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Graduate Studies

THE EFFECTS OF A PUTATIVE *STAPHYLOCOCCUS AUREUS* TWO-COMPONENT REGULATORY SYSTEM ON *SRTA* TRANSCRIPTION AND BIOFILM FORMATION

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

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APPENDIX D

REAGENTS FOR PRIMARY ATTACHMENT ASSAY

THE EFFECTS OF A PUTATIVE *STAPHYLOCOCCUS AUREUS* TWO-COMPONENT REGULATORY SYSTEM ON *SRTA* TRANSCRIPTION AND BIOFILM FORMATION

By Amy Wescott

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ABSTRACT

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Staphylococcus aureus bacteria have evolved mechanisms to block the effects of antibiotics. After treatment for methicillin-resistant *S. aureus* (MRSA) infections, persister cells develop that have the ability to survive but not grow in the presence of the drug. Not a lot is known about persister cells, so it is important to study the mechanism for their ability to persist in the presence of antibiotics. Our proposed mechanism for persister formation in *S. aureus* is the ability of a drug or environmental stressor to impact gene regulation by a two-component regulatory system that affects transcription of the sortase A gene *srtA*. In this study, we have looked at the effects of the drug SK-03-92 on biofilm formation by down-regulating a putative two-component regulatory system. To determine how the two-component regulatory system impacts *srtA* transcription and biofilm formation, strains were obtained with mutations in either the response regulator or the sensor kinase gene. Overall biofilm formation, *srtA* transcription, and primary attachment were increased in the sensor kinase mutant strain, while the response regulator mutant strain results were similar to that of the wild-type strain. Overall, the data demonstrated that a mutation in the hypothesized sensor kinase resulted in a 10-fold increase in *srtA* transcription.

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TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

APPENDIX PAGE

INTRODUCTION

Significance and epidemiology

Staphylococcus aureus is a natural inhabitant of the nose or skin in 25-30% of individuals (Health and Human Services, 2015). The organism uses an opportunity, such as a cut or scrape in the skin, to cause infections. *S. aureus* is the leading cause of bacterial infections involving the bloodstream, lower respiratory tract, skin, and soft tissue in developing countries (DeLeo & Chambers, 2009). The number of patients checking into hospitals with *S. aureus* infections has now exceeded those of patients checking in with HIV and influenza combined, costing United States hospitals $1.4-13.8 billion dollars annually (David *et al.*, 2012; Lee *et al.*, 2013). These infections range from superficial skin infections; such as pimples or boils, to severe infections; such as sepsis, endocarditis, or wound infections after surgery (Sibbald *et al.*, 2012). The large increase in *S. aureus* infections is in part due to the ability of the organism to resist antibiotic treatment.

Methicillin-resistant *Staphylococcus aureus*

One prominent group of antibiotic resistant *S. aureus* is methicillin-resistant *S. aureus* (MRSA). In the 1940's, penicillin, a β-lactam antibiotic which impedes enzymes responsible for the formation of peptidoglycan, was used to treat *S. aureus* infections (Shaikh, *et al,* 2015). Within only two years of the introduction of the drug, penicillin resistance soon became a problem due to the enzyme, β-lactamase, which is produced by some strains of *S. aureus* to break down penicillin (Reynolds & Tansey, 2006; Peacock &

Paterson, 2015). Healthcare facilities then turned to methicillin, a semisynthetic penicillin, to treat *S. aureus* infections. Only one year after the introduction of methicillin as a treatment in 1959, MRSA strains were isolated (Deresinksi, 2005; Peacock & Paterson, 2015). The first two isolated MRSA strains were not thought of as a major threat until outbreaks began showing up in hospitals in Europe and the United States around 1968 (Daum, 2010). These strains were referred to as hospital-associated methicillin-resistant *S. aureus* (HA-MRSA). Initial research on the HA-MRSA strains demonstrated that they were resistant not because of a *bla* gene encoding a β-lactamase enzyme, but rather because of acquisition of a *mecA* gene. The corresponding MecA protein has a lower affinity for β-lactams than a non-resistant organism's MecA protein (Enright *et al.*, 2002; Peacock & Paterson, 2015). The *mecA* gene is part of the *mec* gene complex that contains *mecA*, encoding the penicillin binding protein; *mecI*, encoding a repressor protein that binds to the promoter region of *mecA* and prevents transcription; and *mecRI*, that encodes protease activity to hydrolyze *mecI* and activate *mecA* transcription when β-lactam antibiotics are present (Lee, 2013; Enright *et al.*, 2002; Wielders et al., 2002). The staphylococcal chromosome cassette *mec* (SCC*mec)* carries the *mec* gene complex and a cassette chromosome recombinase (*ccr)* gene complex, which encodes the site-specific recombinase needed for the cassette to insert itself into the chromosome at a precise location (Longzhu *et al.*, 2001; Wielders *et al.*, 2002; Ito, 2009). Acquisition of the SCC*mec* cassette is thought to have occurred via horizontal gene transfer from another bacterial species and acquisition of the SCC*mec* now accounts for the most commonly identified antibiotic-resistant pathogen in U.S. hospitals

(Deresinksi, 2005; Ito, 2009). In the United States, MRSA is the number one cause of hospital-associated infections with a mortality rate of 20% (DeLeo & Chambers, 2009).

In the 1990's, cases of serious MRSA infections in young, healthy individuals who had not been recently hospitalized began to surface. These infections were classified as community-associated methicillin-resistant *S. aureus* (CA-MRSA; Herold *et al.*1998). Community-associated-MRSA initially posed a risk factor for those in contact sports, drug users, and those living in crowded, unsanitary environments such as prisons. Today, CA-MRSA strains have become established in healthcare settings, superseding the classic HA-MRSA strains (Mediavilla *et al.*, 2012). While HA-MRSA strains often harbor SCC*mec* types I-III, CA-MRSA strains commonly harbor SCC*mec* type IV.

Another genetic difference between HA-MRSA and CA-MRSA is the presence of the Panton-Valentine Leukocidin (*pvl*) gene in CA-MRSA strains (Shrestha *et al.*, 2014; Johnsson *et al.*, 2004). The *pvl* gene encodes a potent cytotoxin that can cause tissue necrosis and disrupts leukocyte membranes, leading to increased virulence (Shrestha *et al.*, 2014; Adler *et al.*, 2006). The prevalence of the *pvl* gene in CA-MRSA strains allows the strains to be more virulent than HA-MRSA strains, resulting in more severe infections and the increased presence of CA-MRSA in healthcare settings.

The first prominent CA-MRSA lineage to emerge in the 1990's in the U.S. was USA400, which was exemplified by the MW2 strain (CDC, 1999). The USA400 strain remained the predominant strain until 2000 when CA-MRSA outbreaks were reported in U.S. prisons. These outbreaks were caused by a second CA-MRSA lineage, USA300 (Mediavilla *et al.*, 2012). Around 2001, a shift from USA400 to USA300 as the predominant CA-MRSA lineage occurred in the United States (Daum, 2010; Mediavilla

et al., 2012; Montgomery *et al.*, 2014). The USA300 MRSA strains currently account for 98% of the MRSA infections in North America (Gandara *et al.*, 2015). Current research hypothesizes that the shift from USA400 to USA300 may be due to the acquisition of an element called the arginine catabolic mobile element (ACME), which is only present in USA300 strains (Montgomery *et al.*, 2014; Montgomery *et al.*, 2008). The ACME element carries genes thought to be associated with increased virulence and a greater ability to survive against host immune defenses (Montgomery *et al.*, 2014; Montgomery *et al.*, 2008). Strains of USA300 also had increased levels of staphylokinase, enterotoxin K, and serine protease expression (Montgomery *et al.*, 2014; Montgomery *et al.*, 2008). The increase in virulence factors may have accounted for the increased virulence of CA-MRSA USA300 compared to USA400 strains. Currently, a USA300 strain containing the *pvl* gene, SCC*mec* type IV, and the ACME element is a global problem in healthcare facilities which further indicates these factors in the USA300 increased virulence (Uehara, Y. *et al*, 2015). The heightened virulence of CA-MRSA and the rise of multidrug resistant CA-MRSA strains warrants new drugs to treat CA-MRSA infections.

Treatment

Currently, many *S. aureus* strains infecting humans are MRSA. The presence of the *mecA* gene indicates resistance to β-lactam antibiotics and requires alternative treatment, which may include discovering novel targets for antimicrobial drugs. Vancomycin is another commonly prescribed antibiotic to treat MRSA infections (Stryjewski & Corey, 2014). Unfortunately, treatment of MRSA with vancomycin has its own challenges due to the emergence of vancomycin intermediate-susceptible *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) strains in 2002 (DeLeo &

Chambers, 2009; Sharp *et al.*, 2005; Montgomery *et al.*, 2014; Montgomery *et al.*, 2008; Stryjewski & Corey, 2014). Another challenge of antibiotic treatment of *S. aureus* strains (e.g. MRSA, VISA, and VRSA) is the survival of a small subset of the bacterial population. This subpopulation is referred to as persister cells.

Staphylococcus aureus persisters

Persister cells are defined as dormant variants of regular cells that do not grow nor die in the presence of bactericidal compounds (Lechner *et al.*, 2012a; Lechner *et al.*, 2012b; Zhang, 2014). Persistence is a non-heritable property and depends on multiple factors such as the phase of growth of the organism, concentration of the antibiotic, exposure time, and aeration of the medium (Zhang, 2014). While evidence of persister cells was described in 1944, it is only more recently that persisters have become of interest in current research (Conlon, *et al.*, 2014).

Two types of bacterial persister cells have been identified. Type I persister cells are non-growing cells and increase in number by phenotypic switching at the end of the exponential phase and onset of stationary phase based on environmental signaling. Type II persister cells are defined as slow-growing cells undergoing continuous phenotypic switching regardless of the environmental stimuli (Lechner *et al.*, 2012a; Canas-Duarte *et al.*, 2014). Phenotypic switching is when a wild-type cell switches to a persister cell without a genetic change. While type I and II persister cells are always present in the population, persister cell formation occurs at a higher frequency during mid-to-late exponential phase than early exponential phase, forming a heterogeneous population that acts as a "bet-hedging strategy" for the organism (Lechner *et al.*, 2012a; Lewis, 2007). A "bet-hedging strategy" allows the organism a means of survival when the organism

encounters an unforeseen even that could kill off the population. For example, if exposed to bactericidal antibiotics, the majority of the population will be killed, but the drug persister population will remain dormant until the selective pressure is removed. Once the drug threat is removed, the organism can resume normal growth, leading to chronic, recurring infections (Geoghegan, 2010; Conlon, 2014).

