INSIGHTS INTO THE *ArlR/S* MEDIATED PATHOGENESIS
OF *STAPHYLOCOCCUS AUREUS*

by

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A thesis submitted in partial fulfillment of the requirements for the degree
of
Master of Science
in
Immunology and Infectious Diseases

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2013
of a thesis submitted by

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This thesis has been read by each member of the thesis 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 Graduate School.

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Susan Irene Meyer

July 2013
I would first like to thank my advisor and mentor, Dr. Jovanka Voyich-Kane. I have learned a great deal from her and her guidance and support through this process have been incredibly important to my success. I would also like to thank my committee, Dr. Mark Quinn, Dr. Josh Obar, and Dr. Mike Franklin, for their assistance, advice, and understanding through this process. Thank you to all of the members of the Voyich lab, past and present, including, but not limited to, Kyler Pallister, Tyler Nygaard, Danyelle Long, Rob Watkins, Oliwia Zurek, Fermin Guerra, Meet Patel, Julia Mead and Cassy Cooper – you have all been such wonderful colleagues, friends, and collaborators, and I appreciate all of the help I’ve been given and all of the fun I’ve had with all of you. Finally, I would like to express my appreciation for the continued support of my friends and family – without them I would not be where I am today and I am so lucky and grateful to have all of them in my life.
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Staphylococcus aureus (S. aureus) is a gram-positive pathogen capable of causing a wide range of disease from relatively simple soft tissue infections to severe life-threatening disease like sepsis and endocarditis. Historically, most S. aureus infections were associated with healthcare settings and a majority of cases were seen in patients with compromised immune systems. In the past decade, however, infections caused by S. aureus have become more common in healthy individuals. These community-associated strains are an even bigger problem because a large percentage are resistant to antibiotics and are have an incredible ability to incur antibiotic resistance. The ability of this bacterium to subsist and thrive in a wide range of environmental conditions is partly due to the pathogen’s use of two-component signal transduction gene-regulatory systems that have the ability to sense external conditions and regulate gene transcription appropriately. This study investigates the role of one of these two-component regulatory systems, ArlR/S. An isogenic deletion mutant of the arlR/S operon was created and was tested in several in vitro assays as well as in vivo murine models of infection. Using in murine models of soft tissue infection and invasive infection, it was determined that arlR/S is important to the virulence of S. aureus. A murine model of dissemination showed that S. aureus dissemination is altered with the deletion of the ArlR/S two-component regulatory system. To determine whether the decreased pathogenicity was caused by a change in the interaction between S. aureus and immune cells of the body, in vitro assays with human whole blood and human PMNs were performed with both S. aureus and bacterial supernatants. Interestingly, no differences were seen between the wild type S. aureus and the mutant in these assays. An oligonucleotide microarray was performed and showed strong regulation of ebh (ECM-binding protein homologue), which codes for the giant staphylococcal surface protein (GSSP). Together, this study demonstrates the importance of arlR/S to the regulation of ebh and to the virulence of S. aureus in a PMN-independent manner.
INTRODUCTION

Background

*Staphylococcus aureus* is a Gram-positive, facultative anaerobe capable of causing a wide range of disease from simple skin infections that present as small pimples to serious disease like septicemia, pneumonia, and endocarditis\(^1\)-\(^4\). When visualized, the bacteria presents as grape-like clusters of golden-pigmented cocci\(^5\). It was discovered in a knee wound by the surgeon Sir Alexander Ogston in the early 1880s; he also described the pathogen’s ability to form abscesses and infect the blood\(^6,^7\). From its discovery until the present day this pathogen has been very difficult to treat because of its genetic variability between strains and ability to evolve\(^8\). Because of this, it has become a leading cause of bacterial infections in the past several decades\(^9\)-\(^12\).

Part of the problem with treating *Staphylococcus aureus* disease is its ability to asymptptomatically colonize the nasal passages of healthy individuals\(^13\). It is estimated that upwards of 50% of healthy individuals are colonized with *S. aureus* and that 10-20% are persistently colonized, never clearing the bacteria completely\(^14,^15\). Colonized individuals are much more likely to succumb to *S. aureus* infection and those that are persistently colonized are more likely to have recurring infections that are difficult to treat\(^16\).

*Staphylococcus aureus* Antibiotic Resistance

Another important factor in *S. aureus* pathogenesis is its incredible ability to incur antibiotic resistance through uptake of mobile genetic elements\(^10\). When penicillin was
discovered in the 1940s, it was considered a miracle drug because *S. aureus* infections are usually fatal without proper antibiotic treatment\(^\text{17}\). By the 1950s, however, the bacteria incorporated a plasmid encoding \(\beta\)-lactamase and penicillin-Resistant *S. aureus* became pandemic across the country even in community settings\(^\text{10,11,18}\). With the introduction of methicillin in the late 1950s a similar trend was seen and methicillin-Resistant strains of *S. aureus* developed rapidly. By the mid 1990s Methicillin-resistant *Staphylococcus aureus* (MRSA) had become a huge problem in health care settings and was emerging as a pandemic problem in community settings as well. With the use of a new antibiotic, vancomycin, to treat these antibiotic-resistant strains, there have been documented cases of the first vancomycin resistant strains emerging in health care settings around the world\(^\text{10,19-21}\). This steady progress of antibiotic resistance has made finding new non-antibiotic based therapies to treat disease caused by *S. aureus* very important.

Historically, as MRSA became a widespread problem, it was mostly localized in health care settings. In the past decade, however, there’s been a large number of *S. aureus* isolates being found in community settings. Interestingly, these community-associated *S. aureus* strains are genetically distinct from their nosocomial counterparts\(^\text{22-25}\). MRSA isolates from hospital settings tend to carry staphylococcal cassette chromosome type II and are usually multidrug resistant. In contrast, isolates collected from community settings are most often vulnerable to non-\(\beta\)-lactam based antibiotics and carry staphylococcal cassette chromosome type IV\(^\text{26}\). The most common MRSA strain isolated from the community is pulse-field type USA300, or LAC\(^\text{27}\). Community isolated strains of *S. aureus* tend to be more virulent and can progress to very severe infections
such as necrotizing fasciitis, septicemia, toxic shock syndrome, necrotizing pneumonia and infective endocarditis\textsuperscript{1,2,28}.

The hyper-virulence of community-acquired MRSA (CA-MRSA) makes studying these strains very important. CA-MRSA isolates have become the most frequent cause of skin and soft tissue infection presenting in emergency departments in the United States\textsuperscript{29}. In a study done over a 7 year period ending in 2004, Wisplinghoff, et al. found that approximately 20% of blood stream infections recorded in nosocomial settings were caused by \textit{S. aureus}\textsuperscript{30}. Of those, isolates collected from those infections that were methicillin resistant increased from 22% in 1995 to 57% in 2001\textsuperscript{30}. Using the clinically relevant and very virulent strain of \textit{S. aureus}, USA300, is important to this study because of its profound and increasing presence in hospital and community settings\textsuperscript{31-33}. USA300 has become the dominant isolate causing skin and soft tissue infections seen in emergency rooms in the United States\textsuperscript{29,34}. It is problematic because it has the ability to infect relatively healthy individuals with no pre-existing conditions and causes disease that was not previously associated with \textit{S. aureus}\textsuperscript{35-37}. How USA300 promotes this hyper-virulence and has the ability to cause such severe disease is not well understood; we wish to promote better understanding of this CA-MRSA isolate through completion of the outlined experiments.

