl peptide receptor or the C5a receptor on the surface of PMNs to prevent their migration during inflammation [24]. Although expression of SAK has been associated with noninvasive *S. aureus* infections [25], the direct influence of SAK and CHIP in CA-MRSA skin and soft-tissue infections remains to be determined.

### Arginine catabolic mobile element

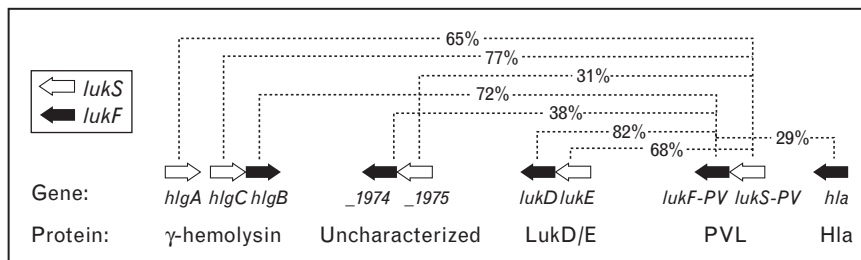
ACME is found primarily in *S. aureus* isolates classified as USA300 and was shown by Diep *et al.* [19<sup>••</sup>] to integrate into the chromosome at the SCCmec attachment site (*orfX*). More recent studies indicate that ACME is found in all USA300 isolates containing SCCmec subtype IVa, but not in other subtypes tested (e.g., SCCmec IVb and IVc, which are sequence variants). Tenover and colleagues [2<sup>••</sup>,26] also identified the element in a small percentage of PFGE type USA100 isolates, a healthcare-associated genotype that has found its way into the community. ACME encodes an arginine deiminase pathway (*arc* cluster), a putative oligopeptide permease operon (Opp-3), and several genes of unknown function [19<sup>••</sup>]. USA300 and other *S. aureus* strains contain an arginine deiminase (ADI) operon in the core genome, but this operon is dissimilar in gene sequence and orientation to the *arc* cluster of ACME [19<sup>••</sup>]. The ADI operon allows *S. aureus* to utilize arginine as an energy source [27]. Examination of the ADI operon in laboratory strains of *S. aureus* indicates this metabolic pathway is critical for survival under anaerobic conditions where arginine is the sole source of energy [27]. Redundancy in the arginine catabolism pathway of USA300 may provide a fitness advantage; expression of multiple ADI pathways could enhance arginine catabolism and further bacterial survival in anaerobic environments rich in arginine, but lacking glucose or other sources of energy.

Oligopeptide permeases of *S. aureus* are important for many general functions, including growth, adhesion to host cells, and expression of virulence determinants [28], and therefore, Opp-3 may contribute to the overall fitness of USA300. Because ACME is common in *S. epidermidis*, a widespread skin commensal, this genetic element may promote transmission or survival of CA-MRSA during skin and soft-tissue infections. Although the specific role of ACME in CA-MRSA pathogenesis has yet to be established, future studies in this area are likely to be revealing.

### Pore-forming leukocidins

PMNs are primary effectors of innate immunity and the ability of *S. aureus* to survive after phagocytosis is thought to contribute significantly to the virulence of this pathogen [29]. A comprehensive study examining the interaction of clinically relevant *S. aureus* strains with PMNs revealed that the most prominent CA-MRSA strains have enhanced survival after phagocytosis compared with HA-MRSA [29]. In addition, these strains are more adept at lysing PMNs following phagocytosis relative to HA-MRSA strains [29]. Comprehensive transcriptome analysis of clinically relevant *S. aureus* strains after PMN phagocytosis revealed that the pathogen undergoes abrupt changes in gene expression profiles [29]. Further, differences in gene expression profiles between clinical strains of *S. aureus* revealed not only the presence or absence of specific virulence factors, but also subtle variations in gene regulation between strains that might explain the increased survival of CA-MRSA following PMN phagocytosis [29]. Molecules that allow *S. aureus* to survive and consequently lyse PMNs following phagocytosis are likely important during skin and soft-tissue infections. Recent studies have examined the importance of different pore-forming leukocidins or other cytolytic molecules produced by CA-MRSA during skin infections using isogenic USA300 mutant strains in mouse models of abscess formation.

Prophage  $\phi$ SA2usa in the USA300 genome encodes PVL, a two-component pore forming leukocidin with cytolytic activity specific for granulocytes, monocytes, and macrophages [30,31]. The role of PVL in *S. aureus* pathogenesis has been the subject of extensive debate [11,30,32]. There is an epidemiological association of SCCmec IV and PVL with CA-MRSA infections [7,18,33], and PVL is not yet common in HA-MRSA isolates. Due to the primary clinical presentation of CA-MRSA as a skin and soft-tissue infection, it was inferred that PVL plays a significant role in pathogenesis of these infections. Mouse models, however, directly testing the influence of PVL during abscess formation, indicate this toxin is not essential for CA-MRSA skin and soft-tissue infections [34<sup>••</sup>]. Specifically, an isogenic PVL deletion mutant strain of USA300 caused dermonecrosis identical to the wild-type

**Figure 1 Multiple LukS and LukF subunits are encoded by the USA300 genome**

Comparison of each *lukS* (white arrows) and *lukF* (black arrows) subunit is presented as percentage amino acid identity (inferred). Amino acid sequence comparisons were made using position-specific iterated BLAST (PSI-BLAST) and the nonredundant protein database at the National Center for Biotechnology Information (NCBI). Encoded proteins are listed below each gene. The leukotoxin listed as an uncharacterized protein is encoded by *SAUSA300\_1975* and *SAUSA300\_1974* (*\_1975* and *\_1974*). Alpha-hemolysin (Hla) is a single component toxin with homology to LukF subunits.

parental strain [34<sup>••</sup>]. Furthermore, the isogenic PVL mutant strain lysed PMNs after phagocytosis at levels indistinguishable from the wild-type strain [34<sup>••</sup>]. Notably, the USA300 genome encodes at least three other pore-forming leukotoxins that have up to 82% identity with PVL subunits,  $\gamma$ -hemolysin (*hlgA*, *hlgB*, and *hlgC*), Luke/D (*lukD* and *lukE*), and an uncharacterized LukS/F homologue (*SAUSA300\_1975* and *SAUSA300\_1974*) (Fig. 1). The role of these molecules in CA-MRSA pathogenesis has yet to be defined.

Wang *et al.* [35<sup>••</sup>] recently used a reverse-genetics approach to discover small (approximately 20 amino acids) cytolytic peptides that play a major role in the pathogenesis of skin and soft-tissue infections caused by CA-MRSA strains. The peptides, related in part to the phenol-soluble modulins (PSM) peptides of *S. epidermidis*, exist as several novel subtypes in *S. aureus* (e.g., PSM $\alpha$ 1-3, PSM $\beta$ 1 and 2) and are encoded by the genomes of prominent HA-MRSA and CA-MRSA; however, they are produced at much higher levels *in vitro* by CA-MRSA strains [35<sup>••</sup>]. The importance of PSMs in skin and soft-tissue infections was tested directly by using isogenic PSM deletion strains of USA300 in mouse models of abscess formation. The mutant strain lacking PSM $\alpha$  peptides had significantly decreased ability to cause skin lesions relative to the parental USA300 strain, clearly demonstrating the importance of this PSM subtype in CA-MRSA skin and soft-tissue infections [35<sup>••</sup>]. Inasmuch as these novel PSMs contribute significantly to the cytolytic activity of CA-MRSA by destroying PMNs and monocytes [35<sup>••</sup>], the peptides promote CA-MRSA pathogenesis by directly assaulting primary innate immune defenses.

### Regulation of virulence

*S. aureus* is a highly adaptable organism capable of colonizing and causing infection at multiple sites within the human host. How this pathogen overcomes different environmental challenges encountered during pathogen-

esis is not entirely understood, but its ability to sense and respond to different environmental conditions via sensory/regulatory systems is clearly important. Analyses of *S. aureus* genomes has identified 16 putative two-component regulatory systems, several of which have been shown to regulate virulence factors [36]. The quorum sensing accessory gene regulator (Agr) system has been shown to be important during abscess formation in mouse models [37<sup>••</sup>,38]. When *S. aureus* reach a critical density during growth, one of four strain specific autoinducing peptides (AIPs) are produced and activate the Agr system, thereby leading to altered gene transcription. Interference of the Agr machinery can alter production of virulence molecules, such as surface adhesins and secreted toxins, and causes a marked reduction of abscess formation in mouse models [38]. The therapeutic potential of Agr interference has recently been explored using antibodies specific to a *S. aureus* AIP [37<sup>••</sup>]. These antibodies act by specifically binding to AIPs to inhibit their signaling ability. Using a mouse model of abscess formation, Park *et al.* [37<sup>••</sup>] showed that treatment of the AIP specific antibody to *S. aureus* culture prior to infection significantly reduces abscess formation. These studies did not involve clinically relevant strains of *S. aureus*, but they offer promise towards novel therapeutic treatments against CA-MRSA skin and soft-tissue infections.

Although the Agr quorum sensing system has been well studied, work directed to understand the roles of other two-component systems and their concerted influence on gene expression is relatively new or underway [36,39,40<sup>•</sup>]. A complete understanding of these sensory/regulatory systems will help to identify specific environmental cues that determine gene regulation as well as define combinations of virulence factors that promote CA-MRSA survival in different host tissues. Such information may prove invaluable toward revealing novel treatment strategies for CA-MRSA infections as well as clarifying which genes are important for skin and soft-tissue pathogenesis.

## Conclusion

Within the last decade there has been a surge of hyper-virulent MRSA strains that cause disease outside of the healthcare setting. Notably, MRSA isolates classified as PFGE type USA300 have emerged as the primary cause of skin and soft-tissue infections in the United States. Given the diminishing effectiveness of our current antibiotics, it is clear we must improve our understanding of molecular mechanisms fundamental to CA-MRSA virulence to control disease caused by this potentially life-threatening pathogen. Putative virulence factors required for skin and soft-tissue infections, such as ACME, and novel virulence molecules such as PSM peptides, are encoded within the genome of USA300, a genotype now epidemic in the United States. Because the regulation of virulence is under the collective influence of sensory/regulatory systems, a full comprehension of these gene-regulation mechanisms (in clinically relevant strains of CA-MRSA) will refine our understanding of factors required to cause disease and may facilitate development of novel therapeutics.

## Acknowledgements

This work was supported by NIH-NRRI grant P20RR020185 (T.K.N. and J.M.V.), P20RR16455-07 (T.K.N.) and the Intramural Program of the National Institutes of Allergy and Infectious Diseases, NIH (F.R.D.).

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 202).

- 1 Kuehnert MJ, Kruszon-Moran D, Hill HA, *et al.* Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001-2002. *J Infect Dis* 2006; 193:172-179.
- 2 Klevens RM, Morrison MA, Nadle J, *et al.* Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 2007; 298:1763-1771.
- This multicenter study provided a comprehensive overview of the incidence, burden and distribution of invasive MRSA disease in the United States. The study highlighted the overwhelming emergence of CA-MRSA strain USA300 and identified a potential health disparity in the observed increase in invasive MRSA infection among blacks.
- 3 Fitzgerald JR, Sturdevant DE, Mackie SM, *et al.* Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci U S A* 2001; 98:8821-8826.
- 4 Appelbaum PC. Microbiology of antibiotic resistance in *Staphylococcus aureus*. *Clin Infect Dis* 2007; 45 (Suppl 3):S165-S170.
- 5 Okuma K, Iwakawa K, Turnidge JD, *et al.* Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J Clin Microbiol* 2002; 40:4289-4294.
- 6 Enright MC, Robinson DA, Randle G, *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci U S A* 2002; 99:7687-7692.
- 7 Moroney SM, Heller LC, Arbuckle J, *et al.* *Staphylococcal cassette chromosome mec* and Panton-Valentine leukocidin characterization of methicillin-resistant *Staphylococcus aureus* clones. *J Clin Microbiol* 2007; 45:1019-1021.
- 8 Davis SL, Rybak MJ, Amjad M, *et al.* Characteristics of patients with healthcare-associated infection due to SCCmec type IV methicillin-resistant *Staphylococcus aureus*. *Infect Control Hosp Epidemiol* 2006; 27:1025-1031.
- 9 Adem PV, Montgomery CP, Husain AN, *et al.* *Staphylococcus aureus* sepsis and the Waterhouse-Friderichsen syndrome in children. *N Engl J Med* 2005; 353:1245-1251.
- 10 Kravitz GR, Dries DJ, Peterson ML, Schlievert PM. Purpura fulminans due to *Staphylococcus aureus*. *Clin Infect Dis* 2005; 40:941-947.
- 11 Lowy FD. Secrets of a superbug. *Nat Med* 2007; 13:1418-1420.
- 12 Miller LG, Perdreau-Remington F, Rieg G, *et al.* Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N Engl J Med* 2005; 352:1445-1453.
- 13 Sifri CD, Park J, Helm GA, *et al.* Fatal brain abscess due to community-associated methicillin-resistant *Staphylococcus aureus* strain USA300. *Clin Infect Dis* 2007; 45:e113-e117.
- 14 Elston DM. Community-acquired methicillin-resistant *Staphylococcus aureus*. *J Am Acad Dermatol* 2007; 56:1-16.
- 15 Kazakova SV, Hageman JC, Matava M, *et al.* A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. *N Engl J Med* 2005; 352:468-475.
- 16 Johnson JK, Khoie T, Shurland S, *et al.* Skin and soft tissue infections caused by methicillin-resistant *Staphylococcus aureus* USA300 clone. *Emerg Infect Dis* 2007; 13:1195-1200.
- 17 Moran GJ, Krishnadasan A, Gorwitz RJ, *et al.* Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med* 2006; 355:666-674.
- 18 Diep BA, Carleton HA, Chang RF, *et al.* Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 2006; 193:1495-1503.
- 19 Diep BA, Gill SR, Chang RF, *et al.* Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 2006; 367:731-739.
- This study reported the genome sequence of USA300. The study revealed that USA300 is genetically similar to MRSA strain COL and revealed the presence of arginine catabolic mobile element (ACME) in USA300. The study also emphasized how horizontal transfer from other species of staphylococci may be responsible for significant changes that enhance the increased fitness and pathogenicity of this CA-MRSA strain.
- 20 Goerke C, Wirtz C, Fluckiger U, Wolz C. Extensive phage dynamics in *Staphylococcus aureus* contributes to adaptation to the human host during infection. *Mol Microbiol* 2006; 61:1673-1685.
- 21 Tenover FC, McDougal LK, Goering RV, *et al.* Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. *J Clin Microbiol* 2006; 44:108-118.
- 22 Hauck CR, Ohlsen K. Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. *Curr Opin Microbiol* 2006; 9:5-11.
- 23 Orwin PM, Leung DY, Donahue HL, *et al.* Biochemical and biological properties of *Staphylococcal enterotoxin K*. *Infect Immun* 2001; 69:360-366.
- 24 Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol* 2005; 3:948-958.
- 25 Bokarewa MI, Jin T, Tarkowski A. *Staphylococcus aureus*: Staphylokinase. *Int J Biochem Cell Biol* 2006; 38:504-509.
- 26 Goering RV, McDougal LK, Fosheim GE, *et al.* Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. *J Clin Microbiol* 2007; 45:1981-1984.
- 27 Makhlin J, Kofman T, Borovok I, *et al.* *Staphylococcus aureus* ArcR controls expression of the arginine deiminase operon. *J Bacteriol* 2007; 189:5976-5986.
- 28 Coulter SN, Schwan WR, Ng EY, *et al.* *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Mol Microbiol* 1998; 30:393-404.
- 29 Voyich JM, Braughton KR, Sturdevant DE, *et al.* Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* 2005; 175:3907-3919.
- 30 Boyle-Vavra S, Daum RS. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab Invest* 2007; 87:3-9.
- 31 Narita S, Kaneko J, Chiba J, *et al.* Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. *Gene* 2001; 268:195-206.
- 32 Wardenburg JB, Bae T, Otto M, *et al.* Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat Med* 2007; 13:1405-1406.

- 33** Said-Salim B, Mathema B, Braughton K, *et al.* Differential distribution and expression of Panton-Valentine leucocidin among community-acquired methicillin-resistant *Staphylococcus aureus* strains. *J Clin Microbiol* 2005; 43:3373–3379.
- 34** Voyich JM, Otto M, Mathema B, *et al.* Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J Infect Dis* 2006; 194:1761–1770.
- This study used human neutrophil assays and animal skin and sepsis models to analyze virulence of Panton–Valentine leukocidin (PVL)-positive and PVL-negative CA-MRSA strains as well as isogenic deletion knockouts of *lukS/F-PV* (encoding PVL) in strains USA300 and USA400. Findings from the study demonstrate that PVL is not the major virulence determinant of CA-MRSA.
- 35** Wang R, Braughton KR, Kretschmer D, *et al.* Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 2007; 13:1510–1514.
- This study linked production of cytolytic peptides (PSMs) with enhanced virulence of CA-MRSA. CA-MRSA strains showed increased production of PSMs and isogenic deletion of PSM $\alpha$  (in strains USA400 and USA300) significantly attenuated skin lesions in mice. This study also demonstrated that wild-type PSMs caused significant lysis of human neutrophils.
- 36** Cheung AL, Bayer AS, Zhang G, *et al.* Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 2004; 40:1–9.
- 37** Park J, Jagasia R, Kaufmann GF, *et al.* Infection control by antibody disruption of bacterial quorum sensing signaling. *Chem Biol* 2007; 14:1119–1127.
- This study explored interfering with quorum sensing signaling and subsequent virulence factor production as a means to ameliorate staphylococcal virulence. The study used an antibody specific to the autoinducing peptide (AIP) of the accessory gene regulator *agr* to demonstrate that interfering with the *agr* signaling mechanism disrupts normal *agr* function rendering *S. aureus* less virulent. This observation advances the search for potential novel antistaphylococcal therapeutics.
- 38** Wright JS III, Jin R, Novick RP. Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci U S A* 2005; 102:1691–1696.
- 39** Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* 2003; 48:1429–1449.
- 40** Xiong YQ, Willard J, Yeaman MR, *et al.* Regulation of *Staphylococcus aureus* alpha-toxin gene (*hla*) expression by *agr*, *sarA*, and *sae* in vitro and in experimental infective endocarditis. *J Infect Dis* 2006; 194:1267–1275.
- This study investigated regulation of *hla* expression *in vitro* and *in vivo* using a rabbit experimental infective endocarditis model. Results demonstrate the complexity of staphylococcal regulation of virulence and highlights differences between *in-vitro* and *in-vivo* regulation of virulence.

CONTRIBUTION OF AUTHORS AND CO-AUTHORS

Chapter 3: SaeR BINDS A CONSENSUS SEQUENCE WITHIN VIRULENCE GENE PROMOTERS TO ADVANCE USA300 PATHOGENESIS

Author: Tyler K. Nygaard

Contributions: Conceived the study, designed and performed experiments, collected and analyzed output data, and wrote manuscript.

Co-authors: Kyler B. Pallister, Peter Ruzevich, and Shannon Griffith.

Contributions: Contributed to data collection.

Co-author: Dr. Cuong Vuong

Contributions: Generated USA300 $\Delta$ *saeR/S* used in study

Co-author: Dr. Jovanka M. Voyich

Contributions: Conceived the study and edited/commented on the manuscript at all stages.

MANUSCRIPT INFORMATION PAGE

SaeR BINDS A CONSENSUS SEQUENCE WITHIN VIRULENCE GENE PROMOTERS TO  
ADVANCE USA300 PATHOGENESIS

Tyler K. Nygaard, Kyler B. Pallister, Peter Ruzevich, Shannon Griffith, Cuong Vuong, and Jovanka M.  
Voyich

Journal Name: Journal of Infectious Disease

Status of Manuscript:

Prepared for submission to a peer-reviewed journal

Officially submitted to a peer-reviewed journal

Accepted by a peer-reviewed journal

Published in a peer-reviewed journal

Publisher: University of Chicago Press

Issue in which manuscript appears in: January 2010, Volume 201, Issue 2: 241-254.