Persisters differ from true antibiotic resistant organisms. In a persister state, the metabolic activity is greatly decreased or non-existent and therefore, the antibiotic has no effect on its normal target (Lewis, 2007). For example, if the antibiotic targets a step in peptidoglycan synthesis but the persisters are not actively synthesizing peptidoglycan, then the antibiotic will not have the desired effect. To decrease metabolic activity, a toxin-antitoxin system is often utilized. Under normal conditions, the antitoxin neutralizes the toxin, but when the bacterial cell is stressed, the anti-toxin is degraded by a host protease and the toxin exhibits toxic effects on the cell (Conlon *et al.*, 2014). The toxic effects often include amino acid starvation which leads to down-regulation of transcription in genes involved in protein machinery and amino acid biosynthesis (Conlon *et al.*, 2014). This ability to decrease or stop metabolism makes the persister cells tolerant to multiple drugs, making treatment of the recurring infections difficult (Zhang, 2014; Lewis, 2007; Prax & Bertram, 2014). Toxin-antitoxin systems were originally characterized in gram negative bacteria, specifically *E. coli*, but have been identified in gram positive bacteria including *S. aureus*. While gram positive toxin-antitoxin systems have not been as well characterized, they are associated with persister formation under stressful environmental conditions (Schuster *et al.*, 2015; Prax & Bertram, 2014).

A study by Lechner *et al.* (2012a) demonstrated that *S. aureus* persister cells are a constant subpopulation in an exponential phase culture. It was shown that the addition of bactericidal antibiotics, such as ciprofloxacin, at 10-fold minimum inhibitory concentration (MIC) resulted in a rapid decrease in CFU counts ranging from 94%-99% of the starting population. The subpopulation of ciprofloxacin persisters remained stable for up to 24 h. This experiment was repeated with tobramycin at 100-fold MIC and the same result occurred, demonstrating that bacterial cultures are a heterogeneous population of persisters and regular cells.

The rate of phenotypic switching from regular cells to persister cells has also been examined by challenging the *S. aureus* cultures with multiple antibiotic treatments. Phenotypic switching rates varied considerably between different antibiotic treatments, indicating multiple subpopulations of persisters. To confirm this finding, dose-dependent killing assays were performed where *S. aureus* cultures were first treated with 100-fold MIC of ciprofloxacin and the surviving persister population treated with 10-fold MIC of daptomycin (Lechner *et al.*, 2012b). When the ciprofloxacin persister cells were exposed to daptomycin, their population was reduced by two logs, demonstrating that *S. aureus* cultures are heterogeneous with multiple persisters exhibiting tolerance to many antibiotics (Lechner *et al.*, 2012b).

Singh *et al.* (2009) further demonstrated the importance of the heterogeneous *S. aureus* populations by showing that *S. aureus* was able to form biofilms resistant to antibiotic treatment. This study compared the antibiotic-resistance of *S. aureus* planktonic cells to *S. aureus* biofilm cells using various antibiotics. The results demonstrated that *S. aureus* planktonic cells were effectively killed by clinically

significant MIC levels of oxacillin, ciprofloxacin, and vancomycin, while the viability of *S. aureus* biofilm cells were not significantly reduced 24 h after of antibiotic exposure. Singh *et al.* (2009) confirmed the presence of persisters by performing dose-dependent killing assays against the biofilm population. For example, dose-dependent treatment of a *S. aureus* culture with oxacillin resulted in an initial reduction in cells followed by a plateau of 1.8% of cells remaining. The remaining cells are persister cells. The combination of the inherent antibiotic-resistance of *S. aureus* biofilms and the presence of biofilm-associated persister cells may contribute to the ability of this organism to cause chronic infection.

Biofilm formation

Recurring infections caused by *S. aureus* may be due to drug persister cells hiding within a biofilm (Bacteriality, 2008). *S. aureus* is considered the leading cause of biofilm-related infections, accounting for 65% of these infections per year (Lewis, 2007; Otto, 2009). Biofilms are surface-associated, multicellular communities in which the cells are held together by a self-produced extracellular matrix (Foulston *et al.*, 2014). The sticky extracellular matrix is composed of exo-polysaccharides, proteins, teichoic acids, and extracellular DNA from lysed cells (Foulston *et al.*, 2014; Archer *et al.*, 2011; Periasamy *et al.*, 2011). The matrix provides the cells with protection from the surrounding environment, specifically from host defense mechanisms and antibiotic pressure. The matrix is capable of obtaining nutrients, such as carbon and nitrogen, and concentrating the nutrients for the bacteria near the top of the biofilm (Archer *et al.*, 2011). The bacteria at the base of the biofilm experience a lack of nutrients. A lack of

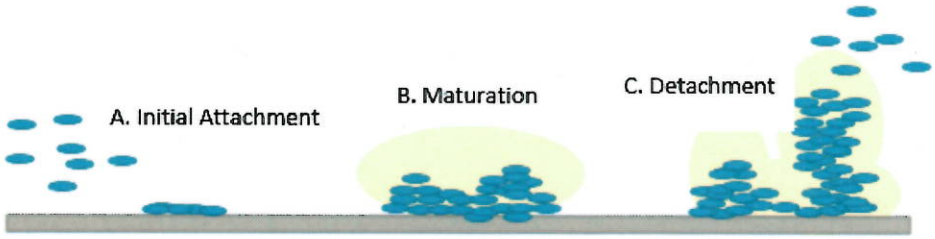

Fig 1. The phases of biofilm formation. A) Planktonic cells attach to an abiotic or biotic surface. B) Intercellular aggregation begins and structure of the biofilm forms. C) Cells detach from the biofilm and begin to recirculate.

nutrients available to a bacterial cell can also induce a persister cell state indicating that the large majority of cells at the base of a biofilm are persister cells. Therefore, these cells remain in a quiescent state and effectively block the antimicrobial targets rendering the antimicrobial ineffective.

S. aureus biofilm formation occurs in three phases (FIG. 1). Phase I, or the primary attachment phase, involves an individual cell attaching to almost any biotic or abiotic surface (Periasamy *et al.*, 2011; Lister & Horswill, 2014). Implanted medical devices are a common abiotic surface that exhibit *S. aureus* attachment. Current treatment involves removal of the implanted device, antibiotic treatment of the patient, and reinsertion of the medical device (Walsh *et al.*, 2008). *S. aureus* can also indirectly attach to medical devices by attaching to human matrix proteins that are already attached to the device. The expression of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) by *S. aureus* allows the organism to attach to matrix proteins: fibrinogen, fibronectin, and collagen. MSCRAMMs maintain a common structure that include an exposed binding domain, a cell-wall spanning domain, and a domain that binds to the surface of the bacterial cell wall (Archer *et al.*, 2011; Walsh *et*

al., 2008). MSCRAMMs are covalently linked to the cell wall by a membrane-bound transpeptidase called sortase A (FIG. 2). This enzyme contains an N-terminal hydrophobic segment that functions as a signal peptide for secretion and membrane anchoring (Marraffini *et al.*, 2006). The N-terminus of sortase A remains inside the cytoplasm while the C-terminal enzymatic portion is located across the plasma membrane (Marraffini *et al.*, 2006). Sortase A enzymatically folds into an eight-stranded β-barrel that includes two helices and several loops (Marraffini *et al.*, 2006). The enzyme active site is made up of the β7 and β8 strands containing conserved Cys and His residues crucial for substrate binding. Replacement of the Cys or His in the active site results in complete abolishment of sortase activity, indicating the importance of these two residues in substrate binding and recognition (Marraffini *et al.*, 2006).

Sortase A recognizes an LPXTG motif on the C-terminus of the MSCRAMM (Archer *et al.*, 2011). Sortase A cleaves the peptide bond between the Thr and Gly residues of the LPXTG motif when activated by Ca^{2+}commonly found in human tissue (Naik *et al.*, 2005). The β3-β4 and β6-β7 loops of the sortase A enzyme contain sets of acidic residues involved in calcium binding, which activates the sortase enzyme 8-fold (Marraffini *et al.*, 2006). Cleavage of the peptide bond allows for formation of an amide bond between the carboxyl group of the Thr residue of the surface protein and the free amino end of a pentaglycine bridge in peptidoglycan precursors, resulting in covalent protein attachment to the cell wall (FIG. 2).

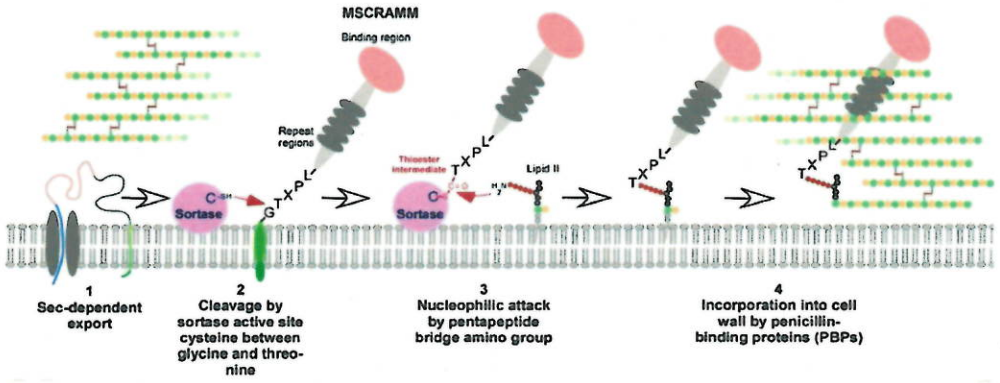

Fig 2. Mechanism of anchoring MSCRAMM to the cell wall of *S. aureus* by sortase A. Image courtesy of Dr. Matt Otto (National Institute of Health Laboratory of Human Bacterial Pathogenesis; Bethesda, Maryland).

S. aureus strains lacking the *srtA* gene do not have the ability to display LPXTG proteins on their cell surface, resulting in decreased virulence of these strains. In a mouse model of infection, a *srtA* mutant strain and wild-type strain were injected into mice. The *srtA* mutant strain displayed a 1.5-log increase in the 50% lethal dose, indicating *srtA* mutations cause a reduction in virulence (Marrafini *et al.*, 2006). Additionally, in a mouse organ abscess model, the *srtA* mutant strain displayed a 3-log-unit reduction in bacterial growth within abscesses compared to that of wild-type (Marrafini *et al.*, 2006). The loss of virulence demonstrates the importance of sortase A in *S. aureus* infections. Twenty one LPXTG-linked MSCRAMMS that bind to host matrix proteins can be expressed by *S. aureus*. The six most common MSCRAMMS are associated with human infection and are listed in the table below (Table 1; Walsh *et al.*, 2008; Foster *et al.*, 2013).