\textit{Staphylococcus aureus} and the Innate Immune System

The ability of the bacteria to avoid the host innate immune response allows it to cause severe disease in humans. CA-MRSA strains have developed ways to evade host antimicrobial peptides and avoid killing by phagocytic immune cells, especially the first
line of defense, human polymorphonuclear leukocytes (PMNs). PMNs are the most abundant leukocyte in the body, with ~$10^{11}$ being produced per day in the bone marrow. The importance of these cells has been shown through various studies looking at *S. aureus* infections in neutropenic patients (less than 500,000 PMNs per mL of blood). Patients lacking the proper neutrophil response have more severe disease and increased mortality associated with bacterial infection\(^{38-40}\). Danger signals and chemical messages produced during infection and the inflammation that follows signal to the body to produce and activate more neutrophils in the bone marrow by up-regulating the production and maturation of precursor cells\(^{41}\). When an infection is detected, PMNs are recruited to the site of infection through a series of chemical signals produced when resident immune cells recognizes foreign material through their pattern-recognition receptors (PRRs). Inflammatory cytokines and chemokines are produced by cells in the infected or damaged tissue and attract neutrophils as they circulate through the bloodstream. This signaling allows PMNs to extravasate from the capillaries into the tissue at the site of infection to phagocytose and kill with antimicrobial peptides (AMPs)\(^{42,43}\). PMNs have many ways to kill bacteria; during phagocytosis they use hydrogen peroxide, reactive oxygen species (ROS) and antimicrobial peptides contained within neutrophil granules that can be released into the phagosome. *S. aureus* has the ability to evade killing by PMNs in several different ways. One method is through the release of neutralizing factors that can block the action of oxygen dependent antimicrobials. One of the major neutralizing factors is the carotenoid pigment of *S. aureus* that can neutralize ROS. *S. aureus* can also degrade AMPs and can up-regulate
other “defense” mechanisms such as changing the overall charge of the bacterial membrane allowing the bacteria to escape detection by AMPs\textsuperscript{44,45}. Through the production of toxins such as hemolysins and leukocidins, the bacteria can cause lysis of the neutrophils instead of a controlled apoptosis, allowing for the release of toxic bacterial products, bacteria, and host cytotoxic molecules\textsuperscript{46-48}. All of these strategies allow \textit{S. aureus} to cause severe disease in the host.

\textbf{\textit{Staphylococcus aureus} Biofilm Formation}

Proper surface associated formations of bacterial biofilms are important to bacterial pathogenesis \textit{in vivo} and host innate immune evasion. Some evidence shows that biofilm growth may increase bacterial resistance to host immune responses and to many antibiotics\textsuperscript{49}. Biofilm formation occurs in several steps including attachment to host tissue, maturation of the biofilm, followed by proper detachment and dissemination of the bacteria\textsuperscript{50}. In the initial attachment phase of biofilm formation, important bacterial molecules known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) begin the attachment process. Examples of important \textit{S. aureus} MSCRAMMs are clumping factor A (ClfA), fibronectin binding proteins A and B (FnA, FnB), and the giant staphylococcal surface protein (GSSP). After initial attachment of the bacteria to host matrix molecules the next step involves bacterial aggregation and maturation of the biofilm. During this phase it is necessary for the bacteria to down-regulate expression of the MSCRAMMs necessary for initial attachment and to up-regulate genes necessary for proper quorum sensing, like \textit{agr}, and aggregation of bacterial cells. As the bacteria adhere and attach to host tissue, they also start to form
mature biofilms, the next step in biofilm formation. An important molecule necessary for this step is the polysaccharide intercellular adhesion (PIA)\textsuperscript{51-54}. Through the de-acetylation of N-acetylglucoasmine residues in this molecule, a positive charge is introduced that interacts with the negative charge on the bacterial cell surface. This interaction allows the bacteria to adhere to one another with PIA acting like glue between the bacterial cells\textsuperscript{55}. During this process, as the bacteria proliferate and the biofilm grows, disruptive forces are also introduced that allow the biofilm to properly form channels to allow nutrients access to the bottom layers of bacterial cells\textsuperscript{56}. These disruptive forces are also important in the final step of the biofilm formation, which involves bacterial detachment and dissemination through host tissues. An important system involved in regulation of bacterial detachment is \textit{agr} regulatory system. Vuong et al. found that when \textit{agr} is knocked out of \textit{Staphylococcus aureus}, biofilm formation is increased\textsuperscript{55}. Despite this phenotype, deletion of \textit{agr} attenuates pathogenesis\textsuperscript{57}. It is important that the bacteria are able to escape the biofilm and disseminate through the body, allowing more bacterial growth in different parts of the body. If one part of the biofilm formation process breaks down, biofilm does not development properly and bacterial dissemination and growth can be halted.

\textbf{Two-Component Gene Regulatory Systems in \textit{S. aureus}}

Many factors lead to MRSA being such a well-established pathogen. It has an incredible ability to live in a variety of environments, surviving and multiplying in diverse conditions such as on the skin, in the nares, or in the blood and tissues of individuals. Bacterial adaptation to different stimuli is one of the main factors that has
led *S. aureus* to become such a widespread pathogen. This ability to adapt can be seen at four levels: individual genetic control to regulate specific transcriptional changes, global regulation of multiple genes, whole-cell modifications, and cell-to-cell interactions including aggregation, biofilm formation, and quorum sensing. On the transcriptional level, the cell wall associated proteins and secreted virulence factors that USA300 produces can partially explain its hyper-virulence. These proteins can be designated into five distinct groups including adhesion proteins, super antigens, pore-forming toxins, ADP-ribosylating toxins, and proteases. It has been shown through previous work that these virulence factors are regulated differently depending on the growth phase of the bacteria and environmental conditions that *S. aureus* encounters.

Much of this regulation takes place on the transcriptional level based on environmental stimuli and it is believed these transcriptional changes are regulated on a large scale by two-component gene-regulatory systems (TCSs). TCSs are composed of membrane bound sensor histidine kinases that sense extracellular signals and transfer that signal to an internal response regulator. When the sensor kinase recognizes a specific extracellular environmental stimulus, it auto-phosphorylates a specific histidine residue on the extracytosolic portion of its structure. Now activated, the sensor kinase transfers the phosphate group to the aspartate residue of the cytosolic response regulator. With this change in the phosphorylation state of the response regulator, it will bind to specific portions of the bacterial genome to modify gene regulation based on the original external stimuli. This is a broad characterization of a prototypical TCS, many TCSs have not been fully characterized or understood. Some response regulators have even been
known to possess other functions like enzymatic activity, being able to bind RNA directly, or the ability post-transcriptionally regulate different cellular functions through direct interaction with proteins\textsuperscript{60,68}. In \textit{S. aureus} there are 16 putative two-component gene-regulatory systems that have been discovered through sequence homology\textsuperscript{64,65}. This study investigates a relatively uncharacterized two-component regulatory system, autolysis-related locus (ArIR/S), to determine its role in the pathogenesis in the clinically relevant CA-MRSA strain, USA300.