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

Tyler K. Nygaard,<sup>1</sup> Kyler B. Pallister,<sup>1</sup> Peter Ruzevich,<sup>1</sup> Shannon Griffith,<sup>1</sup> Cuong Vuong,<sup>2</sup> and Jovanka M. Voyich<sup>1</sup>

<sup>1</sup>Department of Veterinary Molecular Biology, Montana State University–Bozeman, Bozeman; <sup>2</sup>Department of Anesthesiology, University of Massachusetts Medical School, Worcester

This investigation examines the role of the SaeR/S 2-component system in USA300, a prominent circulating clone of community-associated methicillin-resistant *Staphylococcus aureus*. Using a *saeR/S* isogenic deletion mutant of USA300 (USA300Δ*saeR/S*) in murine models of sepsis and soft-tissue infection revealed that this sensory system is critical to pathogenesis of USA300 during both superficial and invasive infection. Oligonucleotide microarray and real-time reverse-transcriptase polymerase chain reaction identified numerous extracellular virulence genes that are 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. Electrophoretic mobility shift assay experiments illustrated direct binding of SaeR<sup>His</sup> to promoter regions containing the conserved consensus sequence. Collectively, the findings of this investigation demonstrate that SaeR/S directly interacts with virulence gene promoters to significantly influence USA300 pathogenesis.

*Staphylococcus aureus* is a prominent bacterial pathogen that is responsible for a wide range of disease in humans [1]. During the past decade, virulent methicillin-resistant *S. aureus* (MRSA) that is distinct from previously characterized hospital-associated MRSA (HA-MRSA) has emerged [2]. Known as “community-associated MRSA” (CA-MRSA) because of apparent origins outside the healthcare setting, these strains are alarming in their ability to cause life-threatening infections in healthy individuals as well as to 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 >18,000 deaths during 2005 in the United States alone [7]. The recent identification of at least 2 separate *S. aureus* lineages that are resistant to vancomycin [8], the antibiotic of choice for the treatment of MRSA infection, emphasizes the necessity of gaining further understanding of the fundamental mechanisms of *S. aureus* pathogenesis to promote novel treatment strategies against this bacteria.

The *S. aureus* genome encodes 16 putative 2-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], but 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, the findings of this investigation demonstrate

Received 13 May 2009; accepted 3 September 2009; electronically published 15 December 2009.

Potential conflicts of interest: none reported.

Presented in part: Annual Wind River Conference on Prokaryotic Biology, 3–7 June, Estes Park, Colorado.

Financial support: National Institutes of Health (NIH; grant NIH-PAR98-072); NIH–National Center for Research Resources (grants P20RR020185 and P20RR16455-07 [to T.K.N. and J.M.V.]); M. J. Murdock Charitable Trust and the Montana State University Agricultural Experimental Station.

Reprints or correspondence: Jovanka M. Voyich, Dept. of Veterinary Molecular Biology, Montana State University–Bozeman, Bozeman, MT 59717 (jovanka@montana.edu).

The Journal of Infectious Diseases 2010;201:241–54

© 2009 by the Infectious Diseases Society of America. All rights reserved.

0022-1899/2010/20102-0011\$15.00

DOI: 10.1093/infdis/jiq170

that 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 toward clearly defining the SaeR/S-mediated transcriptome in *S. aureus*.

## MATERIALS AND METHODS

**Bacterial strains and culture.** *S. aureus* strains were cultured and harvested, as described elsewhere [14–17], at midexponential (ME) and early stationary (ES) growth defined by an OD<sub>600</sub> (ie, optical density measured at 600 nm) of 0.75 or 2.0, respectively. *Escherichia coli* BL21 and Top10 cells (Invitrogen) were grown in Luria-Bertani broth (EMD Chemicals) supplemented with 100 mg/L ampicillin (LBA). *S. aureus* pulse-field gel electrophoresis type USA300 (strain LAC) [18] was used to generate USA300Δ*saeR/S*, as described elsewhere [14, 16, 19] (Figure 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 described elsewhere [14–16]. All animal studies conformed to National Institutes 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. Survival statistics were performed using a log-rank test (Prism software, version 4.0; GraphPad).

**Oligonucleotide microarray and TaqMan real-time reverse-transcriptase polymerase chain reaction analysis.** Oligonucleotide microarray analysis and TaqMan real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis were performed as described elsewhere [14, 15, 17]. For oligonucleotide microarrays, the protocol for Affymetrix prokaryotic target preparation was followed [20] using the standard format hybridized to GeneChip *S. aureus* genome arrays (Affymetrix). Each experiment was performed in triplicate. Real-time RT-PCR was performed using the primers shown in Table 1, for at least 2 separate experiments, with triplicate analysis conducted for each sample.

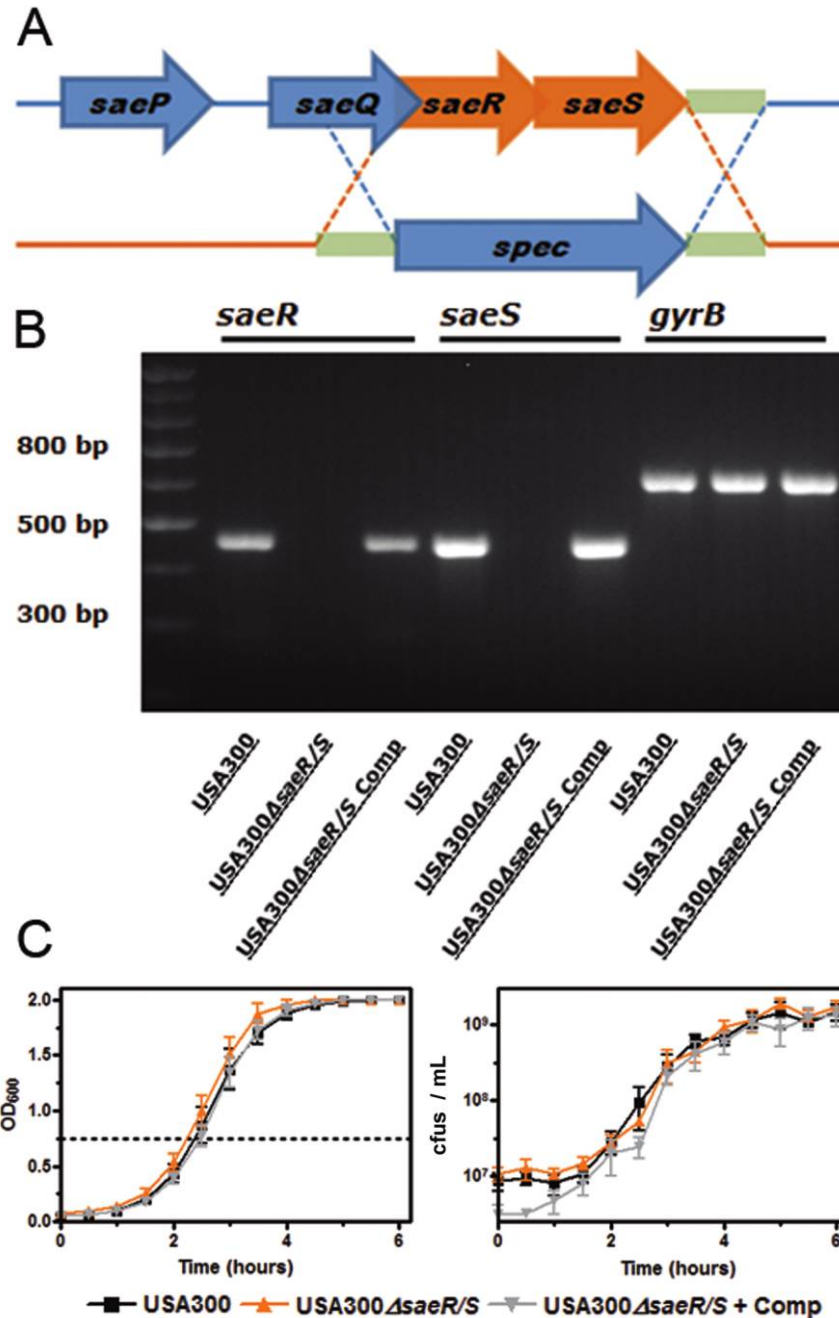
**5'-RACE.** Rapid amplification of 5' complementary DNA ends (5'-RACE) analysis was performed in accordance with previously defined protocols [21], with the use of SuperScript III Reverse Transcriptase (Invitrogen Life Technologies), terminal transferase (New England Biolabs), and gene-specific primers (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 the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence alignment of 5'-RACE products against the

USA300 genome (GenBank accession no. NC\_007793) was performed using nucleotide Basic Local Alignment Search Tool (BLAST) [22]. Multiple sequence alignment was performed using CLUSTALW [23] and a consensus sequence logo derived using WebLogo [24]. Virtual Footprint Bacterial Regulon Analyzer [25] and nucleotide BLAST were used to examine intergenic regions within the USA300 genome.

**Purification of SaeR and EMSAs.** Expression of recombinant SaeR (SaeR<sup>His</sup>) 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 of LBA to an OD<sub>600</sub> of 0.8, induced with 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for 6 more hours. Cells were harvested by centrifugation (4000 g at 4°C for 10 min), resuspended in 20 mmol/L Tris [pH 8] (Tris-HCl), and then lysed via sonication on ice for 15 min. After lysed cells underwent centrifugation (15,000 g at 4°C for 10 min), samples were resuspended in 60 mL of 8 mol/L urea, 0.5 mol/L sodium chloride, and Tris-HCl; sonicated on ice for 15 min; and purified using nickel–nitriloacetic acid affinity (Qiagen) and diethylaminoethyl cellulose anion exchange chromatography (GE Healthcare) with a BioLogic LP Chromatography System (Bio-Rad). Purified SaeR<sup>His</sup> underwent dialysis with 3 L of Tris-HCl and concentrated using a Centricon Plus-20 centrifugal filter (Millipore). Concentration of SaeR<sup>His</sup> was determined using a Micro BCA Protein Assay Kit (Pierce). Electrophoretic mobility shift assays (EMSAs) were performed as described elsewhere [26], using primers and probes in Table 1. SaeR<sup>His</sup> was combined with the indicated quantity of promoter sequence and 500 ng of salmon sperm DNA, when indicated, in 20 μL of binding buffer (100 mmol/L potassium chloride, 1 mmol/L EDTA [ethylenediaminetetraacetic acid], 0.1 mmol/L dithiothreitol, 5% vol/vol glycerol, and 10 ng/mL bovine serum albumin) and incubated at room temperature for 15 min. Samples were run on a 10% native gel and either 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

**Importance of SaeR/S in USA300 pathogenesis.** Because USA300 is a major cause of skin infections, we examined the role of the SaeR/S TCS in this strain by use of a murine model of soft-tissue infection (Figure 2). In contrast to mice inoculated with USA300, mice inoculated with USA300Δ*saeR/S* had 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 (Figure



**Figure 1.** Generation of an isogenic *saeR/S* deletion mutant in USA300. *A*, Construction of USA300Δ*saeR/S* via allelic gene replacement. *B*, Polymerase chain reaction verification of mutant genotype, as described elsewhere [14]. *C*, In vitro growth of USA300, USA300Δ*saeR/S*, and USA300Δ*saeR/S* supplemented with a complement plasmid encoding *saeR/S* (USA300Δ*saeR/S*+ Comp). OD<sub>600</sub>, optical density measured at 600 nm.

2A and 2B), similar to findings presented elsewhere [16, 27, 28]. Surprisingly, nearly one-half of the mice inoculated with USA300Δ*saeR/S* did not display any signs of infection, and no dermonecrosis was observed (Figure 2A and 2B). Moreover, the size of skin infections generated by USA300Δ*saeR/S* was decreased relative to the size of those generated by USA300 (Figure 2C). This experiment was repeated to demonstrate that

the bacterial loads of mice infected with USA300Δ*saeR/S* were the same as those of mice infected with USA300 at day 2 after infection but were lower at day 4 after infection (Figure 2D).

A murine model of sepsis was used to examine the influence of SaeR/S during invasive infection caused by USA300 (Figure 2E). In accordance with previous research [16], USA300 invasive infection was typically found to be lethal within 24 h at

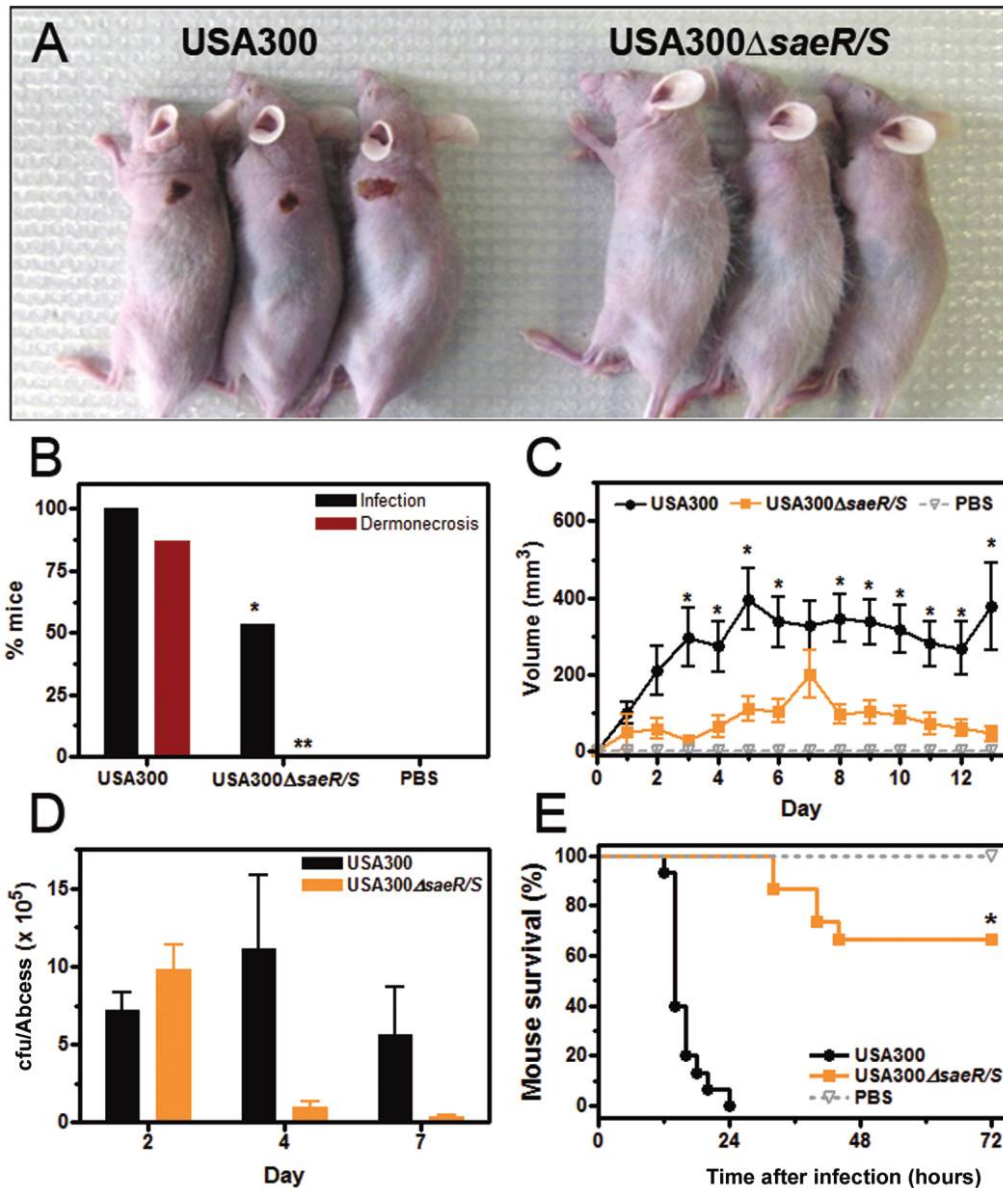
**Table 1. Primer Sequences**

Primer or probe	Sequence
For real-time RT-PCR	
<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'-CGTACATTACAGAGTAGAAAACCTCTCGTAATAC-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'-ATACATCAAACATTACGCGAACAC-3'
<i>sbi</i> rvs	5'-CTGGGTTCTTGCTGTCTTTAAGTG-3'
<i>sbi</i> probe	5'-CAGGTAGCTTTATGTTGCTACAAAAAT-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'-CCTGTTTTACTGTAGTATTGCTTCCA-3'
<i>hla</i> probe	5'-ATGAATCCTGTCGTAATGCCGCAGA-3'
<i>SAUSA300_1975</i> fwd	5'-GCGTCATCATTATCATGTGCAA-3'
<i>SAUSA300_1975</i> rvs	5'-TCTTTCTATTTTGGTCTTGAGAGTCTTT-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'-TGGTCTCAAACAAATGACCCCAATGTAGA-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'-TCCAAACTTTGTTTTTGTGTCTTT-3'
<i>mecA</i> probe	5'-ATCTATAGCGCATTAGAAAA-3'
<i>agrB</i> fwd	5'-GCCATTCTGTGCGACTTA-3'
<i>agrB</i> rvs	5'-GGGCAAATGGCTCTTTGATG-3'
<i>agrB</i> probe	5'-AACCCCTTTTATTATCACACTT-3'
<i>ear</i> fwd	5'-ACCGATCAATAGCGATAGTGTAAAAA-3'
<i>ear</i> rvs	5'-ATGCATCATTTGCGTCAAATCT-3'
<i>ear</i> probe	5'-TGATTTGTCAAGAAAAATCCAATGGAGA-3'
For 5'RACE	
Poly adapter	5'-CACCTGAGCAGAGTGACGAGGACTACGGCTT-CAGCTTTTTTTTTTTTTTTTTT-3'
Poly outer adapter	5'-CACCTGAGCAGAGTGACG-3'
<i>sbi</i> cDNA	5'-GTAATTTGAGTTAGCTTCATATGCTGCTG-3'
<i>sbi</i> PCR	5'-GTCTTTAAGTGATTCAGAGAATACTTCTTG-3'
<i>efb</i> cDNA	5'-TTTGTATTCAAACGAAACTAAGTTGACTGC-3'
<i>efb</i> PCR	5'-AGTACCATCATTGTACTCTACGATATTGTG-3'
<i>agrB</i> cDNA	5'-CCCTAATCGTACTTGCAAAAATTGAATATG-3'
<i>agrB</i> PCR	5'-ATACGTGGCAAACCTGGTCAATTTTATTATC-3'
<i>mecA</i> cDNA	5'-AGCATAAAAATATATACCAAACCCGACAAC-3'
<i>mecA</i> PCR	5'-CCCGACAACACTACAATAAAAATAAGTG-3'
<i>SAUSA300_1975</i> cDNA	5'-GTAACATTACGTTTGTCTTTTGTGAGAC-3'
<i>SAUSA300_1975</i> PCR	5'-CACATGATAATGATGACGCTATTAACAC-3'
<i>lukS</i> cDNA	5'-TTATCGCTACTTGATCTTCTGTTCTTTG-3'