Table 1. Microbial surface components recognizing adhesive matrix molecule proteins and functions.

Protein	Ligand	Function
Clumping factor A (ClfA)	Fibrinogen and complement I	Immune evasion by binding to ligands
Clumping factor B (ClfB)	Fibrinogen	Nasal colonization
Serine-aspartate repeat proteins (SdrC, SdrD, SdrE)	Desquamated epithelial cells	Unknown
Fibronectin-binding proteins A and B (FbpA and FbpB)	Fibronectin	Adhesion to extracellular matrix and adhesion
Collagen adhesion (Cna)	Collagen	Adhesion to collagen tissue and prevent classical complement pathway
SasG	Epithelial cells	Adhesion to nasal epithelial cells

Once the surface proteins have attached to a matrix protein or abiotic surface, phase II of biofilm formation, or maturation, can begin. This phase involves intercellular aggregation and biofilm structuring (Archer *et al.*, 2011). Intercellular aggregation is mediated by the polysaccharide intercellular adhesin protein (PIA) when it is de-acetylated, giving it a positive charge that interacts with the negative charge of the cell wall and the teichoic acids on the cell surface (Archer *et al.*, 2011). This PIA-cell wall interaction gives rise to the sticky matrix of the biofilm. The interactions of the surface proteins, along with phenol-soluble modulins (PSM), lead to the biofilm shape. Phenol soluble modulins are amphipathic peptides that act as surfactants, giving rise to towers and channels in the biofilm that allow for delivery of nutrients to the deeper layers of the biofilm (Archer *et al.*, 2011).

Eventually the towers of the biofilm begin to detach due to liquid shearing, a lack of exo-polysaccharide production, or PSM-related surfactant activity (Periasamy *et al.*, 2011; Picioreanu *et al.*, 2000; Tsompanidou *et al.*, 2013; Peschel & Otto, 2013). Biofilm detachment represents phase III of biofilm formation. Surfactant characteristics of PSMs allow them to disrupt the interaction of biofilm matrix molecules with the bacterial surface (Dastgheyb *et al.*, 2015). The study done by Dastgheyb *et al.* (2015) demonstrated that PIA, an abundant matrix molecule for *S. aureus* biofilms, was released from the bacterial surface in a PSM-dependent manner, indicating that PSMs cause separation of PIA from the bacterial surface. The separation of PIA from the bacterial surface leads to decreased integrity of the biofilm, biofilm detachment, and pathogen dissemination to other locations in the body. Both biofilm-associated and disseminated cells are affected by the environmental cues in the human body. These cues trigger two-component regulatory systems that regulate a variety of virulence factor genes in *S. aureus*.

Two-component regulatory systems

Two-component regulatory systems play essential roles in signaling events in bacteria. These events include, cell-to-cell communication, adaptation to environments, and pathogenesis (Wang, 2012). A two-component regulatory system consists of a sensor kinase (HK) and a response regulator (RR) (Wang, 2012; Mitrophanov & Groisman, 2008; Utsumi, 2008; Jung *et al.*, 2012). The HK is typically an integral membrane protein that detects chemical or physical signals in the environment that lead to auto-phosphorylation of its histidine residue. The phosphate can then be transferred to the aspartate residue on the RR (Utsumi, 2008; Jung *et al.*, 2012; Mitrophanov &

Groisman, 2008). Once the RR is phosphorylated, the RR has the ability to bind DNA, RNA, or other proteins, resulting in transcriptional or enzymatic changes within the cell (Jung *et al.*, 2012; Bijlsma & Groisman, 2003).

S. aureus often adapts to new environments during the infection of a host. Structural genes are often under the control of two-component regulatory systems as these genes encode for proteins that may influence the infection of the host (Groisman & Mouslim, 2006; Mikkelsen *et al.*, 2011). Biofilm formation is also under the control of a pair of two-component regulatory systems, AgrCA and ArlRS (Walker *et al.*, 2013; Toledo-Arana *et al.*, 2005; Table 2). Recently, ArlRS has been shown to be directly involved in agglutination of *S. aureus* cells, a crucial step in the formation of biofilms. When ArlRS is knocked out, the organism is unable to agglutinate and virulence is greatly decreased (Walker *et al.*, 2013). Antibiotic resistance has also been shown to be associated with two-component regulatory systems. For example, GraRS senses and initiates some resistance to specific cationic antimicrobial peptides, including vancomycin (Yang *et al.*, 2012; Mehta *et al.*, 2011). GraRS is now thought to play a role in the rising incidence of VISA strains (Yang *et al.*, 2012).

Two-component regulatory systems are crucial for *S. aureus* virulence and provide the organism with numerous ways to survive adverse environments. Besides the two-component regulatory system described above, thirteen additional chromosomally encoded two-component regulatory systems have been identified in *S. aureus*. Many of these systems allow for rapid adaptation to the environment by controlling the expression of *S. aureus* virulence factors (Cheung *et al.*, 2014; Matsuo *et al.*, 2010; Kawada-Matsuo *et al.*, 2011; Park *et al.*, 2015; Xue *et al.*, 2001). Together, these 16 two-component

regulatory systems control genes coding for extracellular toxins, proteases, coagulases, extracellular proteins, energy metabolism, anaerobic growth, autolysis, and antimicrobial resistance (Table 2; Yarwood *et al.*, 2004; Cheung & Zhang, 2014; 1993; Geiger *et al.*, 2008; Koretke *et al.*, 2000; Traber, *et al.*, 2008).

Although a number of two-component regulatory systems have been identified in *S. aureus,* there are many uncharacterized open reading frames that may encode new two-component regulatory systems and some may be tied to drug persistence and biofilms. As part of a drug discovery program at UW-L, a compound with a stilbene structure was found in the leaves of *Comptonia peregrina*, also known as the sweet fern (Kabir *et al.*, 2007). A structure-activity relationship investigation was undertaken and the drug coded SK-03-92 was identified as the best lead candidate based on MIC activity against *S. aureus* (Schwan *et al.*, 2011). An RNA microarray was performed testing RNA from untreated *S. aureus* strain MW2 cells compared to RNA from SK-03-92 treated cells (Schwan, W.R., unpublished data). A number of genes were down-regulated by exposure to the SK-03-92 drug, including two genes, *MW2284* (downregulated 14.1-fold) and *MW2285* (downregulated 26.9-fold) that may comprise a novel two-component regulatory system and are 100% conserved across *S. aureus* strains. Nomenclature for the genes of this putative two-component regulatory system was adopted from the network on antibiotic resistance in *S. aureus*. Mutant strains of the *MW2284* and *MW2285* genes were ordered from the Network on Antibiotic Resistance in *S. aureus* and RNA analysis demonstrated an increase in *srtA* transcription relative to the wild-type strain. Preliminary analysis of a SK-03-92 drug persister strain showed a down-

Table 2. *Staphylococcus aureus* chromosomally encoded two-component regulatory systems.

Two-component regulatory system	Function	Reference
AgrCA	global regulator of staphylococcal virulon	Abdelnour *et al.*, 1993
SaeRS	temporal control of staphylococcal virulon	Kato *et al.*, 2011
ArlRS	autolysis and agglutination	Kato *et al.*, 2011
LytSR	Autolysis	Bronner *et al.*, 2004
WalKR	cell wall degradation and inflammation	Delaune *et al.*, 2012
GraRS	cell wall maintenance and cationic antimicrobial resistance	Yang *et al.*, 2012
VraDE	cell wall synthesis	Hiron *et al.*, 2011
HssRS	regulation of iron acquisition	Stauff *et al.*, 2007
NreBC	regulates nitrogen sensing	Schlag *et al.*, 2008
SrrAB	regulates oxygen sensing	Bronner *et al.*, 2004
AirRS	regulates oxygen sensing	Bronner *et al.*, 2004
BraRS	regulates bacitracin resistance	Hiron *et al.*, 2011
KdpDE	potassium transport and intracellular survival	Freeman *et al.*, 2013
HptRS	regulation of hexose phosphate transport	Park *et al.*, 2015
FakAB	fatty acid uptake	Parsons *et al.*, 2014
SspAB	proteolytic destruction of host tissues	Hall *et al.*, 2015

regulation (4.09-fold) of *MW2285* and an increase (5.72-fold) in *srtA*. It is hypothesized that this putative two-component regulatory system may repress transcription of the *srtA* gene encoding for sortase A under non-stressful growth conditions.

If *MW2284* and *MW2285* are truly involved in repressing the *srtA* gene, then mutations in either gene or treatment with SK-03-92 drug should cause greater transcription of the *srtA* gene and presumably more sortase A protein will be present in the cell. As described earlier, the function of sortase A is to link MSCRAMM proteins to the cell wall of *S. aureus*. Therefore, with an increase in sortase A, there should also be an increase in MSCRAMM proteins attached to the cell wall allowing for attachment to cellular matrix proteins, or the first step in biofilm formation. Overall, a stressor added to the *S. aureus* culture, such as the SK-03-92 drug, will lead to an increase in sortase A transcription, an increase in MSCRAMM protein attachment, and finally an increase in biofilm formation. For this study, additional characterization of this putative two-component regulatory system have been done to determine its role in *S. aureus* biofilm formation and virulence. Specific research objectives for this study included; compare overall biofilm formation of each strain, compare primary attachment ability of each strain, measure *srtA* transcription of each strain, and compare overall virulence of each strain using a murine thigh abscess model of infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and antibiotics

The bacterial strains and plasmids used in this study are listed in Table 3. One *S. aureus* parent strain, MW2, is a USA400 CA-MRSA strain (CDC, 1999). The University of Nebraska Medical Center cured another parent strain, the USA300 LAC CA-MRSA strain, (Kennedy et al., 2008) of its plasmids to create JE2 (Fey et al., 2013). Subsequently, transposon mutagenesis was performed on strain JE2, creating the NE671 (*MW2284*) and NE272 (*MW2285*) transposon mutant strains (Fey et al., 2013; Figure 4). Furthermore in this manuscript, the NE671 strain will be referred to as the response regulator mutant strain and the gene as *rssR* (repressor staphylococcal sortase regulator) and the NE272 strain will be referred to as the sensor kinase mutant strain and the gene as *rssS* (repressor staphylococcal sortase sensor). The *rssR* response regulator is preceded by a hypothetical protein and succeeded by the *rssS* sensor kinase gene. The *rssR* and *rssS* genes have a 3 bp overlap and are followed by the *mqo (MW2286)* gene. A bioinformatics analysis has shown that the *rssR/rssS/mqo* series of genes is conserved across all gram-positive cocci species. A bioinformatics analysis also indicated that this two-component regulatory system contains a LytTR sequence that is similar to the LytTR domain in the response regulator AgrA. A study by Rajasree *et al.*, demonstrated that binding by AgrA caused a significant conformational change in the promoter DNA, which may play a role in promoter specificity and transcriptional activity. The study showed AgrA interacting with the LytTR domain to act as an activator of gene

expression. While LytTR domains are associated with being transcriptional activators, we hypothesize that the LytTR domain of the *rssR/rssS* two-component regulatory system may act as a transcriptional repressor. There is not a lot of evidence supporting this hypothesis, but a study by Nikolskaya *et al.* (2002), discusses the discovery of a novel LytTR binding domain in *Streptococcus* that regulates important virulence factors for the organism, such as toxins, extracellular polysaccharides, and sortase A. While the study does not indicate if this DNA-binding domain acts as a transcriptional activator or repressor, it does demonstrate that novel binding domains are being characterized and it is possible the binding domain in the *rssR/rssS* two-component regulatory system may still be uncharacterized.