The ArIR/S Two-Component Gene-Regulatory System

The TCS ArIR/S was discovered in 2000 through random transposon mutagenesis in \textit{S. aureus} strain MT23142\textsuperscript{69}. Fournier, et al., found that insertion of a transposon into the gene, which they named \textit{arlS} for autolysis-related locus, sensor protein, had a slight impact on \textit{norA} expression. The norA protein is important because it confers resistance to fluoroquinolones by mediating the active efflux of these compounds\textsuperscript{70}. Surprisingly, when they characterized this mutant more extensively, they also found differences in autolysis, adhesion properties, and protease activities of the mutant bacteria. The sequence was analyzed and a second open reading frame was found upstream of \textit{arlS} and was named \textit{arlR} for autolysis-related locus, regulator protein. Additional transcriptional analysis revealed that the two were transcribed as a single message\textsuperscript{69}. A preliminary study in 2004 found that disruption of \textit{arlS} decreased bacterial growth of \textit{S. aureus} strain RN4220 \textit{in vivo} in a mixed culture competitive infection as well as in a non-competitive systemic infection\textsuperscript{71}.

Knowing that \textit{arlS} disruption causes a decrease in pathogenesis, we wished to
further define how deletion of the ArlR/S TCS from *S. aureus* influences bacterial virulence in murine models of infection. Using an isogenic deletion mutant of *arlr/S*, this study identifies the ArlR/S TCS as important to the pathogenesis of CA-MRSA strain USA300 *in vivo*. We also found a change in the way the bacteria disseminate through the body when *arlr/S* is disrupted, with the mutant bacteria being cleared more quickly from the body during invasive infection and from skin abscesses. Interestingly, we found that this difference in pathogenesis does not seem to be due directly to a difference in the way the bacteria interacts with human polymorphonuclear leukocytes, immune cells that are normally considered an important host defense against *S. aureus*. To determine what genes played a role in this decrease in pathogenesis a microarray with triplicate samples of each strain was performed and analysis showed that the ArlR/S TCS does not transcriptionally regulate any known virulence genes but does strongly regulate the gene of a huge 1.1 megadalton protein, Ebh, which stands for ECM-binding protein homologue. Collectively, this study identifies *arlr/S* as an important contributor to *S. aureus* pathogenesis and identifies *ebh* as an important target of the ArlR/S system.


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45. Li, M. *et al.* Gram-positive three-component antimicrobial peptide-sensing


56. O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm Formation As Microbial


CHAPTER TWO

THE ArlR/S TWO-COMPONENT SIGNAL TRANSDUCTION REGULATORY SYSTEM IS ESSENTIAL TO THE PATHOGENESIS OF

STAPHYLOCOCCUS AUREUS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Susan I. Meyer

Contributions: With the help and collaboration of several colleagues, Susan I. Meyer designed the study to determine what differences, if any, existed after deletion of the ArlR/S two-component regulatory system. After thorough consideration of the literature and past experimentation, she preformed multiple experiments to correctly identify any phenotypes that may be regulated by this specific two-component regulatory system of S. aureus. With much trial and error and after different phenotypes emerged between the mutant and wild-type strains, she organized the data, plotted figures, and wrote a manuscript describing what had been found.

Co-Author: Tyler K. Nygaard

Contributions: Tyler K. Nygaard helped conceive this study and created the mutant necessary for experimentation, usa300ΔarlR/S. He helped perform several experiments and was instrumental in the problem-solving process. He helped design and organize the manuscript and was an important part of the editing process.

Co-Author: Meet Patel

Contributions: Meet Patel designed and problem solved several experiments involving the ArlR/S mutant. He helped determine that the ArlR/S system differentially regulates S. aureus ability to lyse red blood cells in vitro.

Co-Author: Jovanka M. Voyich

Contributions: Jovanka M. Voyich provided guidance through the whole process of experimentation and manuscript creation. She was instrumental in the problem-solving process and helped design studies and experiments that allowed the study to proceed in the correct direction. She edited and commented on all data that was collected and
helped determine the correct ways to present data in figure or table format. She was also an important part of the design and organization of the manuscript and was instrumental in the editing process.
Abstract

*Staphylococcus aureus* (*S. aureus*) is a bacterial pathogen that can cause a wide range of disease from soft-tissue infection to acute septicemia. Antibiotic resistant strains of this bacterium have been identified and methicillin-resistant *S. aureus* are problematic in and outside of healthcare settings. This study investigates the two-component regulatory system ArlR/S using an *arlR/S* deletion mutant of the community-associated MRSA strain USA300. Murine models of soft-tissue and invasive infections showed that deletion of *arlR/S* significantly attenuated the virulence of *S. aureus*. Skin infections with the *arlR/S* deletion mutant (Δ*arlR/S*) were characterized by significantly smaller abscesses than infections with the parent strain. Additionally, mice infected intravenously with Δ*arlR/S* showed a significant decrease in mortality and dissemination to organs compared to USA300. Interestingly, deletion of *arlR/S* did not influence the ability of *S. aureus* to survive phagocytosis by human polymorphonuclear leukocytes. Using microarray analysis, we identified that *arlR/S* influenced transcription of approximately 0.9% of the *S. aureus* genome and did not alter expression of any known hemolysins or leukocidins. The most prominent finding from the microarray analysis was the up-regulation of *ebh* in the *arlR/S* mutant strain. Ebh codes for the 1.1 megadalton Giant Staphylococcal Surface Protein (GSSP) in the *arlR/S* mutant. This protein has been associated with cell adhesion properties and proper coagulation of the bacteria. We propose that the ArlR/S regulatory system affects regulation of the cell surface protein GSSP, leading to a difference in USA300 interaction with host cells and tissues causing a decrease in overall virulence of the pathogen.
Introduction

