7-1-2011

# Small molecule inhibition of Staphylococcus aureus virulence

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# SMALL MOLECULE INHIBITION OF *STAPHYLOCOCCUS AUREUS* VIRULENCE

**BY**

**ERIN SULLY**

B.S., Biomedical Sciences, University of California, Irvine, 2003

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy**
**Biomedical Sciences**

The University of New Mexico
Albuquerque, New Mexico

**July, 2011**

# ACKNOWLEDGMENTS

My deepest thanks to my advisor and committee chair Dr. Hattie Gresham and my committee member Dr. Pam Hall for their support and guidance during my doctoral education. I also thank my committee members, Dr. Carolyn Mold and Dr. Michelle Ozbun, for their advice and their contributions of time and knowledge to my project.

Thank you to the members of the laboratory who have been invaluable for their support and assistance: Susan Alexander, Martha Terrazas, Cynthia Spang, and Stephany Delgado, as well as former members of the lab who laid the foundation for the work presented in this manuscript: Anny Alsup, Dr. Youhanna Sawires, Dr. Michel Peterson, and Dr. Jacob Rothfork.

Finally, I thank my family and friends who have given me the encouragement and support to persevere and achieve my goals. I thank you all.

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ABSTRACT OF DISSERTATION

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## ABSTRACT

The increasing emergence of antibiotic resistant *Staphylococcus aureus* infections, particularly those caused by a single clone of methicillin resistant *S. aureus* (USA300 MRSA), coupled with the slowing of antibiotic discovery makes research into novel therapies a priority (Lowy, 2007). One strategy evolving is the development of drugs that target bacterial virulence factors as opposed to growth (Cegelski et al., 2008). Due to the lack of selective pressure, bacterial resistance to the drugs would be minimized while the infection, attenuated by the inhibition of virulence factor production, could be cleared by the innate immune factors of the host. Virulence factors identified to date as essential for invasive USA 300 MRSA infection are globally regulated in part by a quorum sensing operon, *agr* (George and Muir, 2007; Novick and Geisinger, 2008; Yarwood and Schlievert, 2003). Host factors like apolipoprotein B provide defense by antagonizing *agr* signaling which demonstrates that host defense against an invasive infection could be accomplished by blocking *agr* signaling (Peterson et al., 2008). Therefore, we hypothesized that screening small molecule inhibitors for inhibition of *agr* signaling could

contribute to drug discovery by providing optimal host defense against quorum sensing dependent *S. aureus* infections. Our work focuses on two small molecule inhibitors, CID# 2333 and CID# 3243271, identified in a screen of over 20,000 compounds for antagonism of *agr* signaling. These compounds demonstrate virulence factor inhibition *in vitro* and in an *in vivo* model of community associated -MRSA dermonecrotic infection.

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# INTRODUCTION

## The Medical Impact of *Staphylococcus aureus* infection

*Staphylococcus aureus*, a common colonizer of the human skin and mucosa, can cause illnesses ranging from minor skin infections and abscesses to life-threatening diseases such as necrotizing pneumonia, endocarditis, and septicemia (Lowy, 1998). The emergence of antibiotic resistant strains has become a healthcare and economic burden associated with a 1.2-2.0-fold increase in length of hospital stay and an increase in hospitalization costs ranging from $5000-$40,000 (Lodise and McKinnon, 2007; Naber, 2009). Given the burden of the disease and the absence of an effective vaccine, efforts have been refocused to discover and develop drugs that can be effective therapeutics against this infection.

## Antibiotic Resistant *S. aureus* Infection

Methicillin-resistant *S. aureus* (MRSA) was first reported in 1961, 2 years after methicillin was introduced to treat penicillin-resistant bacteria (Deleo et al., 2010). The first reports of community-associated MRSA (CA-MRSA) came in 1993 from Western Australia (Udo et al., 1993). These strains were isolated from otherwise healthy people who had no predisposing risk factors for MRSA infection such as wounds, indwelling medical devices, or extended hospital stays. These strains were more virulent than the hospital-associated MRSA (HA-MRSA) due to the presence of various virulence factors that cause aggressive invasive disease (Chambers, 2001). Recently CA-MRSA has been replacing HA-MRSA in healthcare facilities, especially in the United States (Klevens et al., 2006).