All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and were used in the following concentrations: 100 μg/ml ampicillin, 5 μg/ml erythromycin, 10 μg/ml gentamicin, 5 μg/ml tetracycline, and 10 μg/ml chloramphenicol.

Construction of plasmid pAB1-5

The pTGCompTCGm2 plasmid was used as the backbone to clone the *rssR* response regulator open-reading frame. The *putP* gene was removed from the pTGCompTCGm2 backbone by digesting with *Eco*RI and *Bam*HI restriction endonucleases (New England Biolabs, Ipswich, MA) and the DNA fragment was separated from the pTGCompTCGm2 backbone using gel electrophoresis, spun through fibrofil in a microfuge tube for 10 min at 6,000 x *g*, and then concentrated using a centrifugal filter (Millipore; Billerica, MA). The *rssR* response regulator gene (1,000 bp containing 480 bp of upstream DNA) was amplified from strain MW2 chromosomal DNA and cut with *Eco*RI and *Bam*HI restriction endonucleases to ligate into the

*Bam*HI/*Eco*RI cut plasmid backbone. *Escherichia coli* DH5α cells were transformed with the ligation mixture and plated on Luria-Bertani (LB) agar containing 10 μg/ml gentamicin. Plasmid pAB1-5 contains tetracycline and erythromycin resistance genes, an *E. coli* and gram-positive origin of replication, and *Bam*HI, *Eco*RI, and *Hind*III restriction endonuclease sites for cloning (FIG. 3). The pAB1-5 plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen, Germanton, MD) and cut with *Eco*RI and *Bam*HI to determine that the correct size of DNA inserted. One transformant containing the plasmid of the correct size and restriction digest pattern including the response regulator gene was processed further.

Table 3. Bacterial strains and plasmids used in this study.

Bacterial Strain or Plasmid	Description	Reference
***S. aureus* strains**		
MW2	USA400 MRSA strain	CDC, 1999
JE2	USA300 MRSA strain	Fey *et al.*, 2013
NE272	JE2 *rssS* mutant	Fey *et al.*, 2013
NE671	JE2 *rssR* mutant	Fey *et al.*, 2013
RN4220	Transformation efficient strain	Novick, R.P., 1990
***E. coli* strains**		
DH5α	General cloning strain	Hanahan, D., 1989
Plasmids		
pTGCompTCGm2	Cloning vector with tet^r, erm^r, and gen^r genes	Schwan *et al.*, 2004
pAB1-5	Modified pTGCompTCGm2 with *rssR*	This study
pGEX-5X-1	shuttle vector with amp^r and *rssS* genes	Genscript
pAD123	cloning vector with cm^r and ap^r genes	Dunn *et al.*, 1999
pAB5X-1	modified pAD123 with *rssS* gene	This study

Construction of plasmid pAB5X-1

To construct the *rssS* suppression analysis plasmid, the sensor kinase open reading frame (*rssS*) preceded by a *S. aureus* ribosome binding site and flanked by *Bam*HI and *Eco*RI restriction sites was commercially inserted into the shuttle vector pGEX-5X-1 (Genscript, Piscataway Township, NJ). The insertion of a *S. aureus* ribosome binding site was done because the sensor kinase gene is not thought to be preceded by its own ribosome binding site (FIG. 4). The pGEX-5X-1 plasmid was digested with *Eco*RI and *Sma*I and then ligated to *Eco*RI/*Sma*I digested pAD123 plasmid DNA. The pAB5X-1 plasmid DNA was extracted as described above. Plasmid DNA of the correct molecular weight size was cut with *Eco*RI and *Sma*I to confirm the correct size of the insert. *E. coli* DH5α cells were transformed with the ligation mixture and plated on LB agar containing 100 μg/ml ampicillin. One plasmid named pAB5X-1 was identified that contained chloramphenicol and ampicillin resistance genes, a gram-positive origin of replication, a *tac* promoter, and *Sma*I, *Eco*RI, and *Hind*III sites for cloning (FIG. 5).

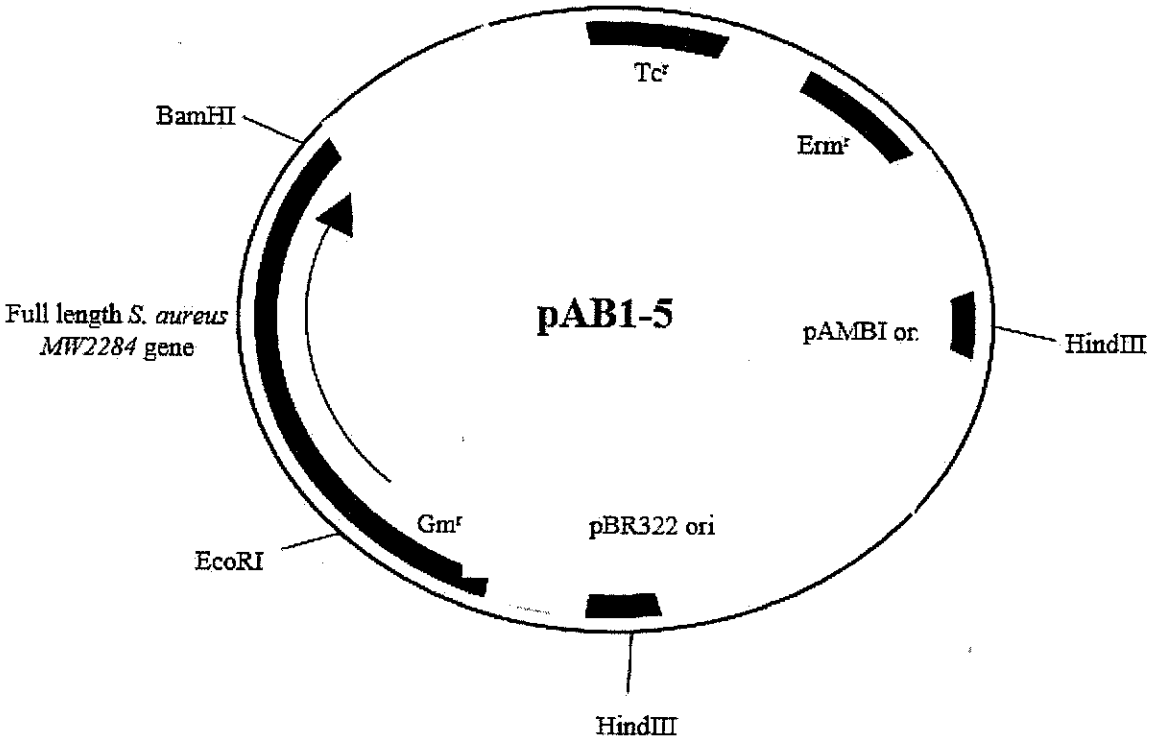

Fig 3. Map of plasmid pAB1-5.

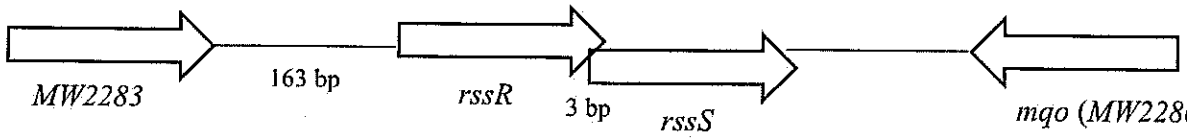

Fig 4. Genetic organization of the two genes comprising the putative RssS/RssR two-component regulatory system in *S. aureus* along with upstream and downstream genes of the putative two-component regulatory system.

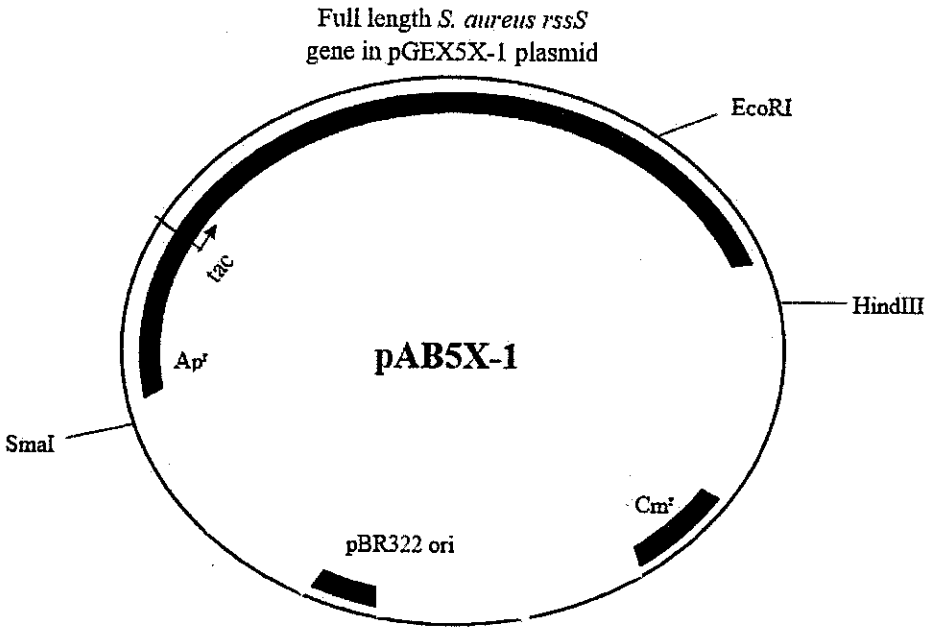

Fig 5. Map of plasmid pAB5X-1.