**Table 1. (Continued.)**

Primer or probe	Sequence
<i>lukS</i> PCR	5'-CAATGTTGCAGCTAATAGTCTTTTTTTGAC-3'
<i>hla</i> cDNA	5'-AAGTGACTAAATCACCTGTTTTACTGTAG-3'
<i>hla</i> PCR	5'-TTAATATGGAACCTAGCAATAGTGTGTTG-3'
<i>hlb</i> cDNA	5'-TGGATACAAAACGGTTCGATAACATATAAAC-3'
<i>hlb</i> PCR	5'-CTAACTAACTTCAAATCAGTATCATCTTTC-3'
For <i>SaeR</i> cloning	
<i>saeR</i> up	5'-CACCATGGTGTATCAATTAGAAGTC-3'
<i>saeR</i> down	5'-GATTATGACGTAATGTCTAATTTGTG-3'
For EMSA	
<i>agrB</i> promoter top	5'-ATTTAACAGTTAAGTATTTATTTCTACAGTTAGGCAATATAATGATAAA-3'
<i>agrB</i> promoter bottom	5'-TTTATCATTATATTGCCTAACTGTAGGAAATAAATACTTAACTGTAAAT-3'
<i>sbi</i> promoter top	5'-CACTAGTTAATCTATTAGTTAACATTAGTTAATAATTAGTTAATTTCCAT-3'
<i>sbi</i> promoter bottom	5'-ATGGAAATTAACATAATTATTAACATAATGTTAACTAATAGATTAACTAGTG-3'
<i>hla</i> probe-1 top	5'-GTAATTAATAAATAGAAATAGAGAAAAAGGGTATTAATTATGTTTGGAA-3'
<i>hla</i> probe-1 bottom	5'-TTCCAAACATAATTAATACCCTTTTTCTCTATTCTATTATTAATTTAC-3'
<i>hla</i> probe-2 top	5'-ATAAAAATTAACATATATTAACATAGTGTAATTAATAATAGAAATAGA-3'
<i>hla</i> probe-2 bottom	5'-TCTATTTCTATTATTAATTTACACTAGTTAATATATAGTTAATTTTAT-3'
<i>hla</i> probe-3 top	5'-AAATCAATTAATTAACATTAATAAATAAATAACTATATTAACATAGTG-3'
<i>hla</i> probe-3 bottom	5'-CACTAGTTAATATATAGTTAATTTTATTTAATAGTTAATTAATTGATT-3'
<i>hla</i> probe-4 top	5'-AAAAATACAAATATCTTAGAATTAATCAATTAATTAACATTAATAAAA-3'
<i>hla</i> probe-4 bottom	5'-TTTATTTAATAGTTAATTAATTGATTTAATCTAAGATATTTGATTTTT-3'
6FAM- <i>hla</i> probe-3 top	5'-6FAM/AAATCAATTAATTAACATTAATAAATAAATAACTATATTAAC-TAGTG-3'
6FAM- <i>agrB</i> pro bottom	5'-6FAM/TTTATCATTATATTGCCTAACTGTAGGAAATAAATACTTAACTGT-TAAAT-3'
6FAM- <i>sbi</i> pro bottom	5'-6FAM/ATGGAAATTAACATAATTATTAACATAATGTTAACTAATAGATTAACT-TAGTG-3'
<i>arIR</i> EMSA fwd	5'-AATTAACAGAATGCCATTAACGATTATTAATATTTG-3'
<i>arIR</i> EMSA rvs	5'-TCACAAATCAATAACCATGTTTTAAATTTTTC-3'
<i>spiA</i> pro fwd	5'-GTCAATTCATGTAAAATAATTAATAAAACGTTAATAAG-3'
<i>spiA</i> pro rvs	5'-CTAGATACACATTTACTATATACATTTTAAATAAATAATTTAA-3'
<i>efb</i> pro fwd	5'-ATAGATAGCTATATTTTGTCTATATTATAAAGTGTATATAG-3'
<i>efb</i> pro rvs	5'-TTACATTTATTAAGTTATCCTATTTATACAACTTTTG-3'
<i>saeP</i> EMSA fwd	5'-CAAATTATTCTTTCTCAATATTAGTTAAGCGA-3'
<i>saeP</i> EMSA rvs	5'-GATAAGTTAAGTTTAAATGAAGGCAATAATATG-3'
<i>mecA</i> pro fwd	5'-TAAAAAAGATGATAACACCTTCTACACCTC-3'
<i>mecA</i> pro rvs	5'-AAGACTACATTTGTAGTATATTACAAATGTAGTATTTATG-3'
<i>hlb</i> EMSA fwd	5'-TACTCAAAAAACATTTACTTAAAAATATAAATTCGAT-3'
<i>hlb</i> EMSA rvs	5'-TTTTATATAGCTTACAACAAAATAGATGCAAAATTG-3'
<i>rot</i> EMSA fwd	5'-GTTTTATGTACTATTATCTTATTTCTAAATATTAACCTATTG-3'
<i>rot</i> EMSA rvs	5'-TTGCTTTCTATTCAATTGTCAAAGTTG-3'
<i>lukS</i> -PV EMSA fwd	5'-ATAGTAAAAAACACATTTGTAGATATTTAAACTC-3'
<i>lukS</i> -PV EMSA rvs	5'-TTACATTTTTATATTAACCTTTTTAACTTTTAATAAATAAATAAT-3'
1975 pro fwd	5'-TTTTAAAGAGGATAAAGTATTTAATAATATTAACAAAATC-3'
1975 pro rvs	5'-GTATCGACATGTGAATAATATCACA AAAAC-3'

**NOTE.** 5'-RACE, rapid amplification of 5' complementary DNA ends; cDNA, complementary DNA; EMSA, electrophoretic mobility shift assay; fwd, forward; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase PCR; rvs, reverse.



**Figure 2.** SaeR/S significantly influences the virulence of USA300 during infection. *A*, Murine soft-tissue infections produced by USA300 and USA300ΔsaeR/S at day 4 after infection. *B*, The percentage of mice exhibiting infection and dermonecrosis after subcutaneous injection of USA300, USA300ΔsaeR/S, or a mock phosphate-buffered saline (PBS) control ( $*P < .001$  and  $**P < .0001$ , as determined by *t* test). *C*, Volume of abscess produced by USA300, USA300ΔsaeR/S, or a mock PBS control ( $*P < .01$ , as determined by *t* test). *D*, A separate experiment in which the no. of colony-forming units per abscess was enumerated for 3 mice from each group on days 2, 4, and 7 after infection. *E*, Survival during invasive infection by USA300 wild type (wt), USA300ΔsaeR/S, or mock PBS control ( $*P < .001$ , as determined by log-rank test). Unless otherwise stated, murine models used 15 mice per experimental group and 10 mice per PBS control group.

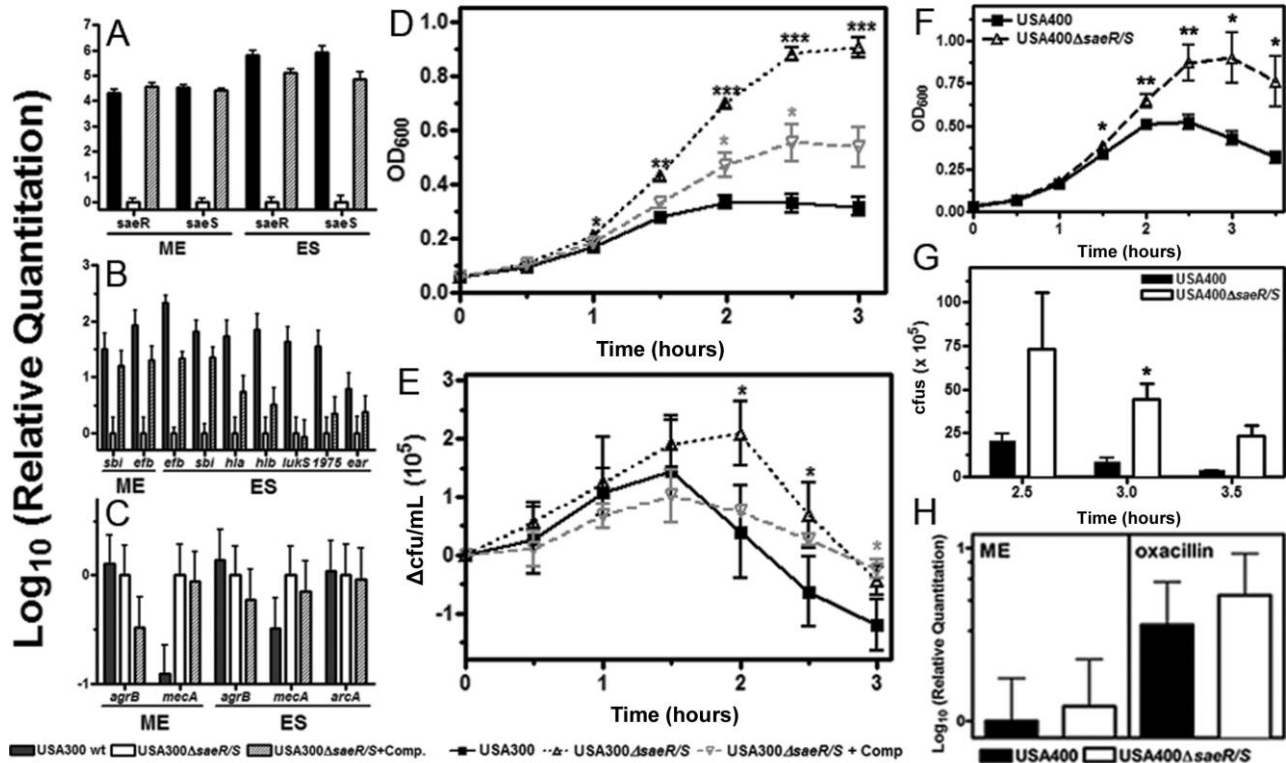
the dose administered. Conversely, mortality was delayed and considerably reduced in mice infected with USA300ΔsaeR/S. These findings demonstrate that SaeR/S significantly influences the virulence of USA300 during both soft-tissue and invasive infection.

**Influence of SaeR/S on transcription of virulence factors in vitro.** Gene transcription profiles of USA300ΔsaeR/S relative to USA300 were defined during in vitro growth, using oligo-

nucleotide microarray analysis (Figure 3). Approximately 1.9% of genes in USA300ΔsaeR/S exhibited a >2-fold change in transcription relative to USA300, including numerous hemolysins, proteases, host immunomodulatory proteins, and adhesion factors. Transcriptional up-regulation of the *mecA* (penicillin-binding protein 2') and *mecR1* genes that impart methicillin resistance was observed in USA300ΔsaeR/S, although such findings were not reported for isogenic *saeR/S* deletion mutants in

<u>Mid-exponential</u>		<u>Early Stationary</u>		<u>Locus Tag</u>	<u>SRS</u>	<u>Gene Annotation</u>
<u>Fold Increase</u>	<u>P Value</u>	<u>Fold Increase</u>	<u>P Value</u>			
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
<u>Fold Decrease</u>	<u>P Value</u>	<u>Fold Decrease</u>	<u>P Value</u>	<u>Locus Tag</u>	<u>SRS</u>	<u>Gene Annotation</u>
		-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>ilvA</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>fmbA</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>fmbB</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>eff</i>
-28.82	1.6E-06	-34.72	1.6E-02	SAUSA300_2364	+	<i>sbi</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>

**Figure 3.** Oligonucleotide microarray analysis. Mean fold-change in USA300 $\Delta$ *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 denoted by a +, 2 SRSs are denoted by ++, and a ~ denotes the presence of the SRS within the putative operon promoter of that gene.



**Figure 4.** Confirmation of oligonucleotide microarray analysis. Relative quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) examining expression of *saeR* and *saeS* (A); *sbi*, *efb*, *hla*, *hly*, *lukS*, SAUSA300\_1975 (1975), and *ear* (B); and *agrB*, *mecA*, and *arcA* (C). TaqMan real-time RT-PCR experiments were performed twice at 2 different growth phases (optical density measured at 600 nm [OD<sub>600</sub>] for midexponential [ME] growth vs early stationary [ES] growth, 0.75 vs 2.0) with triplicate measurement of each sample. The OD<sub>600</sub> value (D) and no. of colony-forming units (cfus) per milliliter (E) noted during growth in tryptic soy broth containing 10 μg/mL oxacillin, for USA300, USA300Δ*saeR/S*, and USA300Δ*saeR/S* supplemented with a complement plasmid encoding *saeR/S* (USA300Δ*saeR/S*+ Comp). The OD<sub>600</sub> (F) and no. of colony-forming units per milliliter (G) during growth of USA400 and USA400Δ*saeR/S* in tryptic soy broth containing 2.5 μg/mL oxacillin. H, Real-time RT-PCR for *mecA* in USA400 and USA400Δ*saeR/S* at ME growth or in the presence of 2.5 μg/mL oxacillin at 3 h. D–G, Experiments performed at least in triplicate (\**P* < .05, \*\**P* < .01, and \*\*\**P* < .001, relative to the parental strain, as determined by *t* test).

other *S. aureus* strains [12, 14, 29]. However, USA300Δ*saeR/S* is primarily characterized by 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 2-component leukotoxins (*hlgB-hlgC*, *lukE-lukD*, and SAUSA300\_1974-SAUSA300\_1975). Down-regulation was also observed for known immunomodulatory genes—namely, *sbi*, *efb*, and *chs* (encoding chemotaxis inhibitory protein of *S. aureus*)—secreted into the extracellular environment to subvert the host immune response [30–32]. The transcriptional regulator *tcaR*, a member of the *tcaR-tcaA-tcaB* operon, was down-regulated in USA300Δ*saeR/S*. In addition to associations with teicoplanin resistance and cell wall biosynthesis pathways [33], *tcaR* is thought to be a repressor of the *ica* locus [34] and to directly regulate *sasF* and *sarS* transcription [35]. Other transcripts down-regulated in USA300Δ*saeR/S* included various host-binding proteins (*fnbA*, *fnbB*, *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 2 genes not included on the oligonucleotide microarray (Figure 4), Pantone-Valentine leukocidin (PVL), and novel arginine catabolic mobile element (ACME) of USA300 [18]. TaqMan real-time RT-PCR confirmed isogenic deletion of *saeR* and *saeS* (Figure 4A); down-regulation of *sbi*, *efb*, *hla*, *hly*, and SAUSA300\_1975 (Figure 4B); and up-regulation of *mecA* (Figure 4C) in USA300Δ*saeR/S*. Complementation with a plasmid encoding *saeR* and *saeS* (USA300Δ*saeR/S* + Comp) partially restored gene transcription in USA300Δ*saeR/S*, reinforcing real-time RT-PCR and microarray results. Transcriptional regulation of *lukS-PV* (PVL component) was down-regulated in USA300Δ*saeR/S*, although USA300Δ*saeR/S* + Comp did not exhibit restored transcription of *lukS-PV* (Figure 4B). Transcription of *arcA* (ACME com-

ponent) was similar between USA300 and USA300 $\Delta$ *saeR/S*, indicating that SaeR/S is not involved in regulating ACME (Figure 4C). The up-regulation of *mecA* and *mecR1* in USA300 $\Delta$ *saeR/S* relative to USA300 was coupled with significantly improved growth in subinhibitory concentrations of oxacillin (Figure 4D and 4E). Improved growth (Figure 4F and 4G) and a subtle increase in *mecA* transcription during growth in oxacillin (Figure 4H) was also observed for USA400 $\Delta$ *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 that 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 $\Delta$ *saeR/S* (Figure 5). Genes shown to be positively regulated by SaeR/S produced more-robust 5'-RACE products in USA300 relative to USA300 $\Delta$ *saeR/S* (Figure 5B). Alternatively, transcription of *agrB* was not influenced by SaeR/S (Figure 4C), and 5'-RACE products for *agrB* were indistinguishable between the USA300 wild type and USA300 $\Delta$ *saeR/S* (Figure 5A). 5'-RACE products for *mecA*, a transcript up-regulated in USA300 $\Delta$ *saeR/S* relative to USA300, were also identical between USA300 and USA300 $\Delta$ *saeR/S* (Figure 5A). These results indicate that the transcriptional down-regulation of *sbi*, *efb*, SAUSA300\_1975, *lukS-PV*, *hla*, and *hly* in USA300 $\Delta$ *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 (Figure 5C and 5D). For *hla* and *agrB*, the TSS was found to be 330 and 18 bp upstream, respectively, from the start codon, in accordance with previous research [38, 39]. 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 USA300 $\Delta$ *saeR/S* corresponded to those obtained from USA300. The 5'-RACE product for *sbi* from USA300 $\Delta$ *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.

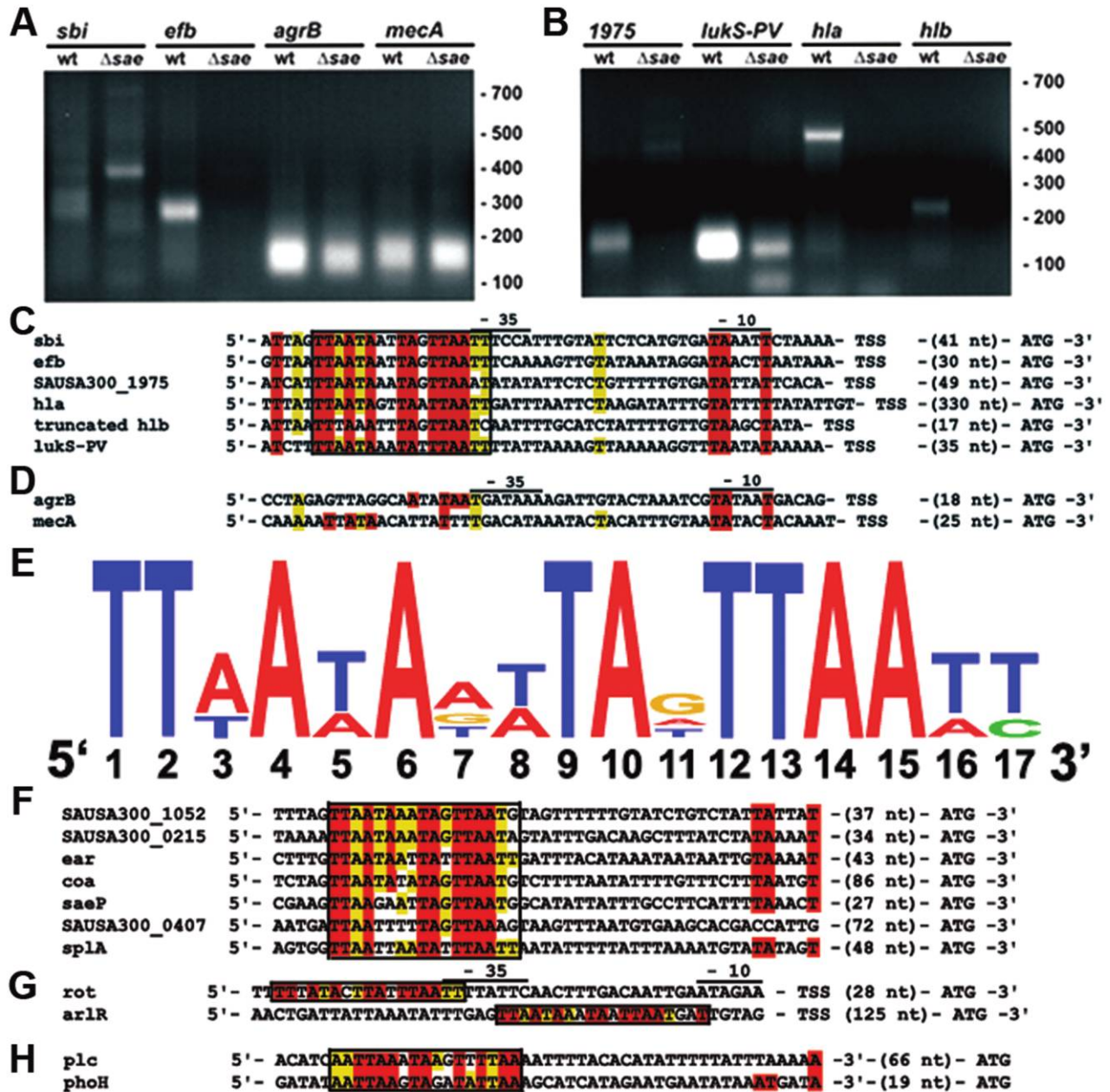
**Identification of a conserved consensus sequence within promoter regions of genes influenced by SaeR/S.** Multiple sequence alignment performed on intergenic sequences immediately upstream from the defined TSS of genes positively regulated by SaeR/S revealed 2 conserved domains corresponding to the -10 and -35 promoter boxes (Figure 5C). In con-

trast, 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 (Figure 5D). A consensus sequence 17 bp in length was derived from the conserved domain immediately upstream from the -35 promoter box (Figure 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 (Figures 3 and 5F). Other investigators have demonstrated that a *sae* deletion mutant of strain COL exhibits reduced expression of Ear and increased expression of Plc [29]. Interestingly, the SRS was found within the putative promoter region of *ear*, whereas the reverse complement of the SRS was located within the putative promoters of *plc* and the phosphate starvation-inducible protein *phoH* (Figure 5H). Subsequent real-time RT-PCR confirmed down-regulation of *ear* in USA300 $\Delta$ *saeR/S* (Figure 4B). The SRS was also found immediately upstream from the -35 promoter region of *rot* defined by primer extension analysis [36] and between the -35 and -10 promoter regions of the *arlR/S* TCS also defined by primer extension analysis [37] (Figure 5G).