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive pathogen capable of mild skin infections as well as invasive diseases including sepsis and endocarditis\(^1\). Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have increased in number and have become the most common cause of bacterial infections in recent decades\(^2-5\). These community-associated MRSA (CA-MRSA) strains have the ability to infect otherwise healthy individuals and cause severe illness\(^6-8\). The ability of *S. aureus* to survive in many different environmental conditions makes it an extremely successful pathogen. Part of the bacterium’s ability to survive is likely due to the concerted influence of 16 two-component gene-regulatory systems (TCSs)\(^9-11\). These systems have the ability to change bacterial gene transcription in response to different environmental stimuli through the transfer of signals from a membrane-bound histidine kinase to an internal response regulator\(^12-14\). It is well accepted that these gene regulatory systems play an important role in the pathogenesis of *S. aureus* disease, however, only a few of these systems (SaeR/S and Agr) have been well studied *in vivo*. In the current study we investigate the contribution of the ArlR/S TCS in staphylococcal virulence. Previous studies have shown that the ArlR/S TCS was involved in adhesion, biofilm formation, and regulation of virulence factors such as Hla. Fournier, et al., found that disruption of the ArlR/S system led to an increase in *hla* transcription and translation of Hla protein\(^15,16\). Fournier, et al. also found that disruption of the sensor portion (*arlS*) of the ArlR/S operon led to a disruption in the ability of the bacteria to form cell clusters, leading to the conclusion that cell-to-cell adhesion seemed to be disrupted\(^15,16\). The
contribution of this TCS to virulence of clinically relevant strains of *S. aureus* is relatively unknown but a study by Benton, et al., found an important role for ArlR/S in the full virulence of *S. aureus* *in vivo*. Using strain RN4220, Benton, et al., investigated several transposon mutants and found that disrupting *arlS* caused a decrease in pathogenesis of *S. aureus*. The study used both competitive and non-competitive invasive models of infection and found decreased virulence in both cases. Knowing that disrupting the sensor protein in this system caused decreased pathogenesis, we wished to further characterize the role of the full *arlR*S operon *in vivo* using the clinically relevant CA-MRSA strain USA300. Using an isogenic *arlR*S deletion mutant in USA300 (strain LAC) we characterized the influence of *arlR*S on *S. aureus* pathogenesis during murine invasive and soft-tissue infections and after phagocytosis by human polymorphonuclear leukocytes (neutrophils or PMNs). Additionally, we defined its influence on the *S. aureus* transcriptome using an oligonucleotide microarray and quantitative real-time PCR.

**Materials and Methods**

**Bacterial Strains and Culture**

*Staphylococcus aureus* pulse-field gel electrophoresis type USA300 strain LAC (USA300) was used to generate USA300Δ*arlR/S* (Δ*arlR/S*) as described previously (Fig 2.1A). To determine whether the mutation caused any significant differences in other parts of the bacterial genome, a complemented strain was created by supplementing Δ*arlR/S* with a plasmid encoding *arlR/S* as previously described. *S. aureus* strains were cultured in tryptic soy broth (TSB) (BD Biosciences) containing 0.5% glucose.
Bacterial cultures were inoculated from overnight cultures at a 1:100 dilution and were harvested at mid-exponential (ME) and early-stationary (ES) growth defined by OD_{600} = 1.5 or OD_{600} = 3.0.

**Murine Models of Infection**

All animal studies were performed in accordance with guidelines set forth by the National Institutes of Health and approved by the Animal Care and Use committee at Montana State University – Bozeman. Female SKH1-hrBR hairless and BALB/c mice (aged 8-10 weeks) were purchased from commercial sources and from the Montana State University Animal Resource Center. USA300 wild-type and USA300\(\Delta\)arlR/S S. aureus strains were cultured to the mid-exponential (ME) phase of growth, washed once in sterile Dulbecco’s phosphate buffered saline (DPBS), and resuspended in sterile DPBS to varied concentrations as described for each model below.

For the murine model of soft-tissue infection, SKH-1 mice were inoculated subcutaneously in the right shoulder with 10^7 CFU/mL USA300 or \(\Delta\)arlR/S bacteria in 50\(\mu\)L sterile DPBS. Mice were weighed and abscess measurements were taken daily for 11 days post-infection. Abscess size was determined using the formula for a spherical ellipsoid \((v + (\pi / 6) x l x w^2)^{21}\). To determine bacterial burden in the abscesses, mice were inoculated with S. aureus as described above and on days 2, 4, and 7 three mice per treatment were euthanized. The abscessed area of skin on each mouse was excised using a 9 mm “punch,” homogenized in 2 mL sterile DPBS, and plated on tryptic soy agar (TSA). Plates were incubated at 37°C and 5% CO_2 overnight and CFUs were enumerated.
on the following day. Statistical analysis was determined using an unpaired t-test (GraphPad Prism, version 6.0 for Mac OS X; GraphPad Software).

For the survival model, BALB/c mice were inoculated intravenously with $3 \times 10^7$ CFU *S. aureus* in 100 μL sterile as was reported previously. Mice were monitored every 3 hours for the first 24 hours post-infection, and every 6 hours for the following 24 hours. Mice were euthanized if they were immobile, were unable to eat or drink, or exhibited signs of labored breathing. Survival statistics were determined using a Log-rank (Mantel-Cox) test (GraphPad Prism, version 6.0 for Mac OS X; GraphPad Software).

To assess dissemination, BALB/c mice were inoculated intravenously with $5 \times 10^7$ CFU bacteria in 100μL sterile DPBS. Mice were checked throughout the day for signs of sickness and were euthanized at ten hours post-infection. Kidneys and hearts were harvested from the mice and homogenized in sterile DPBS and plated on TSA plates. In some mice kidneys and hearts were harvested and processed for histopathology. In brief, tissues were fixed in a 10% formalin solution and processed for 6 hours in a Sakura VIP 6 Tissue Processor and dehydrated using an increasing concentration of ethanol. After processing and dehydration, tissues were infiltrated and embedded in paraffin. Tissues were sectioned at 5 microns, applied to positively charged slides, and stained for H&E as described previously.

**Human PMN Assays**

Human polymorphonuclear neutrophils (PMN) were isolated under endotoxin-free conditions (<25.0 pg/ml) as previously described. Cell preparations contained
~99% PMNs and purity of preparations was assessed by flow cytometry (FACSCalibur; BD Biosciences). To assess lysis, bacteria were grown to the ME or ES phase of growth and opsonized in 50% NHS for 30 minutes at 37°C. Bacteria were washed in DPBS, 25 μL of 10^7 CFU/mL bacteria was combined with 25 μL 10^6 human PMNs, centrifuged at 1500 RPM for 8 minutes, and incubated at 37°C and 5% CO_2. Following phagocytosis of *S. aureus*, PMN lysis was determined by measuring the release of lactate dehydrogenase (LDH) as described by the manufacturer. To determine the influence of *arlR/S* on bacterial survival following phagocytosis, *S. aureus* strains were grown to the ME or ES phase of growth, harvested, and washed in sterile DPBS. Opsonized bacteria (10^7 CFU/mL) were combined with human PMNs (10^6 cells/mL) and synchronized via centrifugation as previously described. Plates were removed from the incubator at designated time points, PMNs were lysed using 0.1% saponin to release ingested bacteria, and *S. aureus* enumerated by plating on TSA. *S. aureus* survival was determined by comparing CFUs at each time point to the CFUs at the start of the assay (0 hour) as previously described.

**S. aureus Survival in Human Whole Blood**

Heparinized venous blood of healthy individuals was collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects, NIAID, and Montana State University, Bozeman, MT. All donors signed a written consent to participate in the study. *S. aureus* strains USA300, *ΔarlR/S*, and *ΔarlR/S+comp* were grown to ME phase of growth, harvested, and washed in sterile DPBS. 100 μL of 10^5 CFU/mL bacteria was added to one mL of heparinized blood and incubated at 37°C and
5% CO₂ and rotated at 20RPM on a Heto Rotamix RX for 1 or 3 hours. At the designated time point, the tubes were vortexed and samples were plated for CFU enumeration. Percent survival of *S. aureus* in blood was determined by comparing the CFUs in the samples at 1 and 3 hours as compared to the start of the assay (0 hour)¹⁸

Statistical analysis was performed using repeated-measures analysis of variance and Tukey’s post- test for multiple comparisons (GraphPad Prism, version 6.0 for Mac OS X; GraphPad Software).