Electroporation of the suppression analysis mutants

In order to adapt the suppression analysis plasmids to *Staphylococcus*, plasmids pAB1-5 and pAB5X-1 were first electroporated into *S. aureus* RN4220 that resists DNA modification. Following plasmid isolation, the *Staphylococcus* adapted plasmids were electroporated into their respective mutant strains. Each strain was grown to an OD_{660} of 0.4 and 200 ml of culture was harvested by centrifugation at 9,000 x *g* for 20 min at 4°C. The bacteria were resuspended in 200 ml ice cold 0.5 M sucrose solution and pelleted by centrifugation as described above. A second resuspension of the pelleted bacteria was done in 100 ml of ice cold sucrose followed by pelleting by centrifugation at 9,000 x *g* for 10 min at 4°C. After centrifugation, the pellet was suspended in 20 ml ice cold sucrose and centrifuged as described above. Each pellet was ultimately resuspended in 300 µl of ice cold sucrose. In separate 0.2 cm gap Gene Pulser cuvettes (BIO-RAD; Hercules, CA), 100 µl of bacterial cells were mixed with approximately 0.1-1 µg of plasmid DNA. Electroporation of *S. aureus* cells were performed under the following conditions; capacitance (100 Ω), resistance (25 µF), and charging voltage (2.5 kV).

Following electroporation, transformed cells were plated on either brain heart infusion agar (BHI agar) containing 5 µg/ml tetracycline for pAB1-5 or BHI agar containing 10 µg/ml chloramphenicol for pAB5X-1 and grown overnight at 37°C. Plasmid DNA was isolated as described above using a QIAprep kit with the addition of 50 µl of lysostaphin (Remel; Waltham, MA) to assist in the breakdown of the cell wall. Plasmids of the correct molecular weight size were digested as described above to confirm the correct insert. Once the plasmid size and digest pattern was confirmed, electroporation of pAB1-5 into the *rssR* response regulator mutant strain (NE671) and

pAB5X-1 into the *rssS* sensor kinase mutant strain (NE272) was performed as described above. Transformed *rssR* mutant cells were plated on BHI agar containing 5 μg/ml tetracycline plus 5 μg/ml erythromycin and transformed *rssS* mutant cells were plated on BHI agar containing 10 μg/ml chloramphenicol plus 5 μg/ml erythromycin. Plasmid DNA was isolated and insert size and digest patterns were confirmed as described above.

Real-time-quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To determine the transcription levels of *srtA* in wild-type, *rssR*, and *rssS* mutant strains as well as the *rssR* suppression analysis mutant; qRT-PCR was performed. Each strain was grown overnight in BHI broth shaking (250 rpm) at 37°C. Total RNA was isolated from each strain using TRizol extraction (Life Technologies; Carlsbad, CA) with an additional lysostaphin treatment to assist in breaking down the cell wall. Briefly, cells were pelleted at 8,000 x *g* for 10 min and then suspended in 400 μl diethylpyrocarbonate (DEPC) water. After a 30 min lysostaphin treatment at 37°C, an equal volume of TRIzol reagent and chloroform were added and incubated at 37°C for 5 min. Bacterial lysates were pelleted by centrifugation at 8,000 x *g* for 10 min at 4°C. Each aqueous layer was extracted once with phenol and twice with chloroform. The RNAs were precipitated by adding 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol and incubated overnight at -20°C. After incubation, the RNAs were pelleted at 14,000 x *g* for 10 minutes and then each pellet was washed (14,000 x *g)* with 70% ethanol (in DEPC water) and allowed to dry completely before being suspended in 100 μl of DEPC-treated water. Contaminating chromosomal DNA was removed by incubating the RNA preparations with 3 μl RNAse-free DNAase (NEB) at 37°C for 30 min. An additional 400 μl TRIzol was added to the solution and incubated again at 37°C for 5 min before the

addition of 350 µl of 100% ethanol. The RNA preparation was spun through a DIRECTzol kit (The Epigenetics Company; Irvin, CA) according to manufacturer's instructions. The quality and concentration of the RNA preparations were determined by taking $OD_{260/280}$ readings and running get electrophoresis.

The cDNAs were synthesized from 2 µg of total RNA from each strain using a First-Strand Synthesis kit (Life Technologies; Carlsbad, CA) according to manufacturer's instructions. All of the qRT-PCRs were performed using the LightCyler FastStart DNA MASTER^{plus} SYBR Green kit according to manufacturer's instructions (Roche; Basel, Switzerland). Primers specific for the *srtA* gene (Table 4) as well as the *ftsZ* housekeeping gene used as standardization controls were used for every assay (Table 4). Strain MW2 chromosomal DNA was used as a positive control and *E. coli* chromosomal DNA and a no template well were used as negative controls. The qRT-PCRs were run in a BIO-RAD CFX (BIO-RAD, Hercules, CA) machine at three different times using two different sets of RNA for each strain. The following parameters were used: initial denaturation for 5 min at 95°C followed by 35 cycles of 95°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min. The level of *srtA* transcript abundance in the cells was compared to the *ftsZ* housekeeping gene. The housekeeping gene was run at the same cycler conditions as described above. Using the BIO-RAD CFX software, crossover points for all genes were standardized to the crossover point for *ftsZ* in each sample using the $2^{-\Delta\Delta C}T$ formula (Livak & Schmittgen, 2001). Standardized transcript levels from mutant and suppression analysis mutant strains were then compared to transcript levels of the wild-type strain.

Table 4. Oligonucleotide primers used in this study.

Primer	Gene	Sequence	Reference
SrtA1	*srtA*	5'- TCGCTGGTGTGGTACTTATC – 3'	This study
SrtA2		5'- CAGGTGTTGCTGGTCCTGGA – 3'	This study
SaFtsZ1	*ftsZ*	5'- GGTGTAGGTGGTGGCGGTAA – 3'	This study
SaFtsZ2		5'- TCATTGGCGTAGATTTGTC – 3'	This study

ANOVA analysis was performed to determine if the mutant strain *srtA* transcript levels are significantly different compared to wild-type or suppression analysis strains.

True biofilm assay

To determine the ability of a strain to form a biofilm, a true biofilm assay was performed (Stepanovic *et al.*, 2001). This total biofilm assay determined if there were differences in biofilm formation between the different strains. The *S. aureus* parent strain (JE2), *rssR* response regulator, and *rssS* sensor kinase mutants, as well as the *rssR/rssR*$^{+}$ suppression analysis mutant strain were grown in BHI broth plus 1% glucose (JE2) and BHI broth plus 5 µg/ml erythromycin and 1% glucose (mutant strains) shaken overnight (250 rpm) at 37°C. Sterile non-tissue culture-treated microtiter plate wells were filled with 250 µl of BHI broth. Twenty microliter aliquots of bacterial suspension were added to corresponding wells. Plates were then incubated statically for 24 h at 37°C. The contents of each well was then removed and wells rinsed three times each with 300 µl of sterile water. The remaining attached bacteria were fixed with 200 µl of methanol per well. After 15 min, wells were emptied and left to air dry. Each well was stained with 160 µl of crystal violet for 5 min and plates allowed to air dry. After drying, the dye was extracted with 160 µl 33% glacial acetic acid per well and the OD_{570} was measured for each well. The total biofilm assay was performed a minimum of 10 times for each strain to achieve statistical significance as per a statistical consultation.

Differences in biofilm formation between wild-type, mutants, and suppression analysis mutant strains were determined by an ANOVA analysis. A p-value < 0.05 was considered significant.

To determine if biofilm forming capabilities of the *S. aureus* strains were altered in the presence of the drug SK-03-92, each strain was treated with different concentrations of the drug that ranged from 2 μg/ml to 64 μg/ml and compared to wells with untreated bacteria.

Primary attachment assay

To further investigate biofilm formation in relation to sortase A expression, a primary attachment assay was performed (Geoghegan et. al, 2010). Primary attachment, or the first phase in biofilm formation, is mediated by sortase A. The primary attachment assay further demonstrates the importance of sortase A in biofilm formation. Wild-type, mutants, and the *rssR* suppression analysis mutant strain were grown shaken overnight (250 rpm) at 37°C in BHI broth plus 1% glucose (JE2) and BHI broth plus 5 μg/ml erythromycin and 1% glucose (mutant strains) and diluted to 300 CFU in a 100 μl volume. One hundred microliter aliquots of diluted broth were spread onto the bottom of empty petri dishes with two replicates per strain. Petri dishes were incubated upright at 37°C for 30 min, washed three times with 5 ml of sterile phosphate buffered saline (PBS), and covered with 0.8% BHI agar. Additional plate counts were also done to determine the true CFU/ml of each culture. All plates were incubated overnight at 37°C and counted the following day. Percent attachment was calculated by dividing the washed plate counts over the true plate counts. The primary attachment assay was repeated a minimum of 10 times for each strain to achieve statistical significance. Differences in primary attachment were determined by ANOVA analysis. A p-value < 0.05 was considered significant.

Mouse thigh abscess model

To determine if mutations in the *rssR* response regulator or *rssS* sensor kinase genes affected the maintenance of an abscess in the thighs of a mouse, a mouse thigh abscess model of infection was used (Benton et al., 2004; Holtfreter et al., 2013). Two cohorts were tested: wild-type strain JE2 versus the *rssS* sensor kinase mutant strain and wild-type JE2 versus the *rssR* response regulator and the *rssR* response regulator suppression mutant. The *S. aureus* strains were grown statically overnight at 37°C, pelleted by centrifugation at 8000 x *g* for one min, and resuspended in one ml of PBS. The resuspended bacteria were diluted 1:100 in PBS and mixed 1:1 with a 20 mg/ml sterile Cytodex beads (Sigma Aldrich; St. Louis, MO). Each thigh was washed with 70% ethanol and 50 µl volumes of the bacteria/Cytodex bead mixture were injected intramuscularly into both thighs of female Swiss Webster mice (Harlan; Indianapolis, IN) ages 6-12 weeks. Five mice were used for each arm of the study. The mice were sacrificed by CO_2 asphyxiation three days post-inoculation. Both thighs were excised from each mouse and homogenized in 1 ml PBS. Each homogenate was ten-fold serially diluted in PBS and 100 µl of each dilution plated onto BHI agar plates. Plates were incubated for 24 hr at 37°C. The next day the bacterial colonies were counted and CFU/g of thigh tissue calculated. This analysis was repeated with an additional five mice per cohort to show reproducibility. An ANOVA analysis with a Bonferroni correction was used for statistical analysis. A p-value <0.05 was considered significant.