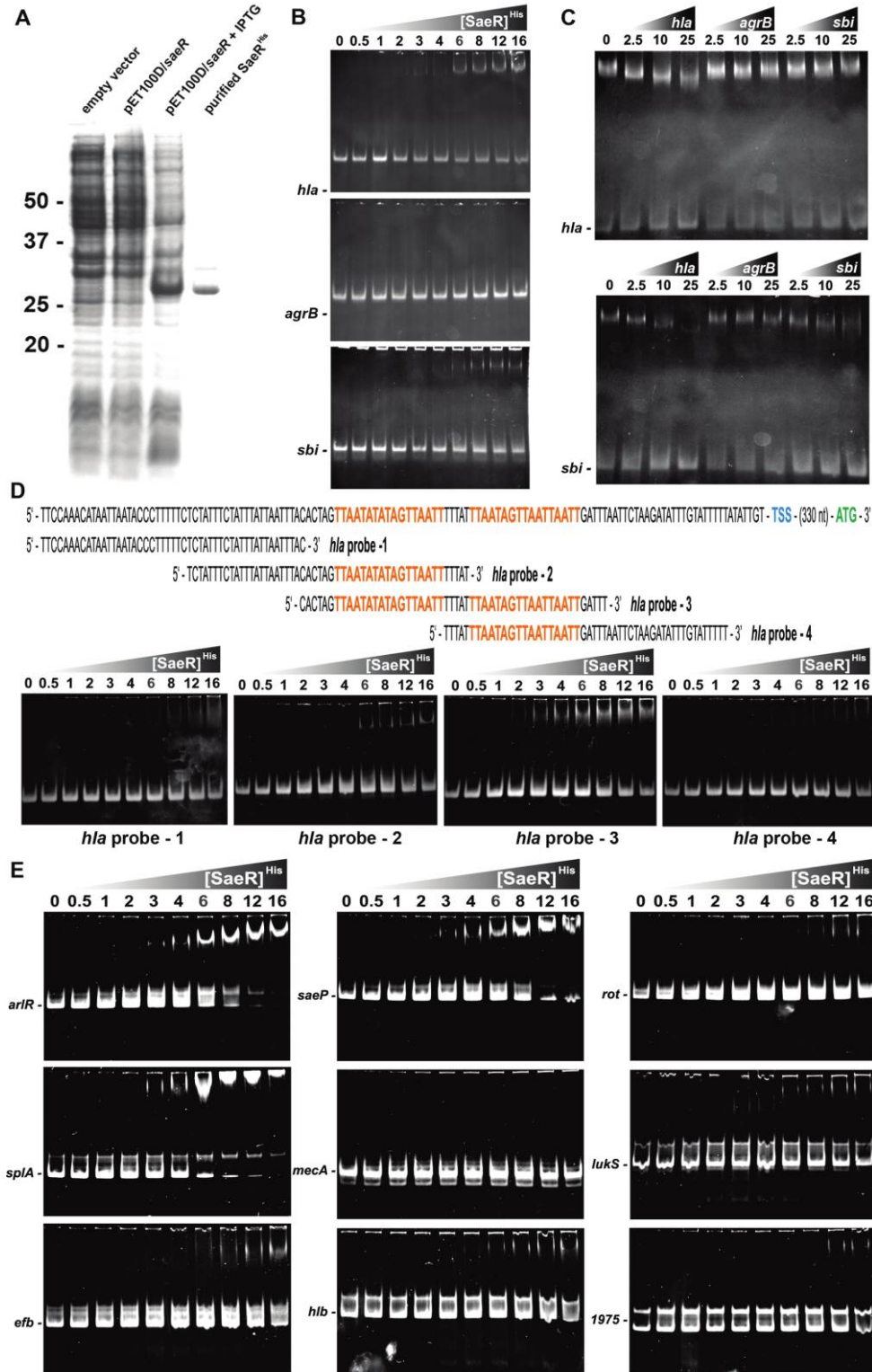
**Binding of putative promoter regions of USA300 virulence factors by SaeR.** EMSAs were used to demonstrate that recombinant SaeR<sup>HIS</sup> binds numerous virulence gene promoter regions in a concentration-dependent manner (Figure 6). Purified SaeR<sup>HIS</sup> (Figure 6) specifically bound the -35 region of the *hla* and *sbi* promoters defined by 5'-RACE in a concentration-dependent manner, whereas no binding was detected for the -35 region of the *agrB* promoter (Figure 6B and 6C). The *hla* promoter probe sequence contains 2 SRSs, and the *sbi* promoter probe contains 1 SRS, whereas the *agrB* promoter probe has no SRS, paralleling the relative affinity of these probes for SaeR<sup>HIS</sup>. Dissection of the *hla* promoter by use of overlapping probe sequences suggests that binding of SaeR<sup>HIS</sup> is most robust to the sequence containing both SRSs immediately upstream from the -35 promoter region (Figure 6D). EMSAs indicate strong binding of SaeR<sup>HIS</sup> to the *arlR*, *saeP*, and *splA* promoter regions (Figure 6E). SaeR<sup>HIS</sup> binding was also observed for the *efb*, *hly*, *rot*, *lukS-PV*, and SAUSA300\_1975 promoter regions, whereas no binding was detected for the *mecA* promoter region (Figure 6E). These findings establish direct SaeR-DNA interaction specific to virulence gene promoters containing the SRS.

## DISCUSSION

Cumulative evidence indicates that *S. aureus* virulence is determined by the coordinated expression of specific virulence gene ensembles that further bacterial survival and dissemination [2, 15, 16, 40–43]. *S. aureus* sensory mechanisms are



**Figure 5.** Sequence homology shared within the promoter regions of genes differentially regulated by *SaeR/S*. Rapid amplification of 5' complementary DNA ends (5'-RACE) products using gene-specific primers for *sbi*, *efb*, *mecA*, and *agrB* (A) and SAUSA300\_1975, *lukS-PV*, *hla*, and *hlb* (B). C, 5'-RACE products were sequenced to define transcript start sites and upstream alignment underscored nucleic acids shared by all sequences (red) and highly conserved between sequences (yellow). Putative -35 and -10 promoter regions are indicated, and the SaeR recognition sequences (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, Consensus sequence generated [24] from the conserved 17-bp sequences immediately upstream of the putative -35 promoter box of *SaeR/S*-regulated genes. F, In silico analysis identifying the SRS within intergenic regions upstream from the start codon of other genes influenced by *SaeR/S*. G, The -35 promoter region of *rot* defined by primer extension analysis [36] and between the -35 and -10 promoter regions of the *arlR/S* TCS [37]. H, Identification of the reverse complement of the SRS upstream of the *plc* and *phoH* start codons.



**Figure 6.** Recombinant SaeR<sup>His</sup> and binding of putative promoter regions of USA300 virulence genes. *A*, Verification of expression and purification of SaeR<sup>His</sup> by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *B*, Demonstration, by use of electrophoretic mobility shift assays (EMSA), of SaeR<sup>His</sup> (0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16  $\mu$ mol/L) binding to 50-bp oligonucleotides encoding the –35 promoter region (0.25 pmol) of *hla*, *agrB*, and *sbi*, in the presence of 500 ng of salmon sperm DNA. *C*, Binding of SaeR<sup>His</sup> (6  $\mu$ mol/L) 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<sup>His</sup> (0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16  $\mu$ mol/L) with 4 overlapping probe sequences (2.5 pmol) of the *hla* promoter. *E*, Examination, by use of EMSAs, of binding of SaeR<sup>His</sup> (0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16  $\mu$ mol/L) to 1 pmol of promoter regions (~100 bp) for *arlR*, *saeP*, *rot*, *splA*, *mecA*, *lukS*-PV, *efb*, *hly*, and SAUSA300\_1975.

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 [44]. Indeed, preliminary investigations by other researchers have demonstrated that targeted inhibition of the *agr* quorum-sensing TCS attenuates soft-tissue infections caused by *S. aureus* [45, 46]. The SaeR/S TCS is thought to be critical during *S. aureus* pathogenesis by up-regulating expression of numerous secreted virulence genes during infection. Because ~22% of the *S. aureus* genome can differ between isolates [47], and because virulence appears to be largely dictated by differences in expression of common genes between strains [41], 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 with 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 >33-fold in USA300 $\Delta$ *saeR/S*, compared with a 4-fold reduction in *hla* transcription in USA400 $\Delta$ *saeR/S* [14]. Others have shown that in vitro transcription of  $\alpha$ -toxin (*hla*), *agr*, *saeR/S*, and *sarA* is higher in USA300 relative to USA400 [42]. Thus, it is likely that differences in genomic content and the extent of the effect of USA300 SaeR/S transcriptional regulation on common virulence gene expression account 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, 29, 48]. We also report an up-regulation of *mecA* and *mecR1* in USA300 $\Delta$ *saeR/S* that corresponds to improved in vitro growth in the presence of subinhibitory concentrations of oxacillin, findings that are supported by the improved growth of USA400 $\Delta$ *saeR/S* relative to USA400 in the presence of oxacillin. Direct binding could not be detected between SaeR<sup>HIS</sup> and the *mecA* promoter region defined by 5'-RACE analysis, suggesting an indirect up-regulation of *mecA* in USA300 $\Delta$ *saeR/S* by SaeR/S. The down-regulation of the transcriptional regulator *tcaR* in USA300 $\Delta$ *saeR/S*, which is involved with cell wall biosynthesis and implicated in methicillin resistance [33], may account for the differential regulation of *mecA* and *mecR1*.

We identified a conserved palindromic consensus sequence

(length, 17 bp) specific to promoters of genes regulated by SaeR/S. EMSAs revealed SaeR<sup>HIS</sup> directly bound various virulence gene promoters containing the conserved consensus sequence, whereas 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, 29], a dramatic down-regulation of *saeP* was observed in USA300 $\Delta$ *saeR/S*. Not surprisingly, the SRS was identified within the previously defined P1 primary start site for the SaeR/S transcript [49]. EMSAs demonstrated direct binding of SaeR<sup>HIS</sup> to this promoter region, supporting a positive feedback loop of SaeR/S transcription suggested by other investigators [12]. The promoter region of *lukS-PV* was defined using 5'-RACE, and weak binding of SaeR<sup>HIS</sup> to this promoter sequence reinforces recent work indicating SaeR/S positively regulates *lukS-PV* transcription [50]. The SRS was also located between the –35 and –10 promoter regions of the *arlR/S* operon [37], and strong binding of SaeR<sup>HIS</sup> 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 [37]. In addition, the SRS specifically mapped to the region immediately upstream from the –35 promoter box of the repressor of secreted virulence genes, *rot* [51], defined by others using primer extension analysis [36]. Recent work on strain COL has demonstrated repression of *hla* expression by *rot* requires *saeR/S* and *rot* down-regulates expression of SaeR/S [52]. Weak binding of SaeR<sup>HIS</sup> to the *rot* promoter supports the hypothesis made by others [52] that SaeR/S-mediated transcription of *rot* acts as a negative feedback loop to curtail overexpression 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 USA300 $\Delta$ *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 *sbj*, *efb*, SAUSA300\_1975, *hly*, *lukS-PV*, and *mecA* and are congruent with previously established TSSs for *hla* and *agrB* [38, 39]. A conserved partially palindromic SRS was identified within virulence gene promoters transcriptionally regulated by SaeR/S, and EMSAs established direct binding of SaeR<sup>HIS</sup> to promoter regions containing the conserved consensus sequence. Collec-

tively, 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.

## Acknowledgment

We thank Kate McInnerney and Mensur Dlakic, Department of Microbiology, Montana State University–Bozeman, for advice and technical support.

## References

- Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* **1998**;339:520–32.
- Nygaard TK, Deleo FR, Voyich JM. Community-associated methicillin-resistant *Staphylococcus aureus* skin infections: advances toward identifying the key virulence factors. *Curr Opin Infect Dis* **2008**;21:147–52.
- Adem PV, Montgomery CP, Husain AN, et al. *Staphylococcus aureus* sepsis and the Waterhouse-Friderichsen syndrome in children. *N Engl J Med* **2005**;353:1245–51.
- Kravitz GR, Dries DJ, Peterson ML, Schlievert PM. Purpura fulminans due to *Staphylococcus aureus*. *Clin Infect Dis* **2005**;40:941–7.
- Miller LG, Perdreau-Remington F, Rieg G, et al. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N Engl J Med* **2005**;352:1445–53.
- Sifri CD, Park J, Helm GA, Stemper ME, Shukla SK. Fatal brain abscess due to community-associated methicillin-resistant *Staphylococcus aureus* strain USA300. *Clin Infect Dis* **2007**;45:e113–7.
- Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* **2007**;298:1763–71.
- Howe RA, Monk A, Wootton M, Walsh TR, Enright MC. Vancomycin susceptibility within methicillin-resistant *Staphylococcus aureus* lineages. *Emerg Infect Dis* **2004**;10:855–7.
- Cheung AL, Bayer AS, Zhang G, Gresham H, Xiong YQ. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* **2004**;40:1–9.
- Bronner S, Monteil H, Prevost G. Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol Rev* **2004**;28:183–200.
- Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* **2003**;48:1429–49.
- Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C. The virulence regulator Sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. *J Bacteriol* **2008**;190:3419–28.
- Giraud AT, Calzolari A, Cataldi AA, Bogni C, Nagel R. The sae locus of *Staphylococcus aureus* encodes a two-component regulatory system. *FEMS Microbiol Lett* **1999**;177:15–22.
- Voyich JM, Vuong C, Dewald M, et al. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J Infect Dis* **2009**;199:1698–706.
- Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* **2005**;175:3907–19.
- Voyich JM, Otto M, Mathema B, et al. Is Pantone-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J Infect Dis* **2006**;194:1761–70.
- Voyich JM, Sturdevant DE, Deleo FR. Analysis of *Staphylococcus aureus* gene expression during PMN phagocytosis. *Methods Mol Biol* **2008**;431:109–22.
- Diep BA, Gill SR, Chang RF, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **2006**;367:731–9.
- Bruckner R. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosum*. *FEMS Microbiol Lett* **1997**;151:1–8.
- Available at: <http://www.affymetrix.com/index.affx> Accessed 19 November 2009.
- Scotto-Lavino E, Du G, Frohman MA. 5' end cDNA amplification using classic RACE. *Nat Protoc* **2006**;1:2555–62.
- BLAST (Basic Local Alignment Search Tool). Available at: <http://blast.ncbi.nlm.nih.gov>. Accessed 19 November 2009.
- Multiple sequence alignment by CLUSTALW. Available at: <http://align.genome.jp/>. Accessed 19 November 2009.
- WebLogo, version 2.8.2 (2005–09-08). Available at: <http://weblogo.berkeley.edu/>. Accessed 19 November 2009.
- Virtual Footprint Bacterial Regulon Analyzer. Available at: <http://prodoric.tu-bs.de/vfp/>. Accessed 19 November 2009.
- Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat Protoc* **2007**;2:1849–61.
- Bubeck Wardenburg J, Palazzolo-Ballance AM, Otto M, Schneewind O, DeLeo FR. Pantone-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis* **2008**;198:1166–70.
- Wang R, Braughton KR, Kretschmer D, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* **2007**;13:1510–4.
- Rogasch K, Ruhmling V, Pane-Farre J, et al. Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. *J Bacteriol* **2006**;188:7742–58.
- Burman JD, Leung E, Atkins KL, et al. Interaction of human complement with Sbi, a staphylococcal immunoglobulin-binding protein: indications of a novel mechanism of complement evasion by *Staphylococcus aureus*. *J Biol Chem* **2008**;283:17579–93.
- Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol* **2005**;3:948–58.
- Hammel M, Sfyroera G, Ricklin D, Magotti P, Lambris JD, Geisbrecht BV. A structural basis for complement inhibition by *Staphylococcus aureus*. *Nat Immunol* **2007**;8:430–7.
- Brandenberger M, Tschierske M, Giachino P, Wada A, Berger-Bachi B. Inactivation of a novel three-cistronic operon *tcaR-tcaA-tcaB* increases teicoplanin resistance in *Staphylococcus aureus*. *Biochim Biophys Acta* **2000**;1523:135–9.
- Jefferson KK, Pier DB, Goldmann DA, Pier GB. The teicoplanin-associated locus regulator (TcaR) and the intercellular adhesin locus regulator (IcaR) are transcriptional inhibitors of the ica locus in *Staphylococcus aureus*. *J Bacteriol* **2004**;186:2449–56.
- McCallum N, Bischoff M, Maki H, Wada A, Berger-Bachi B. TcaR, a putative MarR-like regulator of sarS expression. *J Bacteriol* **2004**;186:2966–72.
- Hsieh HY, Tseng CW, Stewart GC. Regulation of Rot expression in *Staphylococcus aureus*. *J Bacteriol* **2008**;190:546–54.
- Fournier B, Klier A, Rapoport G. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. *Mol Microbiol* **2001**;41:247–61.
- Chien Y, Manna AC, Projan SJ, Cheung AL. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for sar-dependent gene regulation. *J Biol Chem* **1999**;274:37169–76.
- Koenig RL, Ray JL, Maleki SJ, Smeltzer MS, Hurlburt BK. *Staphylococcus aureus* AgrA binding to the RNAPIII-*agr* regulatory region. *J Bacteriol* **2004**;186:7549–55.
- Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol* **2008**;16:361–9.
- Li M, Diep BA, Villaruz AE, et al. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **2009**;106:5883–8.

42. Montgomery CP, Boyle-Vavra S, Adem PV, et al. Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsed-field types USA300 and USA400 in a rat model of pneumonia. *J Infect Dis* **2008**; 198:561–70.
43. Peacock SJ, Moore CE, Justice A, et al. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infect Immun* **2002**; 70:4987–96.
44. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* **2007**; 3:541–8.
45. Wright JS III, Jin R, Novick RP. Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci U S A* **2005**; 102:1691–6.
46. Park J, Jagasia R, Kaufmann GF, et al. Infection control by antibody disruption of bacterial quorum sensing signaling. *Chem Biol* **2007**; 14: 1119–27.
47. Fitzgerald JR, Sturdevant DE, Mackie SM, Gill SR, Musser JM. Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci U S A* **2001**; 98:8821–6.
48. Kuroda H, Kuroda M, Cui L, Hiramatsu K. Subinhibitory concentrations of  $\beta$ -lactam induce haemolytic activity in *Staphylococcus aureus* through the SaeRS two-component system. *FEMS Microbiol Lett* **2007**; 268:98–105.
49. Novick RP, Jiang D. The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. *Microbiology* **2003**; 149: 2709–17.
50. Wirtz C, Witte W, Wolz C, Goerke C. Transcription of the phage-encoded Panton-Valentine leukocidin of *Staphylococcus aureus* is dependent on the phage life-cycle and on the host background. *Microbiology* **2009**; 155:3491–9.
51. Said-Salim B, Dunman PM, McAleese FM, et al. Global regulation of *Staphylococcus aureus* genes by Rot. *J Bacteriol* **2003**; 185:610–9.
52. Li D, Cheung A. Repression of *hla* by *rot* is dependent on *sae* in *Staphylococcus aureus*. *Infect Immun* **2008**; 76:1068–75.

CONTRIBUTION OF AUTHORS AND CO-AUTHORS

Chapter 4: SaeR/S MEDIATED EXPRESSION OF *hla* PLAYS A PROMINENT ROLE DURING USA300 PATHOGENESIS BY INDUCING T CELL PORE FORMATION AND RAPID PROLIFERATION

Author: Tyler K. Nygaard

Contributions: Conceived the study, designed and performed experiments, collected and analyzed output data, and wrote manuscript.

Co-authors: Kyler B. Pallister, Mark Dewald, and Shannon Griffith.