**Oligonucleotide Microarray and TaqMan Real-time RT-PCR Analysis**

Oligonucleotide microarray analysis and quantitative real-time PCR (qPCR) analysis were performed as previously described¹⁸,²²,²⁵. For oligonucleotide microarrays, USA300 or ΔarlR/S bacteria was grown to mid-exponential (ME) or stationary (ES) phase of growth. Using a Qiagen RNeasy Mini Kit, RNA was purified from the cells²⁶ and the protocol for prokaryotic target preparation for Affymetrix was followed to prepare the samples for hybridization²⁷. Samples were hybridized to GeneChip *S. aureus* genome arrays. To compare gene expression between wild type and ΔarlR/S strains, fold changes for each transcript were determined by comparing Affymetrix GeneChips hybridized with cDNA from USA300 with those with cDNA from ΔarlR/S at specific growth phases. Each experiment was performed in triplicate for statistical significance. To confirm microarray results qPCR was performed as previously described. At least three separate experiments were performed with samples tested in triplicate for each primer pair.
Mouse Models of Sepsis and Soft-Tissue Infection Demonstrate that \textit{arlR/S} is Important During \textit{S. aureus} Disease

USA300 can cause a wide range of infections, from relatively mild skin infections to invasive and rapidly fatal disease. To investigate the role of \textit{arlR/S} during skin infection, we used a murine model of soft-tissue infection. Mice infected subcutaneously with USA300\textit{ΔarlR/S} had significantly smaller abscesses as compared to mice infected with wild type USA300 (Figure 2.2A). However, there was no difference in incidence of dermonecrosis in \textit{ΔarlR/S} infected mice compared to wild-type infected mice. To determine if the reduced abscess size was due to a difference in bacterial burden we excised the infected skin on days 2, 4 and 7, and calculated CFUs/gram of tissue. Although there were no significant differences in bacterial burden on days 2 or 4, we found that, by day seven, the amount of bacteria recovered in abscesses from mice infected with \textit{ΔarlR/S} was significantly lower than from mice infected with USA300 (Figure 2.2B).

To determine if \textit{arlR/S} also attenuated virulence in a mouse model of invasive infection mice were infected with USA300 or \textit{ΔarlR/S} intravenously. All mice infected intravenously with USA300 experienced morbidity and 26 of 35 mice died by 24 h after infection. This result is consistent with those of previous studies \cite{17}. In contrast, only 4 of 25 mice infected with \textit{ΔarlR/S} became sick and died by 24 hours after infection (P<0.0001). (Figure 2.2C) \cite{9}. Collectively, these data indicate that deletion of \textit{arlR/S} attenuates virulence in both murine soft-tissue and invasive infections.
ArlR/S Contributes to Dissemination During Invasive *S. aureus* Disease

*S. aureus* must be able to properly coagulate, aggregate, and disseminate for proper tissue abscess formation and disease to take place \(^{28}\). For this reason, we wanted to determine if the decreased virulence seen in the invasive infection was influenced by differential dissemination of the bacteria through the body. Mice were infected intravenously as described for the invasive model and dissemination to kidneys and hearts was assessed ten-hours post infection. This time point allowed tissues to be collected just prior to the onset of mortality and has been shown to be an effective model (Figure 2.2C)\(^{29}\). We found that ∆arlR/S did not disseminate as effectively as the wild-type bacteria (Figure 2.2D). Bacteria recovery in both the heart and kidney of mice infected with ∆arlR/S was reduced when compared to mice infected with USA300, although the trend was significant only in the heart. In summary, these results demonstrate that arlR/S is important to the virulence of *S. aureus* during both superficial and invasive murine models of infection, perhaps by influencing effective bacterial dissemination.

Deletion of the ArlR/S System Causes No Significant Changes in *S. aureus* Survival After PMN Phagocytosis

Previous studies have shown the importance of several TCSs to the survival of *S. aureus* in human whole blood. We sought out to determine if a component of human whole blood might be causing the differences seen in the ∆arlR/S mutant (Figure 2.3C).
Our results indicate that survival in human blood was not affected by deletion of arlR/S (data not shown).

Human polymorphonuclear leukocytes are one of the most important defenses to S. aureus infection. The ability to evade killing and escape phagocytosis by PMNs is a virulence strategy used by S. aureus. To determine whether the attenuation of virulence seen in the arlR/S mutant during murine infection was attributable to enhanced killing by PMNs, we evaluated killing of USA300 and ΔarlR/S following phagocytosis by human PMNs. Interestingly, we saw no significant differences in the ability of the bacteria to survive phagocytosis by human PMNs after 0.5, 1, 3, or 6 hours of culture (Figure 2.3A). Congruent with these results, LDH release by human PMNs after bacterial phagocytosis showed no significant differences between lysis of PMNs exposed to USA300 or ΔarlR/S after 0 or 5 hours (Figure 2.3B). LDH release of PMNs was also determined after exposure to filtered S. aureus supernatants after 0 and 5 hours and no differences were seen in lysis. These results suggest that arlR/S does not regulate factors responsible for survival after exposure to PMNs.

Change in Gene Expression Caused by Deletion of ArlR/S

To better determine mechanisms of the reduced virulence seen in the mice infected with the arlR/S mutant compared to USA300 we compared the transcript abundance of USA300 and USA300ΔarlR/S using a previously described method (Figure 2.4). Analysis of the data showed that deletion of arlR/S from USA300 caused a significant change in regulation of approximately 0.9% of the transcriptome during both
the mid-exponential and early-stationary phase of growth. Of the transcript levels that were significantly different between the mutant and wild type strains, 19 genes had decreased expression, while 41 had increased expression in the ΔarlR/S strain. When arlR/S was disrupted there was transcriptional down-regulation of a group of genes in the purine biosynthesis pathway including pyrC, pyrE, and pyrF by almost 3-fold compared to the wild type. However, most transcriptional changes associated with ΔarlR/S were up-regulation of transcripts encoding genes with uncharacterized function. Serine-aspartate repeat (Sdr) protein genes including sdrC, sdrD, and sdrE were up-regulated in ΔarlR/S compared to wild-type strain. Although the exact function of these genes is not yet fully understood, some literature has provided evidence that sdrE has the ability to bind calcium\(^3\). Additionally, Sdr genes share similar repeats to those found in \textit{S. aureus} fibrinogen-binding clumping factors ClfA and ClfB and have both organizational and sequence similarities \(^3\). One of the most significant changes seen in ΔarlR/S was the dramatic up-regulation of the extracellular matrix (ECM) binding protein homologue (ebh) which was up-regulated 96-fold in ME phase and 288-fold in the stationary phase of bacterial growth. This gene encodes the Giant Staphylococcal Surface Protein (GSSP) which has homology with other ECM-binding proteins and has been shown to be produced during infection \textit{in vivo} in humans by analysis of antibodies post-infection \(^3\). Previous studies looking at specific TCSs involved in virulence have demonstrated that severe dermonecrosis and disease during soft tissue infection can be due to the release of alpha-toxin by \textit{S. aureus} \(^3\). Interestingly, transcript levels of \textit{hla} displayed no significant differences between the strains (Figure 2.5B) and no differences were seen
with any other known hemolytic or cytolytic toxins. Due to the potential for post-transcriptional regulation of Hla, human blood cell assays were performed, and again no significant difference observed between USA300 and ΔarlR/S. These observations indicate that the ArlR/S system does not regulate hla in CA-MRSA USA300 strains. Microarray results were confirmed with TaqMan qRT-PCR of several different genes including hla, ebh, nuc, and sdrD (Figure 2.5A).