RESULTS

The sensor kinase mutant strain had an increase in *srtA* transcription

Previous work has demonstrated that the *rssR* response regulator and *rssS* sensor kinase genes were down-regulated and *srtA* was up-regulated when *S. aureus* strain MW2 was exposed to the SK-03-92 drug (Schwan, W., Medina, S. & Lane, M; unpublished data). Furthermore, mutations in either the *rssR* or *rssS* gene showed an increase in *srtA* transcription compared to the wild-type strain. To confirm the transcriptional up-regulation of *srtA* in the *rssR* and *rssS* mutant strains compared to the wild-type strain JE2, a qRT-PCR assay was performed. The *rssS* sensor kinase mutant strain showed a statistically significant ($p < 0.004$) 10.24-fold increase in *srtA* transcription when compared to the wild-type strain, whereas the *rssR* response regulator mutant strain showed a 2.85-fold increase in *srtA* transcription compared to wild-type ($p < 0.672$; FIG. 5). A qRT-PCR analysis of the *rssR* response regulator suppression analysis strain demonstrated a 1.48-fold increase in *srtA* transcription versus the wild-type strain ($p < 0.988$), which was not significant (FIG. 5).

Multiple attempts were made to electroporate the pAB5X-1 plasmid into the *rssS* sensor kinase mutant strain, but all of the attempts were unsuccessful. In each attempt, either no bacterial colonies grew on the antibiotic medium or the plasmid taken up by the mutant strain was of an incorrect size, indicating the plasmid was unstable. The large size of the plasmid may have played a role in its instability. It is also possible that an

overabundance of the sensor kinase being produced in the bacterial cell was a lethal phenotype and those cells that successfully took up the plasmid were not able to survive.

Therefore, inclusion of the *rssS* sensor kinase suppression analysis strain in the various experiments was not possible.

SK-03-92 drug treatment and a mutation of the sensor kinase gene causes greater biofilm formation in *S. aureus*

To further analyze the effects of the increase in *srtA* transcription, a biofilm assay in microtiter plates was performed after SK-03-92 drug treatment (FIG. 6). Wild-type JE2 cells were tested following SK-03-92 drug treatment (range 2 μg/ml – 64 μg/ml). JE2 cells grown without drug showed an OD_{570} of 2.41. At SK-03-92 drug concentrations of 16 μg/ml to 32 μg/ml, the resulting OD_{570} readings increased. A plateau for biofilm formation was reached at 32 μg/ml (3.43, $p < 0.439$), and treatment with 64 μg/ml of SK-03-92 drug resulted in a decline in biofilm formation compared to 32 μg/ml (2.99, $p < 0.926$). Since higher SK-03-92 drug concentrations led to greater biofilm formation and the SK-03-92 drug negatively impacts the transcription of the *rssR* response regulator and the *rssS* sensor kinase open reading frames, the *rssR* and the *rssS* mutant strains were tested for biofilm formation compared to the wild-type strain.

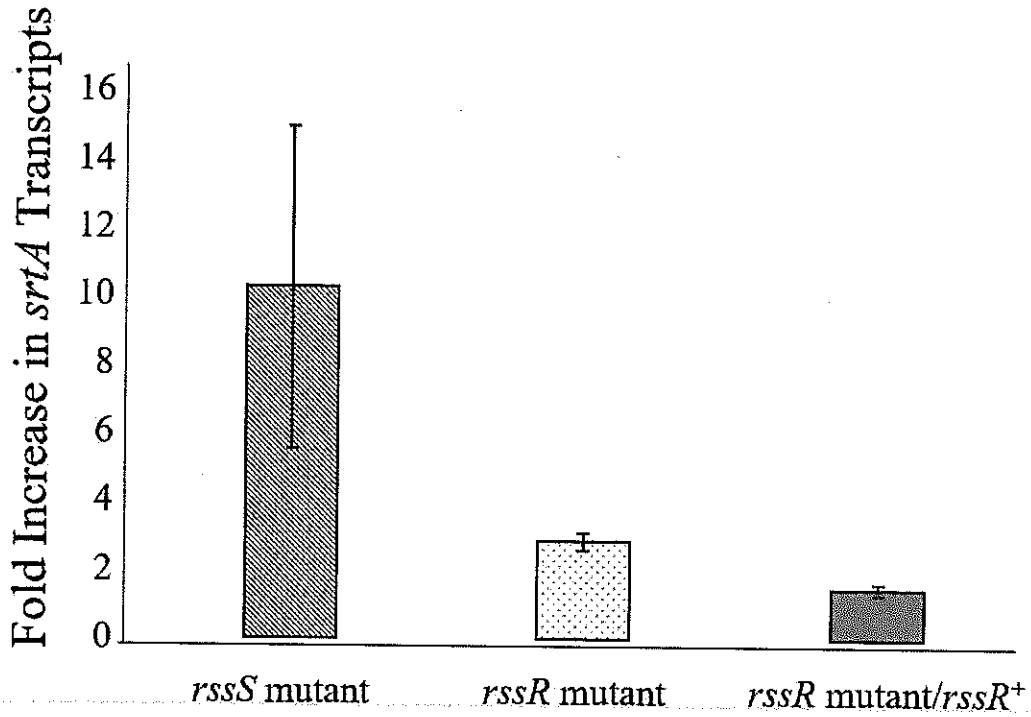

Fig 6. Transcription of *srtA* in the sensor kinase mutant strain (diagonal), the response regulator mutant strain (dots), the response regulator suppression analysis strain (grey) determined by quantitative RT-PCR. Fold difference relative to *S. aureus* JE2 ± standard error. Data represents three separate qRT-PCR runs from two different RNA samples for each strain tested.

The *rssS* sensor kinase mutant strain showed the highest biofilm forming capabilities under all of the conditions tested. In the absence of the SK-03-92 drug, the OD_{570} reading was 3.67, resulting in a statistically significant ($p < 0.01$) 1.5-fold increase compared to the wild-type strain JE2. The *rssS* mutant also showed statistically significantly greater biofilm formation when treated with SK-03-92 drug (2 μg/ml, $p < 0.002$; 4 μg/ml, $p < 0.001$; 8 μg/ml, $p< 0.007$) compared to the drug treated JE2 wild-type strain. Although the *rssS* mutant displayed higher biofilm formation compared to the wild-type strain, the *rssR* mutant strain showed no difference in biofilm formation regardless of the concentration of the SK-03-92 drug added (FIG. 6). A suppression analysis in the *rssR* strain also showed no effect by the SK-03-92 drug. Thus, treatment with SK-03-92 drug or a mutation in the *rssS* sensor kinase gene resulted in an increase in biofilm formation, but the *rssR* response regulator mutation had no effect on biofilm formation.

Mutations in the response regulator and sensor kinase genes do not affect primary attachment

To further analyze the effects of the *rssR* response regulator and *rssS* sensor kinase gene mutations on the steps leading to a biofilm, a primary attachment assay was performed. Primary attachment is mediated by sortase A and therefore, may be an indication of the relative amount of sortase A expressed by each strain. The primary attachment was calculated by dividing the washed plates by the true colony count plates (FIG. 7). Primary attachment of the wild-type strain averaged 63.1%, whereas the *rssS* sensor kinase mutant strain primary attachment was 70.2% ($p < 0.612$), and *rssR*

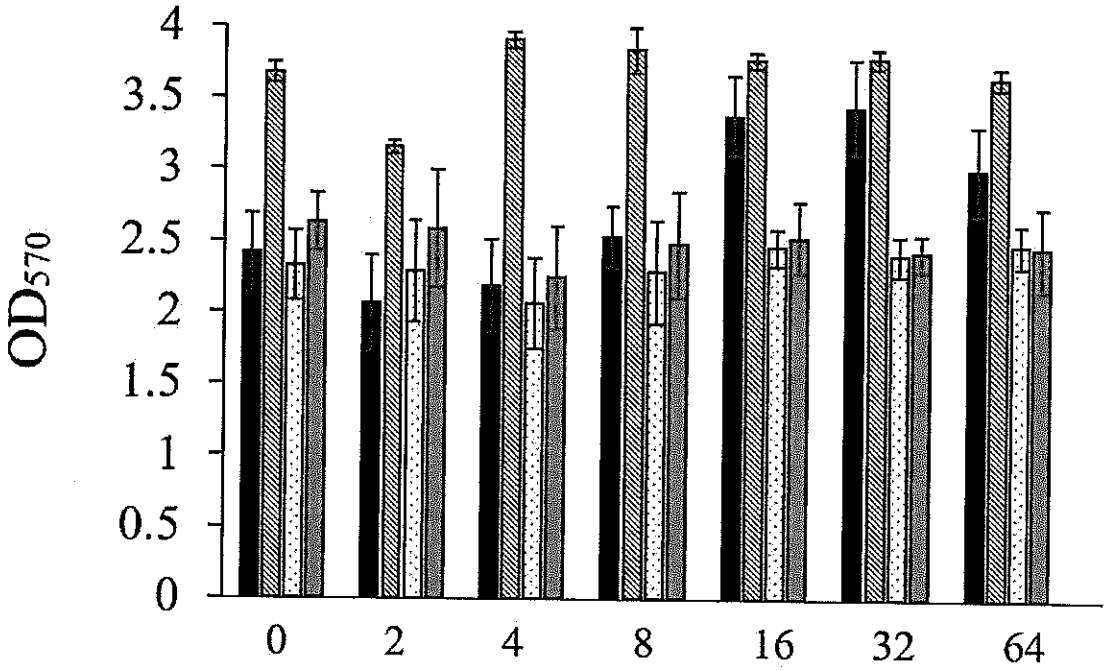

Fig 7. The effects of SK-03-92 drug concentration on 24 h biofilm formation (OD570) for *S. aureus* strains JE2 (WT, black), *rssS* sensor kinase mutant (diagonal), *rssR* response regulator (dots), and rssR/*rssR*$^{+}$ response regulator suppression analysis mutant (grey). All experiments represent an average of at least 10 runs, by recommendation of biostatistician, done in triplicate for statistical significance ± standard error.