Contributions: Contributed to data collection.

Co-author: Dr. Victor Torres & Ashley DuMont

Contributions: Generated Western blot data used in study.

Co-author: Dr. Alexander Horswill, Michael E. Olson, Cherly L Malone

Contributions: Generated USA300 $\Delta$ *agr*, *hla* specific promoter GFP promoter constructs, and the *hla* complement plasmid used in study.

Co-author: Dr. Jovanka M. Voyich

Contributions: Conceived the study and edited/commented on the manuscript at all stages.

MANUSCRIPT INFORMATION PAGE

SaeR/S MEDIATED EXPRESSION OF *hla* PLAYS A PROMINENT ROLE DURING USA300 PATHOGENESIS BY INDUCING T CELL PORE FORMATION AND RAPID PROLIFERATION

Tyler K. Nygaard, Kyler B. Pallister, Shannon Griffith, Mark DeWald, Victor Torres, Alexander Horswill and Jovanka M. Voyich

Journal Name: Journal of Infectious Disease

Status of Manuscript:

Prepared for submission to a peer-reviewed journal

Officially submitted to a peer-reviewed journal

Accepted by a peer-reviewed journal

Published in a peer-reviewed journal

Publisher: University of Chicago Press.

Submitted January 3, 2011

## CHAPTER 4

SaeR/S-MEDIATED TRANSCRIPTION OF *hla* BY USA300 PROMOTES HUMAN T CELL PLASMA MEMBRANE DAMAGE AND RAPID HUMAN T CELL PROLIFERATIONAbstract

This investigation examines the regulation of alpha-toxin (Hla) by the SaeR/S two-component system and the role of this virulence factor during *Staphylococcus aureus* pathogenesis. Strong *saeR/S*-mediated transcription of *hla* corresponded to significantly larger and more severe soft-tissue infections in mice. Survival of an USA300 isogenic deletion mutant of *hla* (USA300 $\Delta$ *hla*) in human blood was comparable to that of the parental wild-type strain and *saeR/S*-mediated cell membrane damage on polymorphonuclear leukocytes did not require *hla*. However, cytokine transcript levels were diminished during infection of human blood with USA300 relative to USA300 $\Delta$ *hla*. Flow cytometry analysis revealed USA300 expressing *hla* generated T cell plasma membrane damage in a concentration-dependent manner. Unexpectedly, rapid *hla*-mediated T cell proliferation was observed following exposure to USA300 supernatants. These findings demonstrate *saeR/S*-mediated transcription of *hla* compromises human T cell plasma membrane integrity and promotes rapid human T cell expansion.

## Introduction

*Staphylococcus aureus* is a common Gram-positive bacterial pathogen that can produce a wide spectrum of disease in humans, ranging from superficial skin abscesses to invasive life-threatening disease. Emerging antibiotic resistant and hypervirulent strains of *S. aureus* continuously compromise our ability to treat these infections, emphasized by a 2005 survey indicating over 18,000 deaths can be attributed to invasive methicillin resistant *S. aureus* (MRSA) infection in the United States alone (1). In particular, strains of community-associated MRSA (CA-MRSA) distinct from previously characterized hospital-associated MRSA (HA-MRSA) are prominent as a major cause of skin and soft-tissue infections and are noted for their enhanced capacity to produce disease in humans (2;3). The mechanisms behind the increased virulence observed for CA-MRSA strains remain incompletely defined.

Currently pulse-field gel electrophoresis (PFGE) type USA300 is a leading cause of soft-tissue infections in the United States (2). This strain exhibits a heightened capacity to elicit human polymorphonuclear leukocyte (PMN) destruction *in vitro* as well as produce more severe dermonecrotic soft-tissue infections in mice relative to other *S. aureus* strains (4-7). Evidence suggests differential expression of core genome-encoded virulence elements common to all *S. aureus* strains is largely responsible for the enhanced virulence observed for USA300 (8;9). Indeed, elevated expression of virulence gene regulators *agr*, *saeR/S*, and *sarA* as well as the pore-forming toxin *hla* in USA300 relative to USA400 is thought to contribute to the increased pathogenicity of this strain during rat models of pneumonia (10). Expression of *hla* directly correlates with *S. aureus* virulence (4;9-12) and immunization

strategies targeting Hla provide effective protection during murine models of *S. aureus* pneumonia and soft-tissue infections caused by USA300 (4;12). However, the factors that dominate the *in vivo* regulation of *hla* in USA300 and the mechanisms by which this toxin promotes *S. aureus* infection are not entirely clear.

The *S. aureus* genome encodes a diverse array of virulence genes that damage host cells, augment bacterial adherence to host tissue, and manipulate the host immune response to promote pathogenesis. The *in vivo* regulation of these genes is believed to be largely coordinated by two-component regulatory systems (TCSs) that recognize extracellular signals and alter gene transcription in response. Recent evidence indicates the *saeR/S* TCS plays a major role recognizing host-associated environmental signals and subsequently inducing transcription of virulence genes critical to *S. aureus* pathogenesis (13-15). In particular, a strong interaction of recombinant SaeR with the *hla* promoter suggests *hla* acts as an effector of this TCS and may play an essential role during *S. aureus* pathogenesis (14;16). The Hla toxin is a major *S. aureus* virulence factor expressed at higher levels by USA300 during infection of human tissue relative to *in vitro* growth (17) and is thought to be largely regulated by the *agr* and *saeR/S* TCSs (18-21). We examined the *in vitro* and *in vivo* regulation of *hla* by *saeR/S* as well as the influence of *hla* during infection of human blood, purified PMNs, and peripheral blood mononuclear cells (PBMCs). This investigation demonstrates that the SaeR/S-mediated transcription of *hla* is important for *S. aureus* pathogenesis during murine models of soft-tissue infection. Moreover, expression of Hla largely contributes to human T cell plasma membrane damage and induces rapid human T cell proliferation.

## Materials and Methods

### Bacterial Strains and Culture

*S. aureus* strains were cultured and harvested as described elsewhere (5;6;14;15). *S. aureus* pulse-field gel electrophoresis type USA300 (strain LAC) (22) was used to generate USA300 $\Delta$ *hla* as described previously using oligonucleotides *hla*-SalI\_upstream\_fwd (5'-TTCAATGTCGACGGTTAGTCAAAG -3'), *hla*-HindIII\_upstream\_rvs (5'-TCGAAATTTTAAAGCTTGATTCAGACTC-3'), *hla*-SacI\_downstream\_fwd (5'-TAATGGAGCTCTAATTAATCCGAAATTAATCATG-3'), and *hla*-BamHI\_downstream\_rvs (5'-TGTTCAAACCTTTTGGATCCCATTATGTG-3') and SalI, HindIII, SacI, and BamHI restriction digest (6;15;23). USA300 $\Delta$ *saeR/S* was generated in previous studies (14). The USA300 isogenic deletion mutant of *agr* (USA300 $\Delta$ *agr*) was generated in previous studies (24). To construct the USA300 $\Delta$ *hla* complementing clone, the *hla* gene was PCR amplified from USA300 genomic DNA using oligonucleotides CLM445 (5'-GTTGTTGGATCCCCTTTCTTGAATTAACAATATAC-3') and CLM446 (5'-GTTGTTGAATTCGCCGAAAAACATCATTTCTG), and cloned into pAH5 using BamHI and EcoRI (25), then transformed into USA300 $\Delta$ *hla* to generate USA300 $\Delta$ *hla* Comp. For the GFP reporter construct specific for *hla*, the *hla* promoter was PCR amplified from USA300 genomic DNA using oligonucleotides CLM429 (5'-GTTGTTAAGCTTAAGATCACCGGT-3') and CLM430 (5'-GTTGTTGGTACCTTTCGTTCTAATAAC-3'), cloned into pCM11 (26) using HindIII and

KpnI, then transformed into *agr* and *saeR/S* isogenic deletion mutants in an erythromycin-sensitive variant of USA300 (strain AH1263) generated as previously described(24;25)

### Murine Models of Infection

Murine models of infection using female Crl;SKH1-hrBR hairless mice (Charles River Laboratories) were performed as described elsewhere (5;6;15). All animal studies conformed to National Institutes of Health guidelines and were approved by the animal care and use committee at Montana State University–Bozeman. Colony forming units (CFUs) were determined as previously described (15) by performing serial dilutions of homogenized tissue sample and plating on tryptic soy broth (TSB) agar. Statistics were performed using GraphPad Prism 4 software.

### Transcription and Expression Analysis

TaqMan real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed using a RNeasy kit (Qiagen) and a 7500 Fast Real-Time PCR system (Applied Biosystems) as described previously (5;15). For *in vitro* transcription analysis, real-time RT-PCR was performed on *S. aureus* in early-stationary growth phase from 2 separate experiments with triplicate analysis conducted on each sample. Western blot analyses for Hla was performed on filtered supernatants from *S. aureus* cultures at 5 hours of growth using a rabbit polyclonal anti-Hla antibody (Sigma). For *in vivo* real time RT-PCR and Western blot analysis, 9 mm punches of murine soft-tissue infections were excised at 8 hours post-infection, homogenized in 2 mL DPBS, and CFUs determined by plating on TSB agar plates. Remaining sample was centrifuged (10,000xg, 5 minutes, 4°C) and the supernatant

was analyzed for Hla production via Western blot as described above. RNA was purified from pelleted bacteria and examined for *hla* transcription as described above.

For GFP reporter construct analysis, *S. aureus* strains with deletions in the *agr* and *saeR/S* systems were constructed in an erythromycin-sensitive variant of USA300 (strain AH1263) generated as previously described (24;25). Reporter plasmid pCM27 was transformed into AH1263 and the mutant strains, and cultures were grown overnight in TSB containing chloramphenicol (10 µg/mL). Overnight cultures were diluted 1:100 into 5 ml of the same media and grown at 37°C with shaking (200 r.p.m.). At designated times, 200 µl of culture was removed from each tube and transferred to a 96-well microtiter plate (Corning 3096) and fluorescence readings were recorded with a Tecan Infinity 200M plate reader with excitation and emission wavelength of 480 nm and 515 nm, respectively. Cell growth was also measured at an OD<sub>600</sub> to calculate relative fluorescence. Statistics were performed using GraphPad Prism 4 software.

#### *S. aureus* Survival in Human Blood and Cytokine Transcription Analysis

Heparinized venous blood samples from healthy donors were collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects, National Institute of Allergy and Infectious Diseases, and Montana State University. All donors provided written consent to participate in the study. USA300 was harvested at the mid-exponential (ME) phase of growth ( $2 \times 10^8$  CFU/mL). Heparinized human blood (3 mL) was inoculated with  $3 \times 10^5$  CFU of *S. aureus* in a 5 mL culture tube and incubated at 37°C with end-over-end rotation (20 rpm). Samples were diluted in sterile deionized water to lyse blood cells and CFUs were determined by plating on tryptic soy agar. Statistics were

performed using GraphPad Prism 4 software. For cytokine transcription analysis, RNA from human blood infected with *S. aureus* as described above was purified at 3 hours post-infection using RNeasy Kit (Qiagen). Purified RNA was analyzed using a Human Inflammatory Cytokine and Receptor RT<sup>2</sup>Profiler PCR array (SABiosciences, Catalog# PAHS-011) following the manufacturer's instructions and analysis software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>) using a 7500 Fast Real-Time PCR system (Applied Biosystems).

### Flow Cytometry

PBMCs and PMNs were isolated from freshly drawn human blood under endotoxin-free conditions (<25.0 pg/mL) as described elsewhere (5). Cell viability and purity of preparations were assessed by flow cytometry (FACSCalibur; BD Biosciences).

Supernatants from *S. aureus* cultured for 5 hours in TSB supplemented with 5% glucose were filtered (0.2  $\mu$ M) and 20  $\mu$ L of undiluted supernatant or supernatant diluted with TSB were added to  $1 \times 10^6$  PBMCs or PMNs in 100  $\mu$ L RPMI with 5 mM HEPES. At indicated times, samples were stained with propidium iodide (Invitrogen, Molecular Probes), FITC mouse anti-human CD3 (BD Pharmingen), APC mouse anti-human CD4 (BD Pharmingen), APC mouse anti-human CD8 (BD Pharmingen), and/or 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, eBiosciences) following the manufacturer's protocol. Cells were analyzed using a FACSCalibur Flow cytometer (BD Biosciences). For infection of PBMCs with *S. aureus*, 1 mL of bacteria grown to ME were harvested by centrifugation (5,000xg, 5 minutes, 4 °C), washed twice with 1 mL DPBS, and resuspended in 100  $\mu$ L DPBS ( $2 \times 10^9$  CFU/mL). PBMCs ( $1 \times 10^6$ ) in 100  $\mu$ L RPMI with 5 mM HEPES were

combined with 5  $\mu$ L of concentrated *S. aureus* ( $1 \times 10^7$  CFU/mL) for an MOI = 10. Samples were incubated at 37 °C for 6 hours and analyzed using flow cytometry as described above. Statistics were performed using GraphPad Prism 4 software.

## Results

### *In vitro* Transcription of *hla* is Dependent upon *saeR/S*.

To examine the role of *hla* during *S. aureus* pathogenesis, we first developed an isogenic deletion mutant of this toxin in USA300 strain LAC using temperature sensitive allelic replacement as previously described (6;15;23). PCR determined the presence of *hla* in USA300, USA300 $\Delta$ *hla* harboring a plasmid expressing *hla* (USA300 $\Delta$ *hla* Comp), and an isogenic deletion mutant of *saeR/S* in USA300 (USA300 $\Delta$ *saeR/S*) but not in USA300 $\Delta$ *hla* (Figure 1A). Western blot confirmed the deletion of *hla* in USA300 $\Delta$ *hla* and rescue of expression in USA300 $\Delta$ *hla* Comp (Figure 1B). Expression of Hla was also diminished in USA300 $\Delta$ *saeR/S*, indicating *saeR/S*-dependent expression of Hla during *in vitro* growth. Real time RT-PCR analysis demonstrated a greater than 100-fold decrease of *hla* transcription *in vitro* for USA300 $\Delta$ *saeR/S* relative to the parental wt (Figure 1C). These findings were supported by an increasing fluorescence signal over time for USA300 and USA300 $\Delta$ *agr* transformed with a GFP reporter construct specific for the *hla* promoter while a significantly decreased signal was observed for USA300 $\Delta$ *saeR/S* and empty GFP vector (Figure 1D). Taken together, these results show strong *SaeR/S*-dependent transcriptional regulation of Hla and support the post-transcriptional regulation of Hla by *Agr* observed by others during *in vitro* growth (20).

SaeR/S-dependent Transcriptional Regulation of *hla* Promotes Virulence of USA300 during Murine Models of Soft-tissue Infection.

Prior work has shown the SaeR/S TCS has a major impact on virulence observed during soft-tissue infections caused by USA300 (14). As *hla* is strongly transcriptionally regulated by *saeR/S in vitro*, we examined the prominence of this toxin *in vivo* using murine models of soft-tissue infection. Overall abscess size was significantly reduced in USA300 $\Delta$ *hla* relative to the parental strain during the majority of infection (Figure 2A) and dermonecrosis exhibited by USA300 infection was completely abolished during infections produced by USA300 $\Delta$ *hla* (Figures 2B and 2C), congruent with recently published results indicating Hla is a prominent cause of dermonecrosis and enhanced virulence observed in USA300 soft-tissue infections (4). Transcription of *hla* was >40 fold less in USA300 $\Delta$ *saeR/S* relative to the parental wt at 8 hours post-infection (Figure 2D) although no significant differences in bacterial burden between these strains at this time point was observed (Figure 2E). The slightly increased transcription of *hla* in USA300 $\Delta$ *agr* further indicates transcriptional upregulation of this gene *in vivo* is largely dependent upon SaeR/S. Western Blot analysis illustrated both *saeR/S* and *agr* are required for Hla production *in vivo*, supporting *saeR/S*-dependent transcriptional regulation of *hla* and *agr*-dependent post-transcriptional regulation of Hla production (Figure 2F). These results demonstrate SaeR/S-mediated transcription of *hla* is a principal component of USA300 pathogenesis during murine models of soft-tissue infection.

SaeR/S-mediated PMN Plasma Membrane Damage does not Require *hla*.

As the capacity of CA-MRSA to evade destruction by PMNs is believed to be an important factor during pathogenesis (5;27), we examined the pore-forming capacity of USA300, USA300 $\Delta hla$ , and USA300 $\Delta saeR/S$  supernatants against PMNs. At 10, 30, and 60 minutes following exposure to USA300 and USA300 $\Delta hla$  supernatants the majority of PMNs (>90%) exhibited compromised plasma membrane integrity as determined using propidium iodide (PI) staining followed by flow cytometry analysis (Figures 3A and 3B). In contrast, significantly less membrane damage was detected in PMNs treated with USA300 $\Delta saeR/S$  supernatants at 10, 30, and 60 minutes. These results demonstrate the USA300 *saeR/S*-mediated membrane damage to PMNs occurs independently of *hla* and is likely generated through the individual or concerted effect of other pore-forming toxins such as gamma-hemolysin, Panton-Valentine leukocidin, or the recently characterized leukocidin annotated as *USA300\_1974/USA300\_1975* in the USA300 genome. (28;29). These findings are congruent with prior studies demonstrating Hla is capable of associating with the human granulocyte cell surface but is unable to induce pores in this cell type (30). In support of these results, we found no significant difference in survival between USA300 and USA300 $\Delta hla$  during infection of whole human blood while survival of USA300 $\Delta saeR/S$  was significantly increased at 1 hr post-infection and significantly decreased at 5 hours post-infection (Figure 3C). The inability of USA300 $\Delta saeR/S$  to recognize and appropriately respond to the human blood environment may account for the initial increase and subsequent decrease in bacterial survival observed for USA300 $\Delta saeR/S$  relative to USA300.

Increased Cytokine Transcript Abundance Observed for USA300 $\Delta hla$  Relative to USA300 during Human Blood Infection.

To further explore the role of *hla* in *S. aureus* pathogenesis, we examined inflammatory cytokine transcription in human blood at 3 hours post-infection with USA300, USA300 $\Delta hla$ , or DPBS control (Figure 4). For both USA300 and USA300 $\Delta hla$  a significant increase in transcript abundance of numerous cytokines relative to the DPBS control was observed. These included the interleukin-1 superfamily (IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RN, and IL-1F9), CC chemokines (CCL3, CCL4, CCL18, CCL20, and CCL23), CXC chemokines (CXCL1, CXCL10, and CXCL11), Tumor Necrosis Factor (TNF), complement component 3 (C3), and interleukin-17 (IL-17). A significant decrease in the CC chemokine receptor 2 (CCR2) for both USA300 and USA300 $\Delta hla$  was also noted while expression of Interleukin-5 (IL-5) was significantly decreased only in USA300 $\Delta hla$  infected blood. The inflammatory cytokine response observed during USA300 and USA300 $\Delta hla$  infection of human blood was similar to that observed during lung infection by USA300 and a USA300 isogenic deletion mutant of the Panton-Valentine leukocidin (USA300 $\Delta pvl$ ) in a rat model of necrotizing pneumonia (31). However, only minimal differences were noted between the cytokine profiles of USA300 and USA300 $\Delta pvl$  while the cytokine profile of USA300 $\Delta hla$  was noticeably exaggerated relative to USA300. In particular, the overall transcription of the interleukin-1 superfamily and CC chemokines was increased in blood infected with USA300 $\Delta hla$  relative to USA300. Collectively these observations suggest Hla does not play a significant role damaging the PMN plasma membrane but instead influences the overall cytokine response to *S. aureus* infection of human blood.