Discussion

Previous studies have found the two-component system ArlR/S to be important in many bacterial functions required for the pathogenesis and proper biofilm formation and coagulation of the bacteria\textsuperscript{15-17,38}. For this reason, we sought out to determine the role arlR/S plays in pathogenesis \textit{in vivo} and \textit{in vitro} and to determine the important genes involved in these changes in virulence. An arlR/S isogenic deletion mutant was created and tested to determine its role during \textit{in vivo} models of pathogenesis (Figure 2.2). We found that without a functioning ArlR/S regulatory system, \textit{S. aureus} has less ability to cause soft tissue infection resulting in significantly smaller abscesses as compared to the wild type bacteria (Figure 2.2A). Also, we saw that the mutant bacteria cleared from the skin more quickly during infection as CFUs were lower in abscesses of mice infected with the mutant bacteria by day seven (Figure 2.2C). During an intravenous infection, virulence of \textit{S. aureus} was reduced with disruption of arlR/S, causing less mortality in mice (Figure 2.2A). Another interesting finding was that deletion of ArlR/S had an effect on the ability of the bacteria to disseminate through the body of mice. An intravenous
murine model of infection was performed in which the kidneys and heart of infected mice were taken at 10 hours to determine bacterial load. We found less mutant bacteria in the kidney and heart of infected mice, perhaps suggesting that the ability of the bacteria to disseminate from the blood had been disrupted (Figure 2.2B).

To determine if the reduced virulence seen \textit{in vivo} was due to an increase in the ability of immune cells to recognize and clear bacteria from the system, several assays with whole blood and human PMNs were performed. The \textit{in vitro} assays indicated no differences in the ability of human PMNs to phagocytize bacteria, nor were there differences in the ability of the bacteria to survive encounters with human PMNs. Although surprising, these results indicate that some other factor in the host is probably involved in the differential virulence seen during infection with the wild type USA300 versus the mutant \( \Delta \text{arlR/S} \). Potentially, this change in pathogenicity is probably due to altered gene expression by the bacteria in response to the host environment or a single host factor as has been seen in previous studies \(^{18,39,40}\).

We performed a microarray analysis on the wild type USA300 and mutant \( \Delta \text{arlR/S} \) strains and found that \textit{arlR/S} negatively regulates transcription of \textit{ebh}, a gene that codes for a protein that has previously been shown to be involved in adhesion and fibronectin binding \(^{34,41}\). The protein encoded by \textit{ebh}, the extracellular matrix (ECM) protein-binding homologue (Ebh), also known as the Giant Staphylococcal Surface Protein (GSSP) \(^{42}\), is a giant 1.1-megadalton protein that has been found only in \textit{S. aureus} and \textit{S. epidermidis} and seems to be species specific \(^{34}\). Studies of its composition have found that this protein has one membrane-spanning region and may be localized at the
cell surface with highly variable module junctions. It has been shown to interact with fibronectin, a eukaryotic ECM that is found throughout the host system. Fibronectin is an important part of the host immune system as it allows proper binding and coagulation of many different host cells. Bacterial species also use fibronectin during host infection and can use it to bind and adhere to host cells and tissue. It has been shown that even human endothelial cells release fibronectin in the extracellular matrix and use it for adherence and coagulation \(^4^3\). A recent study in *S. epidermidis* found that proper biofilm formation and fibronectin binding by the bacteria was dependent on *ebh* expression \(^4^1\).

Because adhesion to host cells is a very important part of *S. aureus* virulence and that adhesion is partly due to interactions between the bacteria and host fibronectin, it could be possible that the improper production of Ebh may be disrupting the bacterial ability to adhere to host tissues to form abscesses and disseminate.

The transcript of *ebh* is significantly increased with deletion of *arlR/S*, leading to an overabundance of GSSP. We believe that this overproduction of such a large protein could cause a breakdown in the bacterial ability to initially adhere during infection and to disseminate properly through the host’s body. The large amount of protein on the cell surface could disrupt other surface associated proteins involved in adherence, quorum sensing, and capsule production. This would explain the faster clearance of the mutant bacteria, as well as the decreased ability of the bacteria to disseminate from blood during the intravenous infection. Interestingly, we also saw changes in genes that code for the fibrinogen binding proteins SdrC, SdrD, and SdrE in the *arlR/S* mutant as has been
previously reported (Figure 2.4)\textsuperscript{44}. Improper production of these proteins could also disrupt the ability of the bacteria to coagulate and disseminate properly during infection.