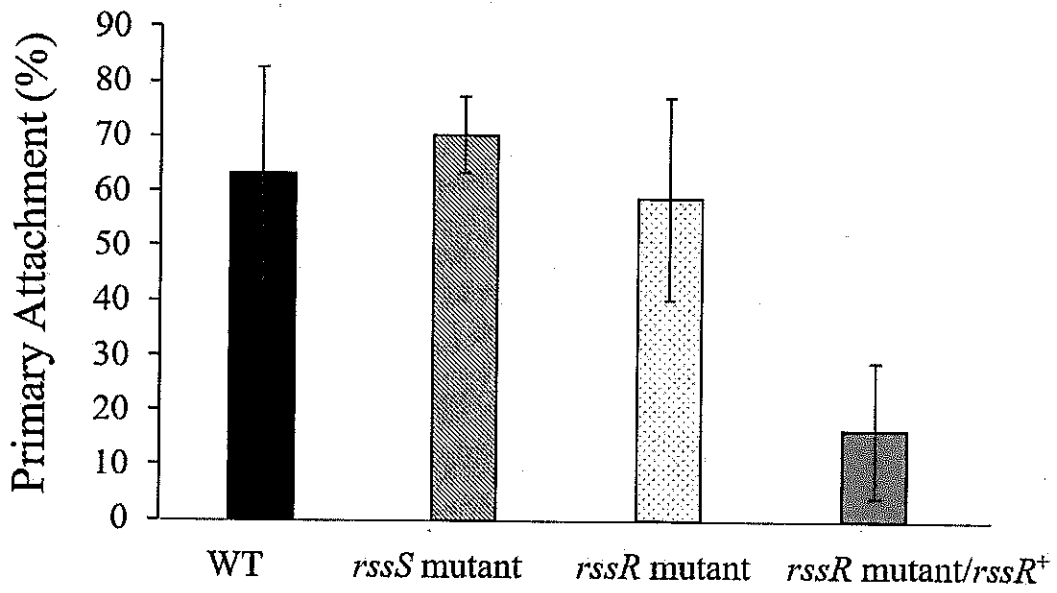

Fig 8. Primary attachment of wild-type (WT) *S. aureus* JE2 compared to the *rssS* sensor kinase and *rssR* response regulator mutant strains grown in BHI broth + 1% glucose. Primary attachment results were reported as an average of at least 10 runs ± standard error.

response regulator mutant strain primary attachment was 58.6% ($p < 0.794$), demonstrating that neither mutation affected primary attachment. However, primary attachment for the response regulator suppression analysis mutant dropped to 16.5% ($p < 0.001$) which might be explained by an over-complementation of the response regulator gene. Thus, mutations in the response regulator and sensor kinase open reading frames did not significantly affect primary attachment.

Mutations in the response regulator and sensor kinase genes do not cause a significant change in bacterial numbers within murine abscesses

The *in vitro* biofilm results showed greater biofilm formation for the *rssS* sensor kinase mutant strain compared to the wild-type strain. In order to determine the *in vivo* effects of the response regulator and sensor kinase mutations in *S. aureus*, a mouse thigh abscess model of infection was used. Two cohorts were used for this analysis. Cohort one included the inoculation of mouse thighs with wild-type strain JE2 or the *rssS* sensor kinase mutant strain. A second cohort of mice was inoculated with strain JE2, the *rssR* response regulator mutant strain, or the *rssR/rssR*$^+$ response regulator suppression analysis mutant. Mice were euthanized three days post-inoculation, infected thighs were removed, homogenized in PBS, 10-fold serially diluted in PBS, plated on BHI agar plates, and bacterial counts per gram thigh tissue (CFU/g) were recorded. For cohort one, the overall median from two trial runs for wild-type infected mice (strain JE2) was 4.80×10^6 CFU/g. The *rssS* sensor kinase mutant strain's overall median was 7.40×10^6 CFU/g that was 0.5-fold higher than the wild-type strain, but was not statistically different than the wild-type strain ($p < 0.345$; FIG. 8A).

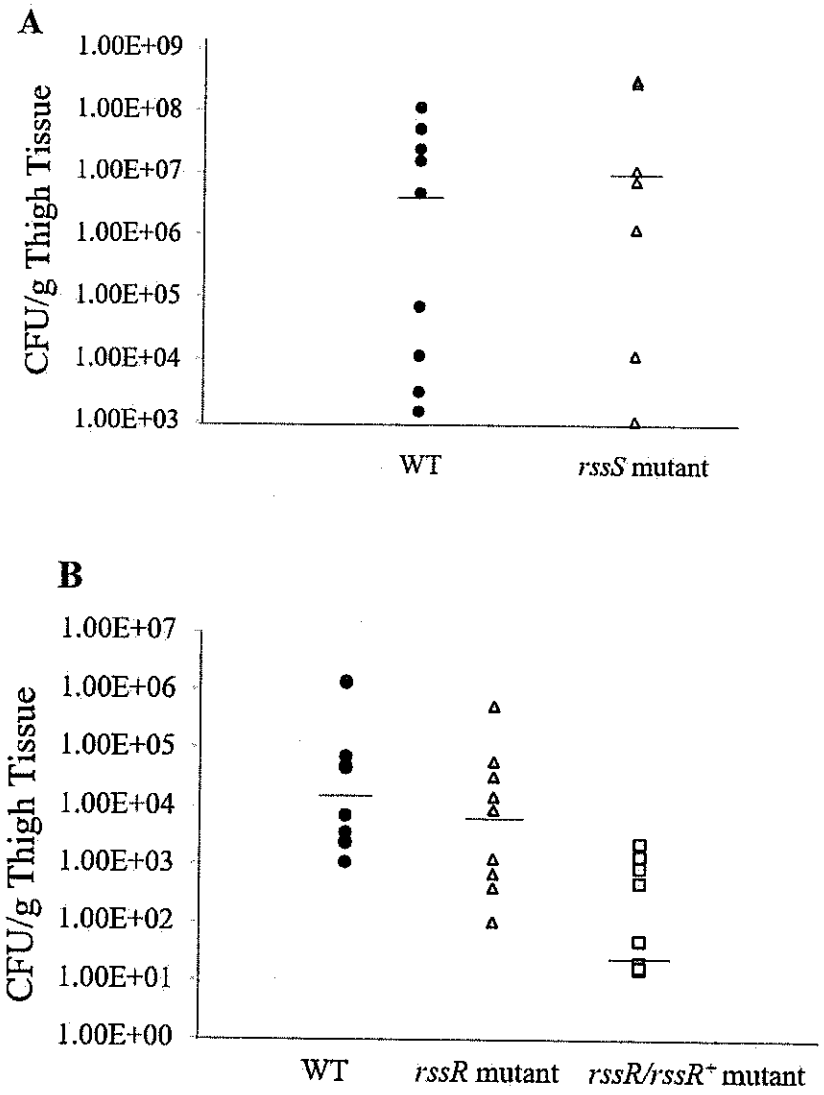

Fig 9. Independent challenge in the thigh of female Swiss Webster mice with *S. aureus* strain JE2 compared to two mutant strains and their suppression analysis counterparts. A) Comparison of wild-type (WT) versus the *rssS* sensor kinase mutant strain and B) comparison of WT versus the *rssR* response regulator mutant strain and the *rssR/rssR*$^+$ response regulator suppression analysis mutant. Each data point represents the CFU/g thigh tissue for one mouse. Horizontal bars represent the median values of the bacterial concentration of the population.

When the *rssR* response regulator mutant strain was tested, the overall median from two trial runs for wild-type infected mice was 2.55×10^4 CFU/g. The overall CFU/g median for the *rssR* response regulator mutant strain was 9.0×10^3 CFU/g, which was not significant ($p < 0.539$), whereas the median for the *rssR/rssR*$^+$ response regulator suppression analysis mutant strain was 2.10×10^1 CFU/g, which was also not statistically different ($p < 0.216$; FIG. 8B). Thus, neither mutation significantly affected CFU counts in infected murine thighs.

DISCUSSION

A previous RNA microarray that compared SK-03-92 drug treated *S. aureus* cells to untreated *S. aureus* cells showed significant down-regulation of two genes that encode for an uncharacterized, putative two-component regulatory system as well as up-regulation of the *srtA* gene that encodes sortase A (W. R. Schwan, unpublished data). We hypothesized that this new two-component regulatory system may be repressing transcription of the *srtA* gene, which in turn could have an effect on biofilm formation in *S. aureus*. *Staphylococcus aureus* uses the sortase A protein to link MSCRAMMs to its cell surface (Archer *et al.*, 2011; Walsh *et al.*, 2008). The MSCRAMMs, crucial players in phase I of biofilm formation, allow for attachment to biotic or abiotic surfaces (Periasamy *et al.*, 2011; Lister & Horswill, 2014). In this study, we have shown that a mutation in the putative sensor kinase but not the response regulator affects *srtA* transcription and biofilm formation as compared to the wild-type strain.

Since we believed that the putative two-component regulatory system had a role in regulating *srtA* transcription, qRT-PCR was done comparing *srtA* transcription in the *rssS* sensor kinase mutant and the *rssR* response regulator mutant strains to the wild-type strain JE2. The *rssS* sensor kinase mutant strain showed a significant increase in *srtA* transcript abundance compared to the wild-type strain, suggesting that the sensor kinase gene product was involved in repressing transcription of the *srtA* gene. Kinase activity is needed in two-component regulatory systems to respond to changes in the environment (Utsumi, 2008; Jung *et al.*, 2012; Mitrophanov & Groisman, 2008). We hypothesized

that the RssS sensor kinase responds to an environmental stressor, becomes phosphorylated, transfers the phosphate group to RssR that in turn binds to a DNA sequence near the *srtA* gene to repress transcription of *srtA*. Inactivation of the *rssS* sensor kinase gene due to a transposon insertion or SK-03-92 drug treatment would mean the response regulator cannot be phosphorylated and therefore, cannot suppress transcription of *srtA*. If the hypothesis is correct, transcriptional repression of *srtA* by the RssR/RssS two-component regulatory system would lead to a decrease in MSCRAMM attachment and biofilm formation due to the lower levels of sortase A available for MSCRAMM anchoring. Under normal growth conditions, biofilm formation is not necessary for a cell, but under stressful environmental conditions, such as exposure to a drug, biofilm formation would greatly benefit a cell. Formation of a biofilm would benefit cells by allowing for the formation of persister cell populations (Wood et al., 2013). When biofilms form, the cells at the base of the biofilm slow or stop most cell metabolism and go into a dormant state, allowing the organisms to survive in the presence of a drug, for example SK-03-92 (Wood et al., 2013). In addition, cells in a biofilm often undergo quorum sensing, which also can lead to the emergence of persister cells. Overall, an increase in *srtA* transcription upon exposure to the SK-03-92 drug or a mutation in either the *rssS* or *rssR* gene would cause an increase in biofilm and persister cell formation increasing a bacterial cell's ability to survive within a human or mouse abscess.