### Hla causes Human T cell Plasma Membrane Damage

The potent hexameric Hla toxin has long been known to produce pores in a variety of human cell types (30;32-36). We hypothesized the observed *hla*-dependent decrease in cytokine transcription in human blood infected with USA300 may be the result of a Hla-mediated killing of cytokine producing lymphocytes. To further explore this hypothesis, we examined the pore-forming capacity of USA300, USA300 $\Delta$ *hla*, USA300 $\Delta$ *hla* comp, and USA300 $\Delta$ *saeR/S* supernatants on CD3<sup>+</sup> PBMCs (Figure 5A). At 1 hour a significant increase was detected in the number of PI<sup>+</sup>CD3<sup>+</sup> PBMCs treated with USA300 supernatant relative to a media control (Figure 5B), indicating *S. aureus* supernatants compromise the plasma membrane of T cells. In contrast, significantly less PI<sup>+</sup>CD3<sup>+</sup> PBMCs were observed following treatment with USA300 $\Delta$ *hla* and USA300 $\Delta$ *saeR/S* supernatants relative to USA300 supernatant, suggesting Hla is a key component of *S. aureus* supernatant-induced pore formation on human T cells. This interpretation is supported by a rescue of the USA300 phenotype using USA300 $\Delta$ *hla* Comp supernatants. Further studies found significant differences in PI<sup>+</sup>CD4<sup>+</sup> and PI<sup>+</sup>CD8<sup>+</sup> PBMCs treated with USA300 $\Delta$ *hla* supernatant relative to USA300 supernatant, a phenotype rescued in USA300 $\Delta$ *hla* Comp (Figure 5A, 5C, and 5D). Supernatants diluted by 50% and 25% illustrated concentration-dependent membrane damage on CD3<sup>+</sup> PBMCs that was significantly reduced in USA300 $\Delta$ *hla* and USA300 $\Delta$ *saeR/S* supernatants relative to USA300 supernatants and was significantly enhanced in USA300 $\Delta$ *hla* Comp supernatants (Figure 5E). Infection of purified PBMCs with USA300 $\Delta$ *hla* and USA300 $\Delta$ *saeR/S* resulted in a significantly reduced membrane permeability of CD4<sup>+</sup> and CD8<sup>+</sup> PBMCs at 6 hours relative to infection with USA300 or

USA300 $\Delta hla$  Comp (Figure 5F). Taken together, these results suggest Hla is a major effector of SaeR/S-mediated pore formation on T cells.

#### Rapid Human T cell Proliferation is Promoted by Hla

Interestingly, a significant increase in the number of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> PBMCs was observed following 1 hour exposure to USA300 and USA300 $\Delta hla$  Comp supernatants but not USA300 $\Delta hla$  or USA300 $\Delta saeR/S$  supernatants (Figures 5B, 5C, and 5D). To further explore the observed *hla*-dependent increase in the number of T cells following incubation with USA300 supernatants, CFSE proliferation assays were performed on PBMCs incubated with diluted supernatants from USA300, USA300 $\Delta hla$ , USA300 $\Delta hla$  Comp, and USA300 $\Delta saeR/S$  (Figure 6). Media (TSB) and 0.1% saponin controls were also tested. At 0, 30, and 60 minutes PI<sup>-</sup>CD4<sup>+</sup> (Figure 6A) and PI<sup>-</sup>CD8<sup>+</sup> (Figure 6C) were gated and mean CFSE fluorescence determined (Figure 6B and 6D). Decreased mean CFSE fluorescence attributable to cell proliferation was observed for PI<sup>-</sup>CD4<sup>+</sup> and PI<sup>-</sup>CD8<sup>+</sup> PBMCs treated with diluted USA300 and USA300 $\Delta hla$  Comp supernatants but absent following treatment with TSB and 0.1% saponin controls or USA300 $\Delta hla$  and USA300 $\Delta saeR/S$  supernatants (Figure 6E and 6F). Further, bacterial supernatant-induced proliferation of PI negative CD4<sup>+</sup> and CD8<sup>+</sup> PBMCs was concentration-dependent and only observed for Hla-containing supernatants (Figure 6E). These results demonstrate that supernatants derived from USA300 producing Hla induce a rapid concentration-dependent expansion of T cells.

## Discussion

During the course of pathogenesis, it is believed that *S. aureus* virulence gene expression is largely controlled by the concerted influence of bacterial TCSs that recognize specific host-associated stimuli and alter gene transcription in response. The *saeR/S* TCS is critical to USA300 pathogenesis during murine models of soft-tissue infection and invasive disease (14) and is believed to be activated by PMN derived components (13). SaeR/S mediates Hla expression (18;19;21), and recombinant SaeR directly binds the *hla* promoter to augment transcription (14;16). These findings are corroborated by microarray analysis of CA-MRSA following PMN phagocytosis or exposure to individual PMN components that demonstrate a marked transcriptional response to PMN-associated stimuli including an immediate increase in *hla* transcription (5;37). This investigation demonstrates SaeR/S strongly mediates *hla* transcription *in vitro* as well as during infection and indicates this toxin is a primary SaeR/S effector contributing to *S. aureus* pathogenesis.

Though *hla* did not have a significant impact on *S. aureus* survival in human blood or induce membrane damage against PMNs, an overall increase in pro-inflammatory cytokines in human blood was observed at 3 hours post-infection with USA300 $\Delta$ *hla* relative to USA300. Given Hla is thought to lyse human lymphocytes (30;32) and a higher population of cytokine producing cells would generate more cytokine transcripts, we examined the capacity of supernatants from USA300 both with and without Hla to cause human T cell plasma membrane damage. Our results demonstrate supernatants from USA300 expressing Hla produce pores in human CD4<sup>+</sup> and CD8<sup>+</sup> PBMCs in a dose-dependent manner within 1

hour of exposure and indicate this toxin is the primary effector of *saeR/S*-mediated lysis of human T cells.

Unexpectedly, a significant Hla-dependent increase in T cell numbers at 1 hour was noted following exposure to USA300 supernatants, and CFSE proliferation assays confirmed a rapid dose-dependent proliferation of PI $CD4^+$  and PI $CD8^+$  PBMCs following exposure only to USA300 supernatants expressing Hla. The proliferation of  $CD4^+$  T cells and keratinocytes, but not Jurkat T cells, in response to purified Hla treatment alone has been observed at times exceeding two days (34;35;38). Others have demonstrated Hla and toxic shock syndrome toxin-1 act synergistically to induce expression of IL-6 and IL-8 in human vaginal epithelial cells (33), and the presence of superantigenic peptides in USA300 supernatants may contribute to the rapid Hla-dependent proliferation of  $CD4^+$  and  $CD8^+$  T cells observed in this study.

Recent work demonstrates Hla strongly binds to a disintegrin and metalloprotease 10 (ADAM10), a cell surface sheddase important for a number of immunological functions, and this binding is required for the initiation of pore formation on eukaryotic cell surfaces (39). Interestingly, ADAM10 has been shown to mediate cleavage of lymphocyte activation gene-3 (LAG-3) that is required for efficient T cell proliferation and cytokine production (40). These findings imply the interaction between Hla and ADAM10 may enhance cleavage of LAG-3 to promote the rapid T cell expansion observed in this investigation. Additionally, Hla has been shown to cause disruption of focal adhesions involved with integrin signaling in A549 human alveolar basal epithelial cells that results in dephosphorylation of focal adhesion kinase (FAK) and c-Src (39). The FAK-Src complex is thought to play a role in various cell

functions including cell motility, cell cycle progression, and cell survival (41). Thus it is also possible that the disruption of focal adhesions caused by Hla stimulates T cell proliferation.

PMNs are essential for defense against *S. aureus* infections; individuals with compromised PMN numbers or function are highly susceptible to *S. aureus* infection and neutropenic mice are significantly limited in their ability to clear both invasive and superficial *S. aureus* infection (42-44). However, evidence also suggests activated CD4<sup>+</sup> T cells elicit an exaggerated CXC chemokine-mediated influx of PMNs to the site of *S. aureus* infection that significantly advances bacterial survival and host tissue damage (42;45-48). Notably, *hla* has been shown to promote CXC chemokine migration of PMNs to the airway during murine models of *S. aureus* pneumonia (45) and facilitates both NOD2 and NLRP3-dependent recognition of *S. aureus* leading to IL-1 $\beta$  expression and a subsequent inflammatory response (36;49-51). This investigation indicates SaeR/S-mediated expression of Hla manipulates T cell function to facilitate a hyperinflammatory response that significantly advances *S. aureus* pathogenesis and provides support for further research exploring the efficacy of therapeutic approaches and immunization strategies that specifically target this toxin.

Literature Cited

- (1) Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 2007 Oct 17;298(15):1763-71.
- (2) Moran GJ, Krishnadasan A, Gorwitz RJ, et al. Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med* 2006 Aug 17;355(7):666-74.
- (3) Nygaard TK, Deleo FR, Voyich JM. Community-associated methicillin-resistant *Staphylococcus aureus* skin infections: advances toward identifying the key virulence factors. *Curr Opin Infect Dis* 2008 Apr;21(2):147-52.
- (4) Kennedy AD, Bubeck WJ, Gardner DJ, et al. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. *J Infect Dis* 2010 Oct 1;202(7):1050-8.
- (5) Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* 2005 Sep 15;175(6):3907-19.
- (6) Voyich JM, Otto M, Mathema B, et al. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J Infect Dis* 2006 Dec 15;194(12):1761-70.
- (7) Wang R, Braughton KR, Kretschmer D, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 2007 Nov 11.
- (8) Li M, Diep BA, Villaruz AE, et al. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* 2009 Mar 17.
- (9) Li M, Cheung GY, Hu J, et al. Comparative Analysis of Virulence and Toxin Expression of Global Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strains. *J Infect Dis* 2010 Nov 4.
- (10) Montgomery CP, Boyle-Vavra S, Adem PV, et al. Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. *J Infect Dis* 2008 Aug 15;198(4):561-70.

- (11) Bubeck WJ, Bae T, Otto M, Deleo FR, Schneewind O. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat Med* 2007 Dec;13(12):1405-6.
- (12) Bubeck WJ, Schneewind O. Vaccine protection against *Staphylococcus aureus* pneumonia. *J Exp Med* 2008 Feb 18;205(2):287-94.
- (13) Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C. The virulence regulator Sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. *J Bacteriol* 2008 May;190(10):3419-28.
- (14) Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 2010 Jan 15;201(2):241-54.
- (15) Voyich JM, Vuong C, Dewald M, et al. The SaeR/S Gene Regulatory System Is Essential for Innate Immune Evasion by *Staphylococcus aureus*. *J Infect Dis* 2009 Apr 17.
- (16) Sun F, Li C, Jeong D, Sohn C, He C, Bae T. 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* 2010 Apr;192(8):2111-27.
- (17) Loughman JA, Fritz SA, Storch GA, Hunstad DA. Virulence Gene Expression in Human Community-Acquired *Staphylococcus aureus* Infection. *J Infect Dis* 2009 Feb 1;199(3):294-301.
- (18) Goerke C, Fluckiger U, Steinhuber A, et al. Role of *Staphylococcus aureus* global regulators sae and sigmaB in virulence gene expression during device-related infection. *Infect Immun* 2005 Jun;73(6):3415-21.
- (19) Li D, Cheung A. Repression of hla by rot is dependent on sae in *Staphylococcus aureus*. *Infect Immun* 2008 Mar;76(3):1068-75.
- (20) Pang YY, Schwartz J, Thoendel M, Ackermann LW, Horswill AR, Nauseef WM. agr-Dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J Innate Immun* 2010;2(6):546-59.
- (21) Xiong YQ, Willard J, Yeaman MR, Cheung AL, Bayer AS. Regulation of *Staphylococcus aureus* alpha-toxin gene (hla) expression by agr, sarA, and sae in vitro and in experimental infective endocarditis. *J Infect Dis* 2006 Nov 1;194(9):1267-75.

- (22) Diep BA, Gill SR, Chang RF, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 2006 Mar 4;367(9512):731-9.
- (23) Bruckner R. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. *FEMS Microbiol Lett* 1997 Jun 1;151(1):1-8.
- (24) Lauderdale KJ, Boles BR, Cheung AL, Horswill AR. Interconnections between Sigma B, agr, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. *Infect Immun* 2009 Apr;77(4):1623-35.
- (25) Malone CL, Boles BR, Lauderdale KJ, Thoendel M, Kavanaugh JS, Horswill AR. Fluorescent reporters for *Staphylococcus aureus*. *J Microbiol Methods* 2009 Jun;77(3):251-60.
- (26) Lauderdale KJ, Malone CL, Boles BR, Morcuende J, Horswill AR. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. *J Orthop Res* 2009 Jul 16.
- (27) Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, et al. Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun* 2010;2(6):560-75.
- (28) Ventura CL, Malachowa N, Hammer CH, et al. Identification of a novel *Staphylococcus aureus* two-component leukotoxin using cell surface proteomics. *PLoS One* 2010;5(7):e11634.
- (29) DuMont A L, Nygaard T K, Watkins R L, et al. Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. *Molecular Microbiology* 2010; Accepted article.
- (30) Valeva A, Walev I, Pinkernell M, et al. Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. *Proc Natl Acad Sci U S A* 1997 Oct 14;94(21):11607-11.
- (31) Montgomery CP, Daum RS. Transcription of inflammatory genes in the lung after infection with community-associated methicillin-resistant *Staphylococcus aureus*: a role for panton-valentine leukocidin? *Infect Immun* 2009 May;77(5):2159-67.
- (32) Jonas D, Walev I, Berger T, Liebetrau M, Palmer M, Bhakdi S. Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect Immun* 1994 Apr;62(4):1304-12.

- (33) Brosnahan AJ, Mantz MJ, Squier CA, Peterson ML, Schlievert PM. Cytolysins augment superantigen penetration of stratified mucosa. *J Immunol* 2009 Feb 15;182(4):2364-73.
- (34) Haslinger B, Strangfeld K, Peters G, Schulze-Osthoff K, Sinha B. Staphylococcus aureus alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. *Cell Microbiol* 2003 Oct;5(10):729-41.
- (35) Haugwitz U, Bobkiewicz W, Han SR, et al. Pore-forming Staphylococcus aureus alpha-toxin triggers epidermal growth factor receptor-dependent proliferation. *Cell Microbiol* 2006 Oct;8(10):1591-600.
- (36) Craven RR, Gao X, Allen IC, et al. Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* 2009;4(10):e7446.
- (37) Palazzolo-Ballance AM, Reniere ML, Braughton KR, et al. Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant Staphylococcus aureus. *J Immunol* 2008 Jan 1;180(1):500-9.
- (38) Breuer K, Wittmann M, Kempe K, et al. Alpha-toxin is produced by skin colonizing Staphylococcus aureus and induces a T helper type 1 response in atopic dermatitis. *Clin Exp Allergy* 2005 Aug;35(8):1088-95.
- (39) Wilke GA, Bubeck WJ. Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci U S A* 2010 Jul 27;107(30):13473-8.
- (40) Li N, Wang Y, Forbes K, et al. Metalloproteases regulate T-cell proliferation and effector function via LAG-3. *EMBO J* 2007 Jan 24;26(2):494-504.
- (41) Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* 2006 Oct;18(5):516-23.
- (42) Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of Staphylococcus aureus inside neutrophils contributes to infection. *J Immunol* 2000 Apr 1;164(7):3713-22.
- (43) Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. *N Engl J Med* 2000 Dec 7;343(23):1703-14.
- (44) Molne L, Verdrengh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous infection caused by Staphylococcus aureus. *Infect Immun* 2000 Nov;68(11):6162-7.

- (45) Bartlett AH, Foster TJ, Hayashida A, Park PW. Alpha-toxin facilitates the generation of CXC chemokine gradients and stimulates neutrophil homing in *Staphylococcus aureus* pneumonia. *J Infect Dis* 2008 Nov 15;198(10):1529-35.
- (46) McLoughlin RM, Solinga RM, Rich J, et al. CD4+ T cells and CXC chemokines modulate the pathogenesis of *Staphylococcus aureus* wound infections. *Proc Natl Acad Sci U S A* 2006 Jul 5;103(27):10408-13.
- (47) McLoughlin RM, Lee JC, Kasper DL, Tzianabos AO. IFN-gamma regulated chemokine production determines the outcome of *Staphylococcus aureus* infection. *J Immunol* 2008 Jul 15;181(2):1323-32.
- (48) Weidenmaier C, McLoughlin RM, Lee JC. The zwitterionic cell wall teichoic acid of *Staphylococcus aureus* provokes skin abscesses in mice by a novel CD4+ T-cell-dependent mechanism. *PLoS One* 2010;5(10):e13227.
- (49) Hruz P, Zinkernagel AS, Jenikova G, et al. NOD2 contributes to cutaneous defense against *Staphylococcus aureus* through alpha-toxin-dependent innate immune activation. *Proc Natl Acad Sci U S A* 2009 Aug 4;106(31):12873-8.
- (50) Miller LS, Pietras EM, Uricchio LH, et al. Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo. *J Immunol* 2007 Nov 15;179(10):6933-42.
- (51) Munoz-Planillo R, Franchi L, Miller LS, Nunez G. A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J Immunol* 2009 Sep 15;183(6):3942-8.

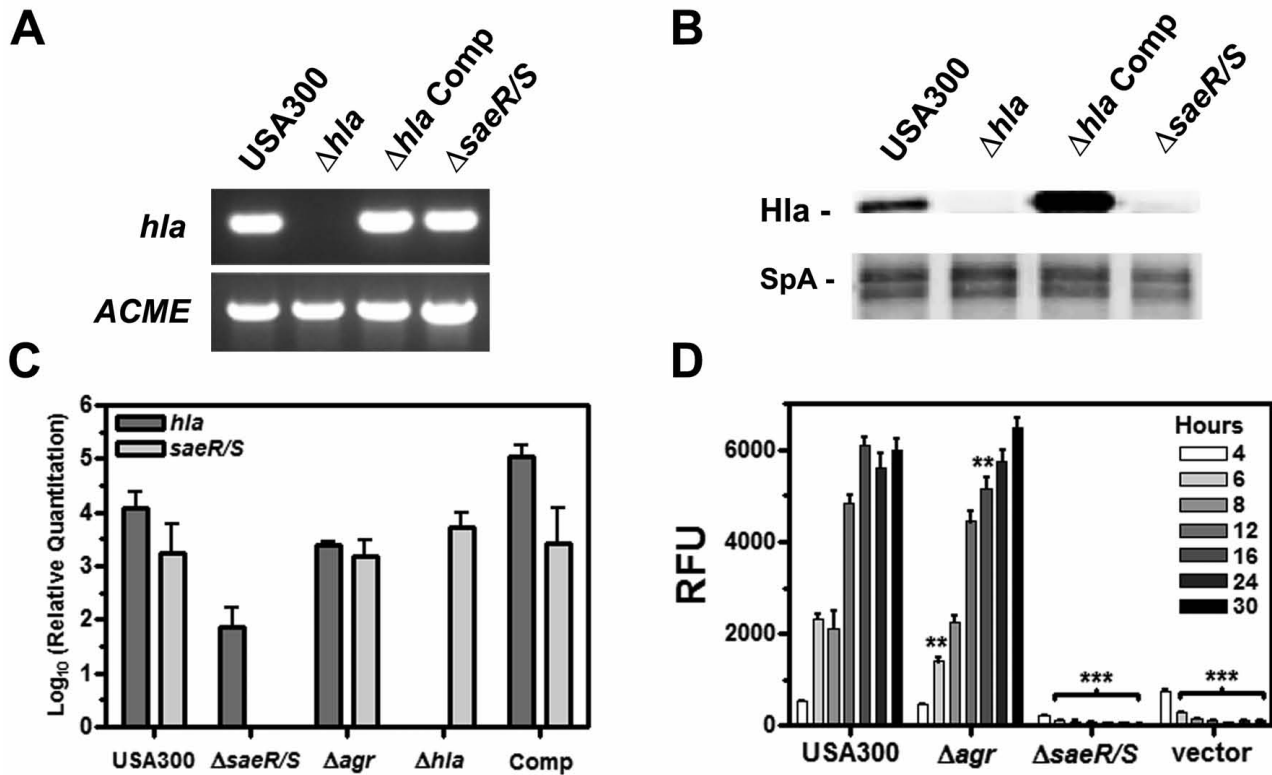


Figure 1. SaeR/S strongly regulates *hla* transcription during *in vitro* growth. A) Colony PCR for *hla* and *ACME* in USA300, USA300 $\Delta hla$ , USA300 $\Delta hla$  Comp, and USA300 $\Delta saeR/S$ . B) Western blot for Hla on 5 hr culture supernatants from USA300, USA300 $\Delta hla$ , USA300 $\Delta hla$  Comp, and USA300 $\Delta saeR/S$ . C) Real-time RT-PCR for *hla* and *saeR/S* during early-stationary growth of USA300, USA300 $\Delta saeR/S$ , and USA300 $\Delta agr$ , USA300 $\Delta hla$ , and USA300 $\Delta hla$  Comp. D) Fluorescence of GFP reporter constructs specific for the *hla* promoter transformed into USA300, USA300 $\Delta agr$ , and USA300 $\Delta saeR/S$  during *in vitro* growth. A promoterless GFP reporter vector was transformed into USA300 as a control. \*\* P < 0.01 and \*\*\* P < 0.001 relative to USA300 as determined by repeated measures ANOVA with Tukey's post-test.