This study sought out to determine the role of $arlR/S$ in pathogenesis of $S. aureus$. Interestingly, we found that when the system is disrupted there is decreased pathogenesis \textit{in vivo} but that this phenotype does not seem to be mediated by interaction with host innate immune cells as bacterial survival when cultured with human blood or PMNs was unchanged. Previous studies have shown this decreased virulence, we are the first to report, however, that deletion of the ArlR/S regulatory system causes a decrease in virulence in several \textit{in vivo} models of infection in the prevalent MRSA strain USA300\textsuperscript{17}. We propose that the ArlR/S system is regulating a series of proteins involved in adhesion of bacterial cells during initial infection. It has been shown that initial attachment of bacteria to host cells and tissues is a very important part of MRSA pathogenesis and if this system is disrupted it could easily decrease virulence. Not only that, but if the bacteria cannot properly adhere to host tissue and to other bacteria, it would cause the pathogen to be more easily cleared from the host after infection as proper bacterial dissemination would not occur. This phenotype is important as even the last line of antimicrobial defense against MRSA is failing. As more bacteria become resistant to every antibiotic on the market, it is important to find therapies that are non-drug related. Understanding the $arlR/S$ system and how it could be influencing the bacterial ability to adhere to host tissue could lead to a new way to disrupt $S. aureus$ growth and dissemination causing decreased pathogenesis and less severe disease.
Figure 2.1. A, Schematic showing the generation of the isogenic arlR/S deletion mutant in USA300. B, Polymerase Chain Reaction (PCR) to verify the deletion of the arlR/S gene from USA300△arlR/S. Analysis of USA300 and USA300△arlR/S was done using the primers for gyrB for a control and arlR (arlR forward 5’- AAG CCT GTT AAA GAT ATA TCT GCA TTA GAC – 3’ and arlR reverse 5’ – AAA CAT CTA CGA CAT TTG TTT CTA CTT CAC – 3’) C, In vitro growth of USA300, △arlR/S and △arlR/S complemented with a plasmid encoding arlR/S. Growth curve experiments were performed in triplicate.
Figure 2.2 The ArlR/S system influences virulence of USA300 during both superficial and invasive infection. A, arlR/S is important to the virulence of USA300 during murine soft-tissue infection. 15 mice per group were injected subcutaneously with 1 x 10^7 CFU of USA300 or USA300ΔarlR/S. Skin abscesses were measured and abscess volumes were calculated as described in materials and methods. B, Bacterial burden in skin abscesses at days 2, 4, and 7 for mice inoculated with USA300 and USA300ΔarlR/S. 3 mice per group per time point were used with * P< 0.05 and **P< 0.01 as determined by paired T test. C, 25 mice per group were infected intravenously (IV) with 10^8 CFU USA300 or USA300ΔarlR/S. ***P < 0.001 as determined by a log-rank test. D, Deletion of the ArlR/S system influences S. aureus dissemination during invasive murine infection. Mice were infected IV with 10^8 CFU bacteria and bacterial burden was determined in kidneys and hearts at nine hours post-infection. Results represent 5 mice per group and statistical significance was determined using a paired t-test with * P < 0.05.
Figure 2.3 ArlR/S does not influence bacterial survival or PMN plasma membrane permeability following PMN phagocytosis of USA300. A, Results are from 7 different donors at 0, 0.5, and 3 hours, 4 at 1 hour, and 2 different donors at 6 hours. No significant differences were found as determined by 1-way analysis of variance with Tukey’s post-test of ΔarlR/S versus USA300. B, Lysis of PMNs following phagocytosis of S. aureus. PMN lysis was determined after 5 hours of culture with USA300, ΔarlR/S, and ΔarlR/S+comp at an MOI of 10:1. Cytotoxicity was determined by the following equation: fluorescence value of PMNs + S. aureus – fluorescence value of PMNs in RPMI media buffered with 10mmol/L of HEPES ÷ fluorescence value of [maximum lactate dehydrogenase level – fluorescence value of PMNs in RPMI with Hepes x 100. Results are from 3 different donors. No significant differences were found by 1-way analysis of Tukey’s post-test. C, USA300, ΔarlR/S, and ΔarlR/S+comp were incubated with freshly collected heparinized human blood. Samples were collected at the specified time points and percent survival was calculated by comparing initial bacterial inoculum at t=0 to CFUs collected at the designated time point.
Figure 2.4 Deletion of arlR/S changes the transcription profile of USA300 during *in vitro* growth. Oligonucleotide microarray analysis of USA300 ΔarlR/S relative to USA300 at of growth in TSB as measured at mid-exponential and early stationary phases of growth. Experiments were performed in triplicate.
Figure 2.5 Confirmation of the oligonucleotide microarray using Quantitative real-time RT-PCR. A, Expression of arlR, ehh, nuc, and sdrD was measured at both mid-exponential and stationary phases of growth in USA300, ΔarlR/S, and ΔarlR/S+comp. B, Expression of hla in vitro in USA300, ΔarlR/S, and ΔarlR/S+comp. Experiments were performed three times at each growth phase and triplicate measurements were taken during each experiment. Relative quantification of S. aureus genes was determined by change in expression of target transcripts relative to that of gyrB and normalized to ΔarlR/S transcript abundance.
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CHAPTER 3

PROSPECTIVE STUDIES AND CONCLUSIONS

Defining the Mechanism by Which the ArlR/S TCS Affects *S. aureus* Pathogenesis

Determining the exact mechanism behind the ArlR/S two-component gene-regulatory system’s (TCS) influence on pathogenesis in CA-MRSA is very important because it may allow for the discovery of new non-antibiotic based therapies against infection\(^1\). Although we understand that deletion of the complete ArlR/S operon (figure 2.2) as well as blocking the action of only the sensor histidine kinase\(^2\) can cause decreased pathogenesis, the way this system adds to the bacteria’s virulence has not yet been completely determined. In a study done by Fournier, et al., it was shown that disrupting the ArlR/S TCS caused an increase in biofilm formation on a polystyrene surface\(^3\). Biofilms are extremely important to bacterial pathogenesis and are large aggregations of surface attached cells bound together by an extracellular matrix. Bacteria in a biofilm are well protected from host immune responses and innate immune cells \(^4\)\(^-\)\(^6\). Biofilm formation occurs in three stages: initial adherence and attachment to host cells and tissue, biofilm maturation and creation of channels to allow nutrients to the bottom layers of bacteria, and detachment and dissemination of biofilm bacteria to allow disease spread in the host\(^7\). For *S. aureus* to properly bind to host tissue to start the biofilm formation, the bacteria produce several different proteins that can bind to and interact with the host’s extracellular matrix components \(^8\)\(^,\)\(^9\). These proteins are either bound to the
bacterial membrane, or released into the extra-cellular space. Collectively, these proteins are known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and are involved in attaching to and colonizing the host tissue, as well as evading the innate immune response of the host. A few of these proteins are fibronectin, fibrinogen, vitronectin, collagen, thrombospondin, bone siaploprotein, elastin, and von Willebrand factor. The ability of the bacteria to correctly adhere to host tissue is vital to the growth and survival of the bacteria. This adherence to host tissue is an important process in initial bacterial colonization, formation of abscesses and biofilms, and dissemination and growth of bacterial cells. If adherence is not being regulated properly at any step, bacterial survival is at risk in the host. During our studies, an interesting finding was that the ArlR/S TCS transcriptionally regulated important adherence factors involved in biofilm and abscess formation of S. aureus. The most highly regulated of these factors was ebh (ECM-binding protein homologue), a gene that is cell surface associated and codes for an ECM-binding protein (figure 2.4). Ebh contains a 1.1 megadalton operon that codes for the giant staphylococcal surface protein, GSSP. This protein has homology with other known extracellular matrix (ECM) binding proteins of S. aureus and S. epidermidis and has been shown to be produced during infection in vivo by analysis of antibodies found in human serum post-infection. Deletion of arlR/S caused a dramatic upregulation of ebh. Previous studies have shown altered binding activity of arl mutants to fibronectin and it has been hypothesized that GSSP regulates this binding.
As a first step to determining the importance of GSSP regulation by arlR/S in *S. aureus*, we performed similar fibronectin binding experiments. Our results supported the findings of Christner et al.\(^9\). When human blood was incubated with the *S. aureus* wild type versus the mutant strain, less fibronectin was seen in the plasma that was infected with USA300ΔarlR/S than in the plasma infected with wild type bacteria. This difference was hypothesized to be due to the overexpression of GSSP in the mutant. We hypothesize the overexpression of GSSP, which binds fibronectin, on the surface of the bacterial cells allowed more fibronectin binding to the bacteria. As the supernatants were separated from the whole bacteria, the fibronectin bound to the bacterial surfaces would be removed with the bacterial fraction (figure 3.1A). To verify that another common blood factor was not playing a role, we also tested fibrinogen binding. Our results were similar to the findings of Clarke et al. and we were able to confirm that overexpression of *ebh* has no effect on fibrinogen binding in the plasma of human whole blood (figure 3.1B)\(^8\). Binding of pathogenic bacteria to fibronectin is common and bacteria produce many factors that target this ubiquitous eukaryotic ECM protein. Interestingly, disrupting the ArlR/S TCS led to increased *ebh* transcription and decreased pathogenesis. Although when *arlR/S* is deleted, the bacteria may have more sites at which to bind fibronectin and other ECM molecules through the overexpression of GSSP on the bacterial membrane, this overexpression could lead to an overabundance of biofilm formation that could be detrimental to bacterial survival *in vivo*. Increased interactions between fibronectin and GSSP on the bacterial cell surface could cause abnormalities in the biofilm and may lead to improper maturation of the biofilms. Without proper maturation, channels, which
allow nutrients to move from the surface of the biofilm to the bottom layers of bacteria, may not form and this could lead to bacterial cell death through nutrient depletion. Also, it is important for bacteria to be able to detach and disseminate from the biofilm. This is probably best demonstrated with studies analyzing Δagr strains that have a more robust biofilm and decreased virulence. With improper regulation, as can be seen with studies of the Agr regulatory system, when Agr is knocked out more robust biofilms are formed and more biomass can be measured.