Although a mutation in the sensor kinase gene led to a significant de-repression of *srtA* transcription, a mutation in the response regulator gene caused only a small increase in *srtA* transcript accumulation compared to the wild-type, indicating that the

response regulator may not be crucial to down-regulating *srtA* transcription. A second *S. aureus* response regulator could be phosphorylated by the rssS sensor kinase if the *rssR* gene is inactivated, which then allows the protein to serve as a repressor of transcription of *srtA*.

The *rssR* response regulator and *rssS* sensor kinase genes have a 3 bp overlap, indicating they are polycistronic. This is an important aspect when mutations are introduced. A mutation in the *rssR* response regulator gene would be a polar mutation, affecting both the *rssR* response regulator and the *rssS* sensor kinase genes. If the RssS sensor kinase and the RssR response regulator are not expressed, it would be expected that biofilm formation may increase in the *rssR* mutant strain as it did in the *rssS* mutant strain, but that is not what the data showed. We hypothesize that there may be an additional two-component regulatory system or global regulator that may be acting upon *srtA* transcription when both the response regulator and sensor kinase are not present in the cell.

With an increase in *srtA* transcript abundance shown by the qRT-PCR results, an increase in biofilm formation would be expected. The *rssS* sensor kinase mutant strain had significantly higher biofilm formation compared to the JE2 wild-type strain, while the biofilm forming ability of the *rssR* response regulator mutant strain and the response regulator suppressor strain was similar to the wild-type strain. The increase in biofilm formation for the *rssS* sensor kinase mutant strain correlated with the qRT-PCR data, indicating an inability of the two-component regulatory system to down-regulate *srtA* expression due to the presumed loss of the sensor kinase protein. Furthermore, primary

attachment, which is the first stage of biofilm creation, was higher but not significant in the *rssS* sensor kinase mutant strain as compared to the wild-type strain.

Our previous unpublished research (W. R. Schwan, unpublished data) has demonstrated that the SK-03-92 drug also appears to act to down-regulate the *rssR/rssS* two-component regulatory system genes. If the SK-03-92 drug is hindering transcription of the *rssR* response regulator and *rssS* sensor kinase genes, then we should see an increase in *srtA* expression and presumably greater biofilm formation. When different SK-03-92 drug concentrations were tested against the wild-type strain in a biofilm assay, we saw an increase in biofilm formation occur up through treatment with 16 μg/ml of the drug. Treatment of wild-type cells with 16 μg/ml of SK-03-92 drug was similar to the effect of an *rssS* sensor kinase gene mutation in terms of formation of a biofilm. Concentrations of SK-03-92 drug that were tested above 16 μg/ml showed no further increase in biofilm formation, suggesting a plateau had been reached. If a *S. aureus* strain with higher biofilm producing capabilities was used in contrast to the wild-type strain JE2, a greater difference may have been observed following treatment with various concentrations of SK-03-92 drug.

The JE2 wild-type strain is not a great biofilm producing strain. Tan *et al.*, attributes the poor biofilm forming abilities of MRSA strains to an active Agr system. The Agr system is a global gene regulator that influences biofilm forming ability partly by regulating extracellular proteases. The group hypothesizes that methicillin-susceptible *S. aureus* inactivate the Agr system, allowing for better biofilm forming capabilities while an active Agr system in MRSA strains leads to an increase in the extracellular proteases (Tan *et al.*, 2015).

The SK-03-92 drug has a biphasic effect on the wild-type strain. At low concentrations the drug does not affect biofilm formation, but as concentrations of SK-03-92 increase, the drug may be inhibiting normal function of the two-component regulatory system to repress *srtA* expression by blocking an environmental trigger. If the necessary environmental trigger for inducing auto-phosphorylation of the sensor kinase is blocked, the regulatory system will no longer be able to repress *srtA* transcription. Thus, SK-03-92 drug treatment appears to mirror the effects of a mutation of the *rssS* sensor kinase gene, triggering an increase in biofilm formation. When higher concentrations of the drug are used, there is an inhibitory effect on the two-component regulatory system that will lead to an up-regulation of *srtA* expression and greater biofilm formation.

A similar finding was observed when *Candida* species were treated with drug concentrations higher than the MIC. *Candida* species grown as a biofilm were exposed to varying concentrations of echinocandin up to the MIC, disrupting the biofilm and killing the fungal cells (Melo *et al.*, 2007). *Candida* treated with varying drug concentrations higher than the MIC did not show a biofilm disruption and in some cases had increased cell density. Echinocandin acting on the *Candida* species has the same effect as our SK-03-92 drug, triggering up-regulation of a specific gene that increases biofilm formation. SK-03-92 drug treatment did not affect the ability of either the *rssS* sensor kinase mutant or the *rssR* response regulator mutant strains to form biofilms, suggesting that the drug's mode of action to de-repress *srtA* may be through this two-component regulatory system. If the response regulator or the sensor kinase have already been rendered nonfunctional due to a transposon insertion, SK-03-92 drug treatment should not affect these strains further.

The identification of the *rssS* gene encoding a putative sensor kinase and its role in biofilm was substantiated by the biofilm forming ability of the mutant. However, the role of the *rssR* gene encoding a response regulator was not as clear in regard to biofilm formation. The *rssR* response regulator mutant had the same biofilm creating ability and primary attachment as the wild-type strain, and biofilm formation remained consistent across all SK-03-92 drug concentrations that were tested. What strengthens the argument that the *rssR* gene may encode a response regulator protein that represses *srtA* transcription, was the suppression analysis experiment that examined primary attachment. We saw a significant decrease in primary attachment in the *rssR*/pAB1-5 response regulator suppression analysis mutant strain compared to the wild-type strain. The pAB1-5 plasmid exists in multiple copies within the *S. aureus* cell, resulting in a potentially higher concentration of the response regulator gene product that would exceed what is necessary to complement the gene mutation. It is possible that the large decrease in primary attachment could be explained by the increase in the response regulator protein in the cell. While this logic fits for the primary attachment assay, the qRT-PCR did not show a decrease in *srtA* expression compared to the wild-type strain.

I hypothesize that the sortase A protein is still being transcribed and translated, but may have some sort of conformational change. The conformational change in the protein would lead to a decrease in primary attachment, because the active site may be rendered inactive and unable to attach MSCRAMMs to the cell surface. With a decrease in MSCRAMMs on the cell surface, a decrease in primary attachment would be expected. It is also possible that the protein is still being translated but not transcribed. This would explain why levels of *srtA* remain similar to that of the wild-type strain but primary

attachment is affected in the *rssR*/pAB1-5 response regulator suppression analysis mutant.

In order to determine if the effects on biofilm formation and *srtA* expression happening in the *in vitro* experiments would also occur *in vivo*, a murine mouse thigh abscess model of infection was performed. The bacterial counts of the *rssS* mutant strains higher within murine abscesses were compared to the wild-type strain, while the bacterial counts within murine abscesses infected with the *rssR* mutant strain were slightly lower than the wild-type strain. However, neither mutant strain showed a significant difference in CFUs compared to the wild-type strain. Although the *rssR* response regulator suppression analysis mutant strain showed a 3-log lower median bacterial count inside murine abscesses versus the wild-type strain, this result was also not significant. If an over-expression of the response regulator does in fact lead to a down-regulation of *srtA*, the reduction in bacterial count correlates with previous studies demonstrated that *S. aureus srtA* knock-out strains are greatly reduced and have the inability to form biofilms.

Visually, murine thighs inoculated with the *rssS* sensor kinase mutant strain displayed white pus-filled abscesses in four of the mice, whereas none of the mice inoculated with the *rssR* response regulator mutant strain or the JE2 wild-type strain displayed pus-filled abscesses. This may suggest that the *rssS* sensor kinase mutant strain may be more effective at forming a biofilm than either the wild-type strain or the *rssR* response regulator mutant strain *in vivo*. As discussed above, MRSA strains are not great biofilm producers. A murine thigh abscess model of infection carried out for six days,

allowing the bacteria an additional three days to form a biofilm, may have resulted in a significant difference between wild-type and either mutant strain.

While the *in vivo* models did not show an increase in overall virulence, the *in vitro* data demonstrated the importance of understanding interactions between an organism and a drug or environmental stressor. As demonstrated by the biofilm formation data, the addition of a drug at higher concentrations can cause an organism to increase biofilm formation and could cause the formation of persister cells in the biofilm. In a clinical setting, treatment of a *S. aureus* biofilm infection may lead to the patient appearing to have been cured, but instead persister cells have remained within the biofilm that was created in the body. As the biofilm breaks up, the persister cells are able to disseminate through the body and begin a new round of biofilm formation. This cycle leads to chronic recurring infections in a patient, making infections difficult to treat. With antibiotic treatments becoming increasingly ineffective, new compounds or methods are necessary for treating infections. With these new compounds or methods, it is imperative to learn the mechanism in which they interact with the organism to determine if in fact the drug is truly beneficial for the patient.

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APPENDIX A

REAGENTS FOR ELECTROPORATION

Appendix A. Reagents for Electroporation

1. 0.5 M sucrose

68.4 g sucrose
400 ml distilled H_2O
-Filter sterilize

2. 2x SMM

85.5 g sucrose
1.15 g maleic acid
2.02 g $MgCl_2*6H_2O$
250 ml distilled H_2O
-pH to 6.5 and filter sterilize

3. 4x PAB

17.5 g antibiotic medium #3 (Difco)
250 ml distilled H_2O
-Autoclave to sterilize

4. SMMP

-Equal volumes of 2x SMM and 4x PAB

APPENDIX B

REAGENTS FOR RNA EXTRACTION

Appendix B. Reagents for RNA extraction

1. 0.1% DEPC-treated Water

300 ml Milli-Q H_2O
300 µl diethylpyrocarbonate
-Incubate shaking overnight at 37°C
-Autoclave

2. 70% ethanol

70 ml 100% ethanol
30 ml 0.1% DEPC-treated water

APPENDIX C

REAGENTS FOR TRUE BIOFILM ASSAY

Appendix C. Reagents for true biofilm assay

1. 0.5 % crystal violet

0.005 g crystal violet
200 ml distilled water

2. 33 % glacial acetic acid

33 ml 100% acetic acid
67 ml distilled H_2O

Appendix D. Reagents for primary attachment assay

1. Phosphate buffered saline

0.23 g NaH_2PO_4
1.15 g Na_2HPO_4
9.0 g NaCl
1 L distilled H_2O
-pH to 7.2
-Autoclave

2. 0.8% BHI agar

14.8 g BHI broth medium
3.2 g Bacto agar
400 ml distilled H_2O
-Autoclave