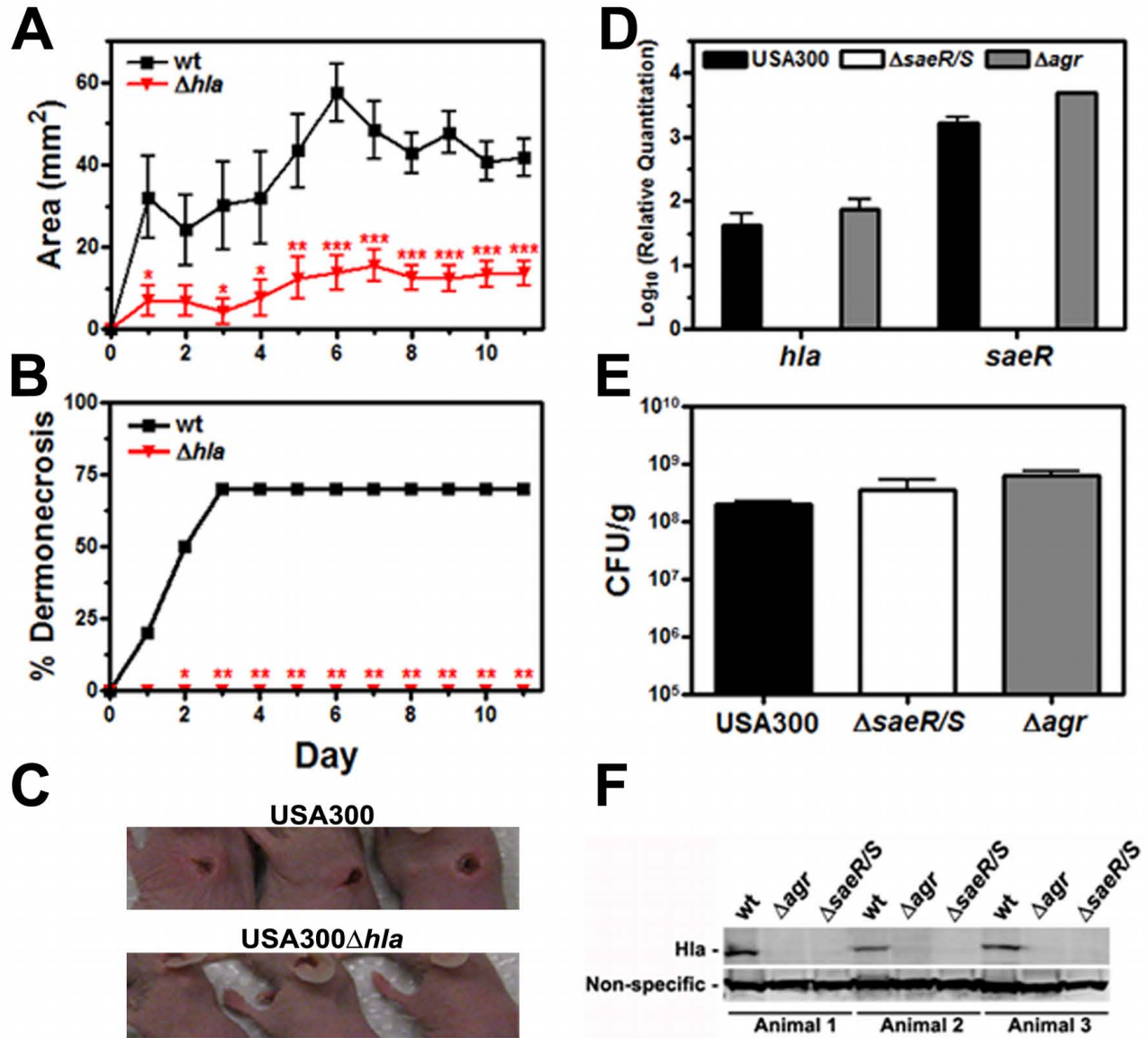


Figure 2. Virulence of USA300 $\Delta hla$  is significantly attenuated during murine models of soft-tissue infection. A) Average area of soft-tissue infection and B) the incidence of dermonecrosis during a murine model of soft-tissue infection caused by USA300 and USA300 $\Delta hla$ . Data represents 10 mice per group. C) Dermonecrosis presented by USA300 soft-tissue infections and absent from USA300 $\Delta hla$  soft-tissue infections at day 4 post-infection. D) Transcription of *hla* and *saeR*, E) bacterial counts and F) Western blot for Hla during soft-tissue infection caused by USA300, USA300 $\Delta saeR/S$ , and USA300 $\Delta agr$  at 8 hours post-infection. Data represents 3 mice per group with \*  $P < 0.05$  \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  relative to USA300 for all figures as determined by paired t-test.

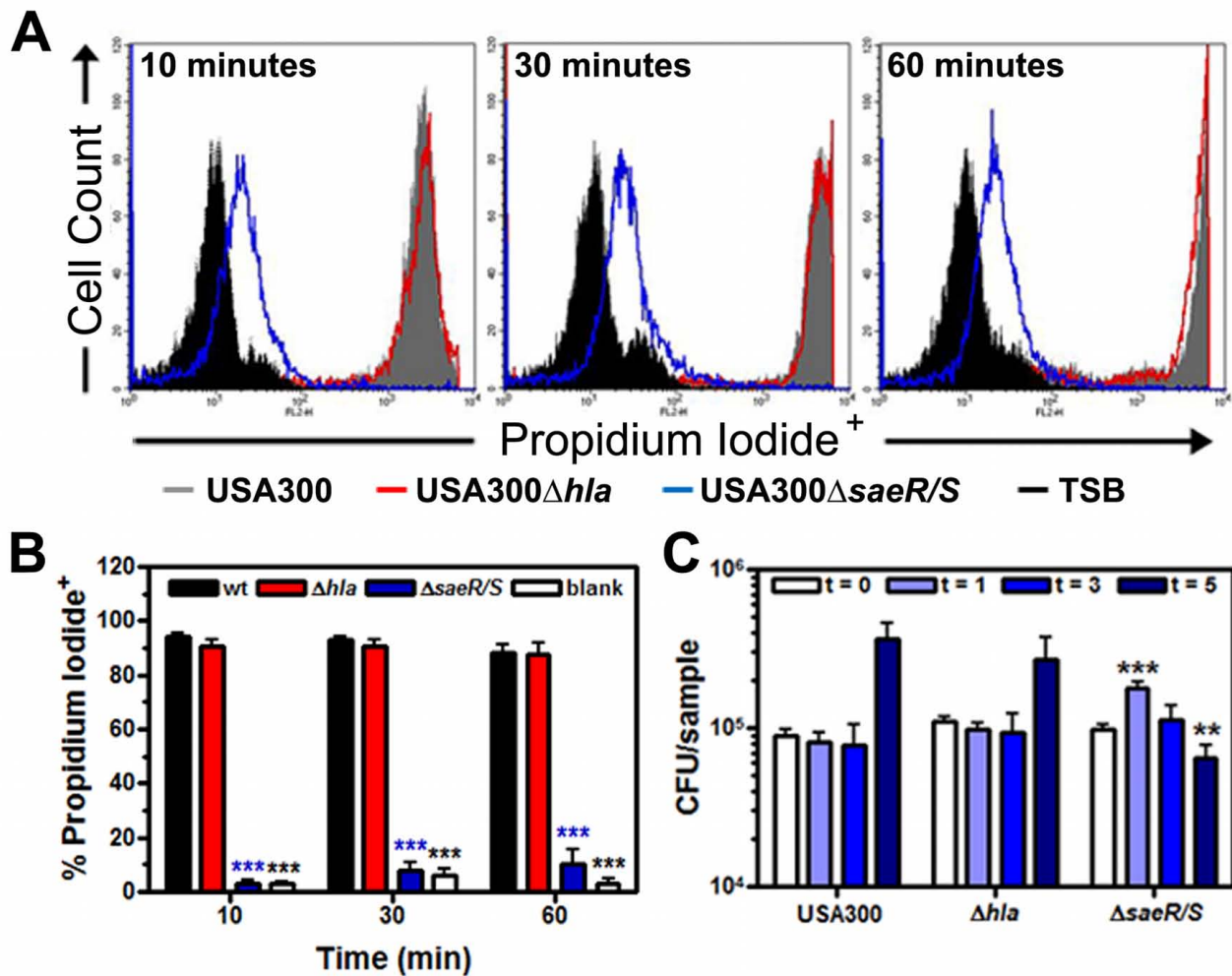


Figure 3. The influence of *hla* during USA300 infection of human blood. A) Representative flow cytometry histograms and B) compiled results from 5 replicate experiments of purified PMNs stained with propidium iodide following exposure to supernatants from USA300, USA300 $\Delta$ *hla*, USA300 $\Delta$ *hla* Comp, USA300 $\Delta$ *saeR/S*, or TSB media control at 10, 30, and 60 minutes. C) Bacterial counts of USA300, USA300 $\Delta$ *hla*, and USA300 $\Delta$ *saeR/S* following exposure to human blood at 0, 1, 3, and 5 hours. Data represents 15 replicate experiments from more than 10 separate blood donors. \*  $P < 0.05$  \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  relative to USA300 for figures B and C as determined by two-tailed unpaired t-test.

Symbol	USA300	Wt P-Value	$\Delta hla$	$\Delta hla$ P-Value	Refseq	Description
IL1A	11.5685	0.0024	17.6532	0.0004	NM_000575	Interleukin 1, alpha
CCL3	10.3738	0.0315	15.049	0.0167	NM_002983	Chemokine (C-C motif) ligand 3
CCL4	9.4166	0.0214	13.4062	0.0026	NM_002984	Chemokine (C-C motif) ligand 4
CCL20	8.885	0.0393	9.8637	0.0132	NM_004591	Chemokine (C-C motif) ligand 20
CCL18	8.4183	0.1293	10.0032	0.0003	NM_002988	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
IL1RN	8.0859	0.0765	12.4715	0.0105	NM_000577	Interleukin 1 receptor antagonist
CXCL11	7.4132	0.0214	7.5139	0.0028	NM_005409	Chemokine (C-X-C motif) ligand 11
TNF	4.8735	0.0133	5.0368	0.0013	NM_000594	Tumor necrosis factor (TNF superfamily, member 2)
IL1B	4.3996	0.0588	5.8654	0.0062	NM_000576	Interleukin 1, beta
CXCL10	4.145	0.0827	4.1231	0.0079	NM_001565	Chemokine (C-X-C motif) ligand 10
C3	3.9785	0.2130	3.1749	0.0441	NM_000064	Complement component 3
IL17C	3.8621	0.0066	3.4875	0.0367	NM_013278	Interleukin 17C
CCL23	3.3627	0.0647	4.0524	0.0106	NM_005064	Chemokine (C-C motif) ligand 23
IL1F9	3.0424	0.0059	3.5523	0.0399	NM_019618	Interleukin 1 family, member 9
CXCL1	2.609	0.0002	3.0007	0.0003	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
LTA	1.9475	0.0092	2.1832	0.0012	NM_000595	Lymphotoxin alpha (TNF superfamily, member 1)
CCL15	1.139	0.9038	2.8184	0.0321	NM_032965	Chemokine (C-C motif) ligand 15
IL5	1.0883	0.7518	-3.5983	0.0353	NM_000879	Interleukin 5 (colony-stimulating factor, eosinophil)
CCR2	-2.4292	0.0037	-2.7161	0.0001	NM_000648	Chemokine (C-C motif) receptor 2

Figure 4. Hla expression modulates pro-inflammatory cytokine transcript abundance during USA300 infection of human blood. Cytokine transcription profiles during USA300 and USA300 $\Delta hla$  infection of human blood at 3 hours post-infection relative to DPBS mock-infected blood. Data represents compiled results from 3 separate blood donors. Fold-changes and P values were calculated using SA Biosciences™ web-based software as described in Materials and Method.

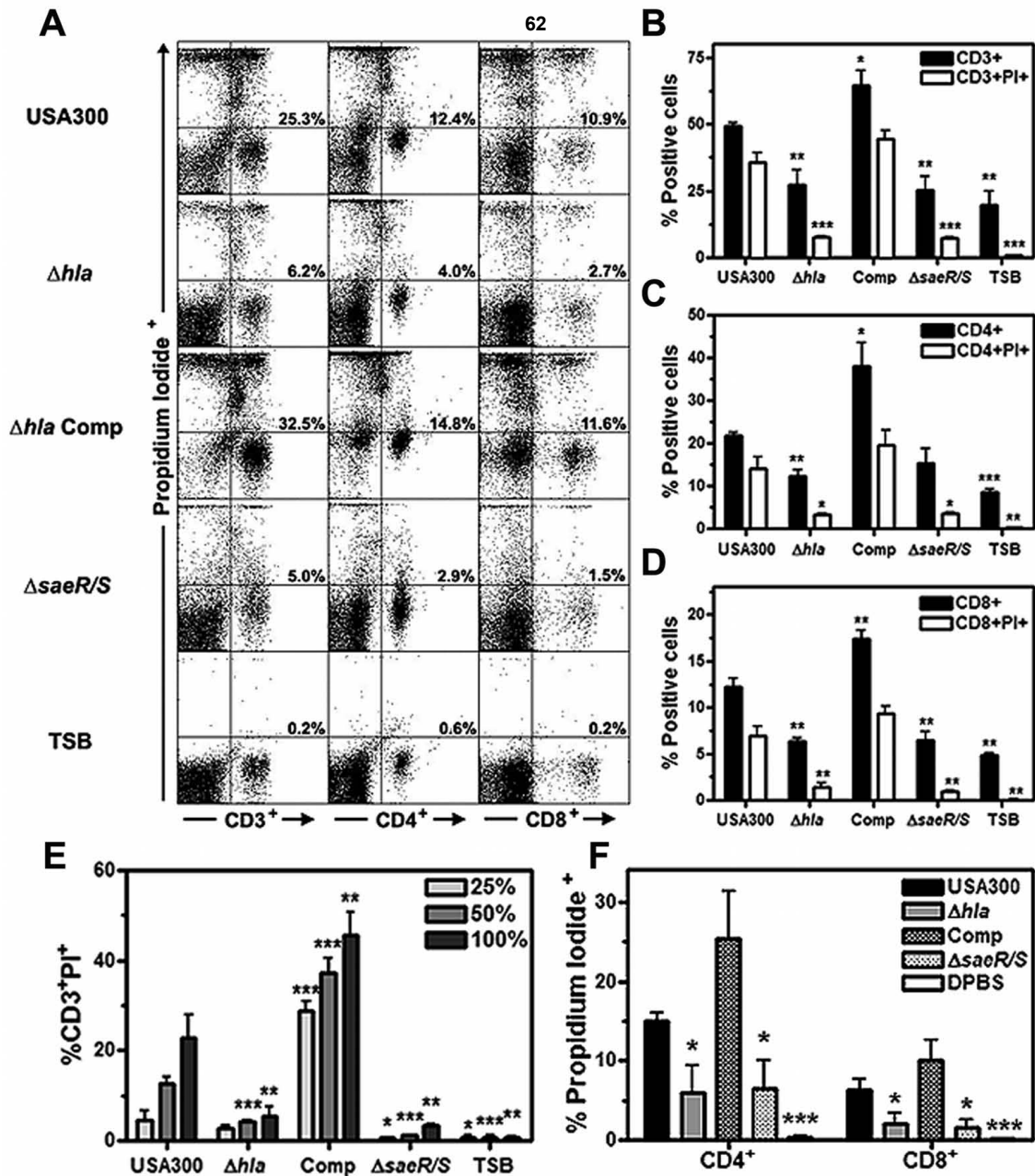


Figure 5. Hla causes T cell plasma membrane damage. A) Representative flow cytometry dot plots of human PBMCs stained with propidium iodide (PI) and anti-CD3, anti-CD4, or anti-CD8 following exposure for 1 hour to USA300, USA300 $\Delta hla$ , USA300 $\Delta hla$  Comp, and USA300 $\Delta saeR/S$  filtered culture supernatants or a TSB control. The percentage of PI+CD3+, PI+CD4+, or PI+CD8+ PBMCs are indicated in the upper right quadrant. Compiled flow cytometry analysis of PBMCs exposed to filtered culture supernatants for 1 hour then stained with PI and antibodies against B) CD3, C) CD4, or D) CD8. E) Compiled flow cytometry analysis of PBMCs exposed to undiluted cell culture supernatants or cell culture supernatants diluted to 50% or 25% with TSB for 1 hour then stained with PI and anti-CD3. F) Compiled flow cytometry analysis of PBMCs infected at an MOI of 10 with USA300, USA300 $\Delta hla$ , USA300 $\Delta hla$  Comp, and USA300 $\Delta saeR/S$  or a TSB control at 6 hours post-infection stained with PI and anti-CD4 or anti-CD8. Figures B, C, D, E, and F represent compiled results from 3 separate blood donors with \*  $P < 0.05$  \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  relative to USA300 as determined by one-tailed unpaired t-test.