This study demonstrates that regulation of ebh by arlR/S is important to S. aureus survival perhaps due to the role it plays in fibronectin binding in vivo. Binding to human matrix proteins is an important step to abscess and biofilm formation. However, the overall S. aureus fibronectin-binding ability is most likely multifactorial and may rely on a host of other fibronectin-binding proteins including FnbB and FnbA. We found that a deletion mutant of ebh was just as pathogenic as the wild type bacteria when put into an invasive model of infection (figure 3.2). Although it seems that deletion of this important fibronectin-binding protein would be detrimental to the bacterial adherence to host tissue through fibronectin, there are several other factors that can bind this important ECM-molecule. Therefore, even if the GSSP protein is non-existent in the system, another fibronectin binding protein could be properly regulated to make up for this deficit. Through the creation of an arlR/S and ebh double mutant in USA300 we could more exactly delineate the role of ebh to the pathogenesis of S. aureus.
Defining the Immune Response to the ArlR/S TCS

The innate immune response is very important to host defense from and clearance of pathogens. The first line of defense against *S. aureus* infection is the human polymorphonuclear leukocytes (PMN), which has several methods to engulf and destroy bacteria. Studies have shown that patients lacking PMNs are more susceptible to severe disease caused by bacteria and those with an overabundance tend to suffer disease from the release of too many immune molecules like cytokines and antimicrobial peptides. Interestingly, the ArlR/S TCS does not seem to affect the interaction of *S. aureus* with human PMNs or any factor in human whole blood during *in vitro* experimentation (figure 2.3). Therefore, a Procarta Immunoassay bead array was performed to determine the influence of the ArlR/S TCS on pro-inflammatory cytokines in human blood plasma. When human plasma was infected with USA300, USA300Δarlr/S, or Δarlr/S+comp there were no significant differences seen in expression of pro-inflammatory cytokines IFN-γ, IL-1α, IL-6, IL-8, MIP-1α, MIP-1β, or TNF-α (figure 3.3). Although the differences were not significant, there were trends seen when looking at expression of IFN-γ, IL-1α, IL-6, MIP-1α, MIP-1β, or TNF-α. Using more donors to characterize this response more effectively would be an important next step in determining if the ArlR/S TCS is affecting the innate immune response in the host.

Conclusions

*Staphylococcus aureus* infection is a serious concern in healthcare settings as well as in the community. Understanding what causes decreased pathogenesis is very
important as we move forward in our understanding of non-antibiotic based therapies to treat disease caused by this pathogen\(^1\). This study demonstrates that the ArlR/S two-component system of the community-associated methicillin-resistant \textit{S. aureus} strain USA300 is essential for virulence during soft tissue and invasive infections. The study also demonstrated that unlike some other two-component regulatory systems (such as \textit{saeR/S}) that show decreased virulence \textit{in vivo}, this decrease in pathogenesis of the \textit{arlR/S} mutant did not seem to be mediated by increased killing by PMNs or other factors in human whole blood \textit{in vitro}\(^{22,23}\).

This study demonstrated \textit{arlR/S} is also important to the regulation of the fibronectin-binding protein GSSP through the transcriptional regulation of the gene, \textit{ebh}. A previous study has shown that a disruption of only the sensor histidine kinase in the ArlR/S system causes increased biofilm formation\(^3\). An up-regulation in the fibronectin binding protein GSSP might be the cause for this increased biofilm formation. An important next step will be the determination of the signal that regulates the ArlR/S TCS. Many two-component systems have multifactorial signals that can activate or deactivate their action and if the signals can be determined for the ArlR/S TCS, we will gain a better understanding of the role of this TCS. Determining the target genes directly regulated by the ArlR/S TCS will also be an important part of future studies. By determining the signal that regulates this system, and the target genes that this system regulates, we may be able to determine the full function of this important TCS and its regulation of bacterial pathogenesis. This study is an important step in the understanding of the ArlR/S two-component gene regulatory system. Using our knowledge of this system and other
putative two-component systems currently being studied in *S. aureus* we may soon be able to uncover possible therapies to treat this dangerous pathogen using non-antibiotic based therapies.
Figure 3.1 Deletion of arlR/S causes a decrease in the concentration of fibronectin in human plasma. Human blood mixed with sodium citrate was collected from three separate donors. Human whole blood was infected with USA300, USA300ΔarlR/S, or ΔarlR/S+comp for 3 hours. Concentrations of fibronectin or fibrinogen in the plasma fraction of the whole blood were determined using a fibronectin or fibrinogen human ELISA kit. A, Although not significant, fibronectin levels were reduced in the arlR/S mutant compared to the wild type USA300. B, No differences were seen in expression of fibrinogen in human plasma.
Figure 3.2 Deletion of *ebh* has no effect on virulence during an invasive model of dissemination compared to a wild type strain, USA300. Mice were infected IV with 5x10^7 CFU bacteria and bacterial burden was determined in kidneys and hearts at nine hours post-infection. Results represent 4 mice per group and statistical significance was determined using a paired t-test with * P < 0.05.

Figure 3.3 The influence of *arlR/S* on pro-inflammatory cytokine production in human plasma. *S. aureus* bacteria were collected at early stationary growth phase. Human whole blood was exposed to USA300, USA300*ΔarlR/S*, or *ΔarlR/S*+comp for three hours. Levels of indicated pro-inflammatory cytokines in the plasma fraction were measured using a Procarta 7-plex Immunoassay bead array. Results represent the concentration of cytokines in human plasma measured in USA300, USA300*ΔarlR/S*, or *ΔarlR/S*+comp-treated plasma to the concentration measured in USA300*ΔarlR/S*-treated plasma, normalized for each donor.
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