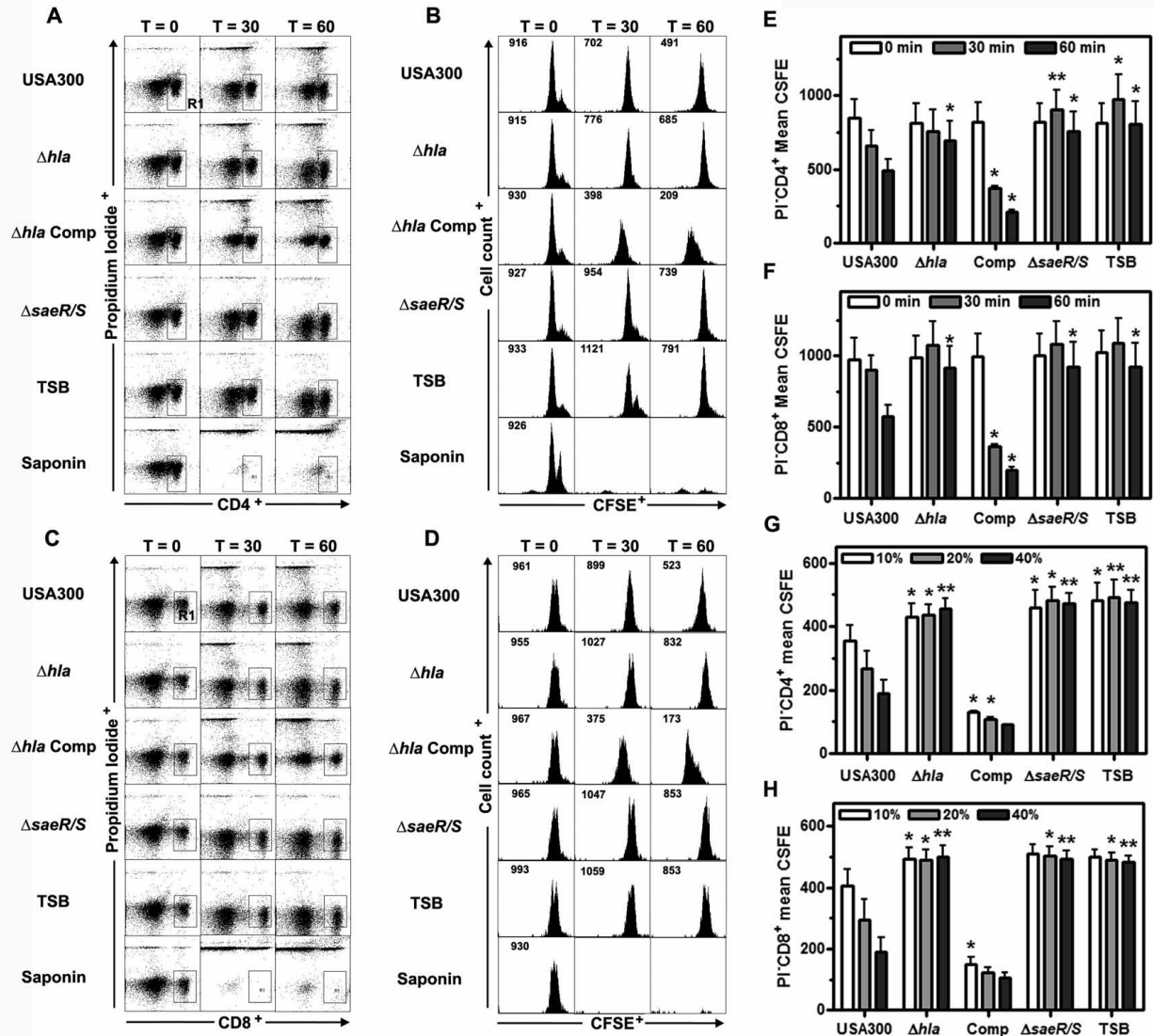


Figure 6. Proliferation of T-cells promoted by Hla. Flow cytometry dot plots of CFSE treated PBMCs at 0, 30, and 60 minutes following exposure to 0.1% saponin, TSB control, or filtered supernatants diluted to 10% with TSB from USA300, USA300 $\Delta hla$ , USA300 $\Delta hla$  Comp, and USA300 $\Delta saeR/S$  were stained with propidium iodide (PI) and A) anti-CD4 or B) anti-CD8. Flow cytometry histograms of PI negative and C) CD4<sup>+</sup> or D) CD8<sup>+</sup> were gated (R1) and examined for CFSE fluorescence with mean CFSE fluorescence indicated in grey. Compiled flow cytometry analysis of mean CFSE fluorescence for PI negative and E) CD4<sup>+</sup> or F) CD8<sup>+</sup> cells at 0, 30, and 60 minutes T following exposure to 10% diluted supernatants from 4 separate blood donors. Compiled flow cytometry analysis of mean CFSE fluorescence for PI negative and G) CD4<sup>+</sup> or H) CD8<sup>+</sup> PBMCs following exposure to 10%, 20%, or 40% diluted supernatants from 3 separate blood donors. For all figures \* P < 0.05 \*\* P < 0.01 and \*\*\* P < 0.001 relative to USA300 as determined by paired one-tailed t-test.

## CHAPTER 5

## PROSPECTIVE STUDIES AND CONCLUSIONS

Defining the SaeR/S Activation Signal

The ability of pathogenic bacteria to recognize and respond to the host immune system is essential to causing disease. This research elucidates the role of the SaeR/S two-component system of *S. aureus* during infection and underscores genes that may be important for pathogenesis of this bacteria. SaeR/S is shown to be crucial for disease using murine models of infection while transcriptional and DNA binding assays identified a conserved SaeR binding sequence within the promoter region of numerous virulence genes. Identification of the SaeR binding sequence has allowed the construction of SaeR/S activation reporter constructs that have enabled experiments exploring the SaeR/S activation signal. Preliminary work has demonstrated a concentration-dependent increase in *hla* transcription following exposure to human  $\alpha$ -defensin but not human LL37 (Figure 1), suggesting human  $\alpha$ -defensin activates SaeR/S in accordance with findings by others (1). Clearly defining the SaeR/S activation signal will provide direction for further studies exploring therapeutic strategies inhibiting SaeR/S activation. For example, we have performed promising preliminary work in collaboration with the Kaufmann lab of the Scripps Research Institute blocking the well studied Agr two-component quorum-sensing system of *S. aureus* during murine models of soft-tissue infection caused by USA300 (Figure 2).

### Investigating the Rapid *hla*-dependent Proliferation of Human T cells.

The SaeR/S two-component system strongly mediates transcriptional regulation of *hla*, a potent pore-forming toxin shown to generate human T cell plasma membrane damage. Unexpectedly, we noted a rapid *hla*-dependent proliferation of human T cells following exposure to USA300 supernatants. The presence of superantigenic peptides in USA300 supernatants may be an important component of the rapid T cell proliferation observed, as the TSST-1 toxin has been shown to work synergistically with Hla to induce cytokine production (2). However, others have shown commercially available Hla alone can induce proliferation of T cells at times greater than 2 days (3;4). Future studies repeating *hla*-dependent T cell proliferation assays and defining the  $\beta$  chain specificity of proliferated T cells using a IOTest Beta Mark kit will help determine if T cell proliferation is poly-clonal and likely superantigen-dependent or monoclonal and possibly specific for Hla. Hla is thought to activate monocytes via NOD2 and inflammasome-mediated pathways (5-8) and these cell types may also be important for the *hla*-mediated T cell proliferation we observed. First isolating CD4<sup>+</sup> and CD8<sup>+</sup> PBMCs using flow cytometry then repeating *hla*-dependent T cell proliferation assays will determine if other cell types such as monocytes contribute to rapid T cell expansion upon exposure to USA300 supernatants containing Hla.

### Defining the Primary Effector of SaeR/S-mediated PMN Plasma Membrane Damage

Though Hla is accountable for SaeR/S-dependent human T cell plasma membrane damage, this toxin is not responsible for the rapid SaeR/S-dependent human PMN plasma membrane damage observed following exposure to USA300 supernatants. SaeR/S also

mediates the transcriptional regulation of other pore-forming toxins, including the Panton-Valentine leukocidin, gamma-hemolysin, and the recently characterized leukocidin annotated as *USA300\_1974/1975*. The individual contribution of each of these toxins to SaeR/S-mediated PMN plasma membrane damage remains unclear and is a subject of further investigation by our lab. However, we have recently published results in collaboration with the Torres Lab of the New York University School of Medicine (9) that illustrate *USA300\_1974/1975* contributes to PMN membrane damage in agreement with findings made by others (10). Currently we are confirming the transcriptional regulation of *USA300\_1974/1975* both *in vitro* and *in vivo* and are exploring the importance of this toxin during murine models of infection.

#### The Role of other SaeR/S Regulated Virulence Genes during USA300 Infection

In addition to the different pore-forming toxins mentioned above, the SaeR/S two-component system regulates other virulence genes including an uncharacterized nuclease (*nuc*), an operon encoding 6 distinct serine proteases (*spl*), and the *arlR/S* two-component system. 5'RACE and EMSAs have been performed to demonstrate the *nuc* promoter region contains the SaeR/S binding sequence (Figure 3) and recombinant SaeR<sup>His</sup> directly interacts with the *nuc* promoter (Figure 4), though with less affinity than the *hla* or *sbi* promoters. Murine models of invasive infection also indicate *nuc* is important for USA300 dissemination (data not shown). Research in collaboration with the Horswill lab of the Roy J. and Lucille A. Carver College of Medicine at the University of Iowa that investigates the role of *nuc* in USA300 pathogenesis comprises a manuscript currently in preparation. The

role of the *spl* operon during USA300 pathogenesis has not been examined, though the promoter region of this operon contains the SaeR/S binding sequence and an isogenic deletion mutant of the entire *spl* operon in USA300 has been generated for further studies (data not shown). The promoter region of *arlR/S* two-component system also contains the SaeR/S binding sequence and EMSAs demonstrated strong binding of SaeR to this promoter that was later verified by others (11) although differential expression of the *arlR/S* two component system was not detected in USA300 $\Delta$ *saeR/S*. Using an isogenic deletion mutant of *arlR/S* in USA300 (USA300 $\Delta$ *arlR/S*), we have demonstrated this two-component system is important during both superficial and invasive infection caused by *S. aureus* (Figure 5) as indicated by others (12). We are currently performing oligonucleotide microarray analysis on USA300 $\Delta$ *arlR/S* to determine genes differentially transcribed by this two-component system that may account for the attenuated virulence observed for this strain.

### Conclusions

*S. aureus* infection remains a serious healthcare concern worldwide despite the advent of antibiotic therapy in the early 1940's (13). A clearer understanding of how *S. aureus* recognizes host tissue during infection and the bacterial response that results in disease may promote novel treatment strategies against this bacteria (14). This investigation demonstrates the SaeR/S two-component system of *S. aureus* PFGE type USA300 is essential to pathogenesis during both soft-tissue and invasive infection by directly upregulating transcription of numerous virulence genes that destroy host cells, augment bacterial adherence to host tissue, and manipulate the host immune response. A conserved consensus

sequence specific to promoter regions of genes under strong transcriptional regulation by *saeR/S* was identified and binding of recombinant SaeR<sup>His</sup> was shown to be specific to this conserved consensus sequence. These results have provided direction towards ongoing investigations characterizing the role of SaeR/S regulated virulence genes such as the  $\alpha$ -toxin gene (*hla*), the leukocidin annotated as *USA300\_1974/1975*, the thermonuclease *nuc*, the *spl* serine protease operon, and the *arlR/S* two-component system.

Robust binding of recombinant SaeR<sup>His</sup> to the promoter region of *hla* correlated with two conserved consensus sequences within the *hla* promoter. Further studies demonstrated SaeR/S-dependent transcription of *hla* during murine models of soft-tissue infection corresponded to more severe disease caused by USA300, suggesting *hla* is a primary effector of *saeR/S*-dependent USA300 pathogenesis. Though this toxin was not responsible for SaeR/S-mediated plasma membrane damage to PMNs, USA300 supernatants containing Hla induced rapid concentration-dependent human T cell plasma membrane damage. Surprisingly, rapid Hla-dependent human T cell proliferation was also observed upon exposure to USA300 supernatants. Taken together, this research furthers our understanding of mechanisms fundamental to *S. aureus* pathogenesis and provides a solid foundation for innovative immunization and therapeutic strategies inhibiting these processes to treat *S. aureus* infection.

Literature cited

- (1) Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C. The virulence regulator Sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. *J Bacteriol* 2008 May;190(10):3419-28.
- (2) Brosnahan AJ, Mantz MJ, Squier CA, Peterson ML, Schlievert PM. Cytolysins augment superantigen penetration of stratified mucosa. *J Immunol* 2009 Feb 15;182(4):2364-73.
- (3) Breuer K, Wittmann M, Kempe K, et al. Alpha-toxin is produced by skin colonizing *Staphylococcus aureus* and induces a T helper type 1 response in atopic dermatitis. *Clin Exp Allergy* 2005 Aug;35(8):1088-95.
- (4) Haslinger B, Strangfeld K, Peters G, Schulze-Osthoff K, Sinha B. *Staphylococcus aureus* alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. *Cell Microbiol* 2003 Oct;5(10):729-41.
- (5) Craven RR, Gao X, Allen IC, et al. *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* 2009;4(10):e7446.
- (6) Hruz P, Zinkernagel AS, Jenikova G, et al. NOD2 contributes to cutaneous defense against *Staphylococcus aureus* through alpha-toxin-dependent innate immune activation. *Proc Natl Acad Sci U S A* 2009 Aug 4;106(31):12873-8.
- (7) Miller LS, Pietras EM, Uricchio LH, et al. Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo. *J Immunol* 2007 Nov 15;179(10):6933-42.
- (8) Munoz-Planillo R, Franchi L, Miller LS, Nunez G. A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J Immunol* 2009 Sep 15;183(6):3942-8.
- (9) DuMont A L, Nygaard T K, Watkins R L, et al. Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. *Molecular Microbiology* 2010; Accepted article.
- (10) Ventura CL, Malachowa N, Hammer CH, et al. Identification of a novel *Staphylococcus aureus* two-component leukotoxin using cell surface proteomics. *PLoS One* 2010;5(7):e11634.

- (11) Sun F, Li C, Jeong D, Sohn C, He C, Bae T. 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* 2010 Apr;192(8):2111-27.
- (12) Benton BM, Zhang JP, Bond S, et al. Large-scale identification of genes required for full virulence of *Staphylococcus aureus*. *J Bacteriol* 2004 Dec;186(24):8478-89.
- (13) Skinner D and Keefer CS. Significance of bacteremia caused by *Staphylococcus aureus*. *Arch Inter Med* 1941;68:851-75.
- (14) Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* 2007 Sep;3(9):541-8.

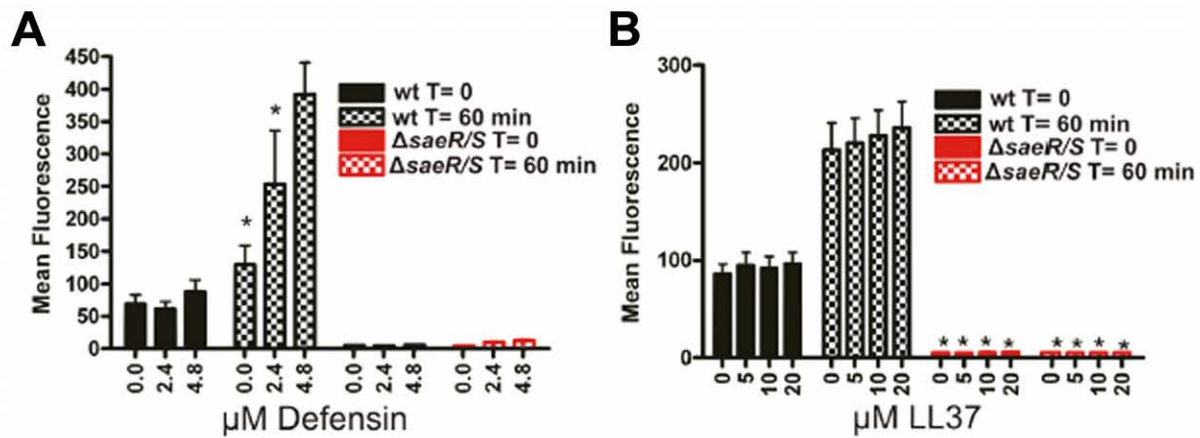


Figure 1. Human  $\alpha$ -defensin induce *saeR/S* mediated transcription of *hla*. GFP reporter constructs specific for the *hla* promoter transformed into USA300 and USA300 $\Delta\text{saeR/S}$  exposed to increasing concentrations of A)  $\alpha$ -defensin or B) LL37 over time. \*  $P < 0.001$  as determined by Anova and Tukey's post-test.

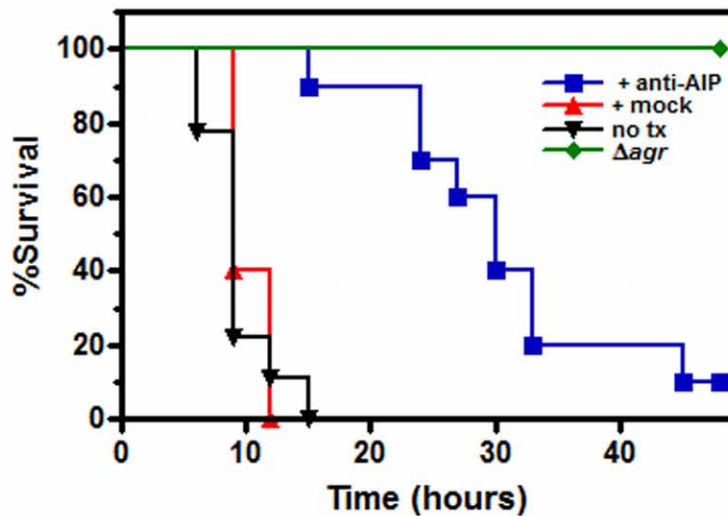


Figure 2. Anti-AIP treatment inhibits USA300 pathogenesis during invasive infection. Mice infected with  $1 \times 10^8$  CFU of USA300 or USA300 $\Delta\text{agr}$  by interperitoneal injection were treated with 2 mg anti-AIP, mock antibody, or DPBS control at 1 hour post-infection and survival monitored every 3 hours for 48 hours.



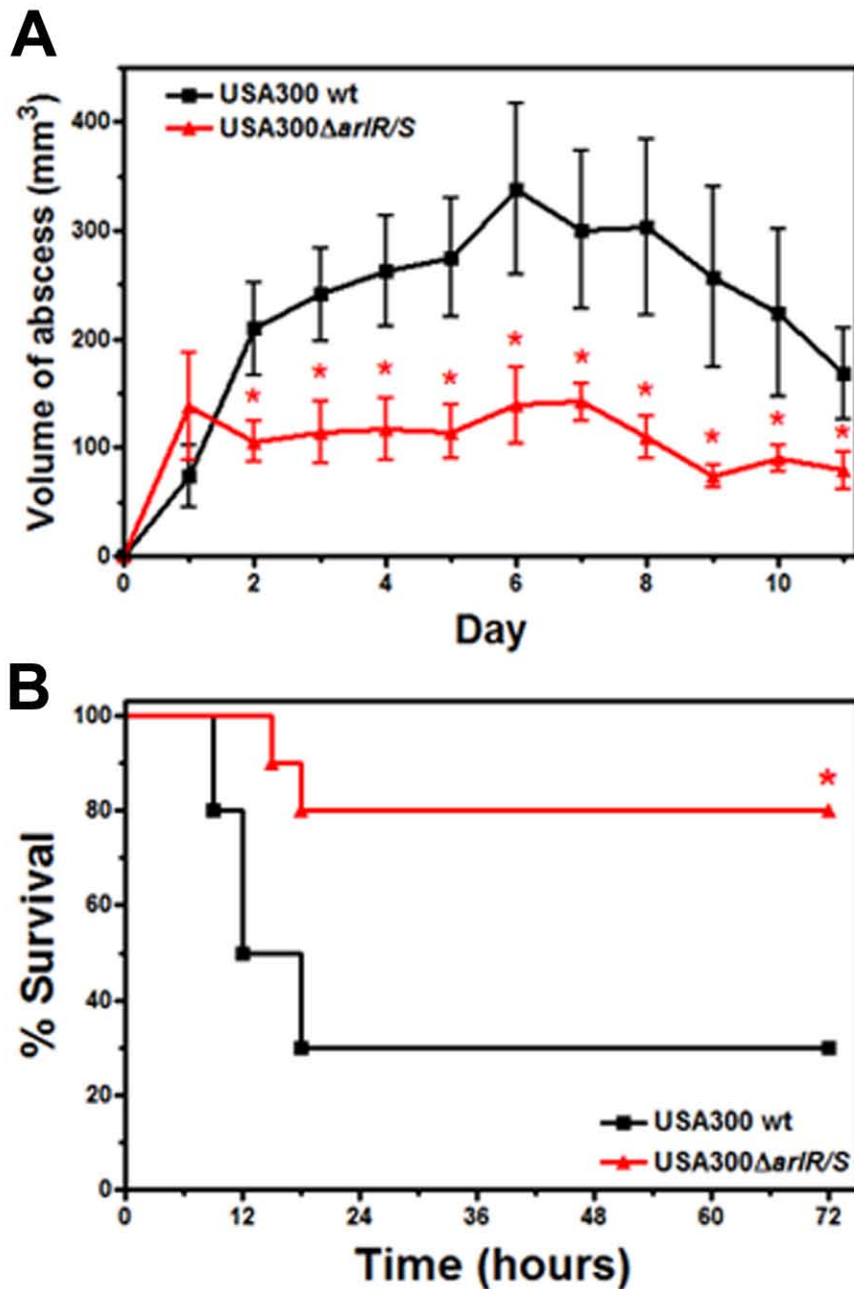


Figure 5. The *ArlR/S* two-component system is important for both superficial and invasive infections caused by USA300. A) Abscess volume over 11 days post-infection for mice infected subcutaneously with  $1 \times 10^7$  CFU of USA300 or USA300 $\Delta$ *arlR/S*. B) Survival of mice over 72 hours following tail-vein injection of  $1 \times 10^8$  CFU of USA300 or USA300 $\Delta$ *arlR/S*.