In addition to being isolated from people lacking the predisposing risk factors described above, CA-MRSA can also be defined based on genetic markers. Unlike HA-MRSA strains which usually contain type I, II, or III staphylococcal cassette chromosome *mec* (SCC*mec*), CA-MRSA isolates contain type IV SCC(Chambers, 2005)*mec* (Chambers, 2005). SCC*mec*IV contains the *mec*A gene which encodes for specific resistance to β-lactam antibiotics including methicillin (Baba et al., 2002; de Lencastre et al., 2007). One of the most discriminative and utilized typing methods for *S. aureus* is pulsed-field gel electrophoresis (PFGE) (Deurenberg and Stobberingh, 2008; Prevost et al., 1991). The chromosomal DNA is digested by the restriction enzyme, *Sma*1, and the DNA fragments of the bacteria are separated out by agarose gel electrophoresis. There are currently eleven CA-MRSA PFGE types found in United States numbered as USA100 to USA1100. Of these eleven types, USA300 and USA400 are responsible for the majority of CA-MRSA disease seen in the United States and USA300 is replacing traditional HA-MRSA in health care settings (Klevens et al., 2007). In 2005, the incidence of invasive MRSA infections in the US was 31.8 out of 100,000, which is greater than the incidence of all other invasive bacterial infections combined, and was the cause of more deaths than HIV/AIDS that same year (Klevens et al., 2007).

CA-MRSA infections were first seen in children and then later in professional football players and other athletes, prisoners, and men who have sex with men (Miller et al., 2005; Otto, 2007). CA-MRSA is transmitted most frequently by direct contact with the organism, most commonly mediated by skin-to-skin contact with a colonized or infected individual or by contacting contaminated surfaces or items (Deleo et al., 2010),

as opposed to HA-MRSA which is primarily caused by the patients' colonizing strain gaining access beyond the skin or mucosal barrier (Wertheim et al., 2005; Wertheim et al., 2008).

The pathogenesis of CA-MRSA disease is much more aggressive than HA-MRSA due to the numerous virulence factors associated with the strains that lyse cells and destroy tissue, resulting in the entry of the bacteria into the blood stream. Coupled with heightened virulence, CA-MRSA strains are highly resistant to standard antibiotic therapy and many strains are multi-drug resistant. The majority of CA-MRSA isolates are susceptible to non-β-lactam antibiotics (eg: clindamycin, tetracycline, doxycycline) but determining the most appropriate treatment for a particular strain of unknown antibiotic resistance profile is time consuming (Deurenberg and Stobberingh, 2008). Glycopeptides (eg: vancomycin, teicoplanin) have been considered the treatment of choice for invasive MRSA infections but concern has arisen due to the emergence and spread of strains with reduced susceptibility to vancomycin (Simor et al., 2010). Linezolid, an oxazolidinone, and daptomycin, a lipopeptide, are FDA-approved for invasive MRSA infections but have not been shown to be more effective than vancomycin and are associated with detrimental side effects (Deleo et al., 2010).

Due to the limitations of conventional antibiotic therapies against MRSA, it is essential to develop new treatment options. One such option may be the targeting of *S. aureus* virulence. Since virulence factors are involved in the pathogenesis of the infection but not bacterial viability, targeting virulence could ameliorate the infection while the lack of selective pressure would not promote resistance development to the treatment.

This approach has focused efforts on understanding the virulence factors used by CA-MRSA to cause disease and how they are regulated.

## Bacterial Quorum Sensing

Intercellular communication between bacteria allows for coordinated activity usually reserved for multicellular organisms (de Kievit and Iglewski, 2000). This capacity for group behavior permits migration ability, adaptation of new modes of growth, and coordinate production of virulence factors. The regulation of gene expression in response to changes in cell-population density is referred to as quorum sensing and is a cell-to-cell signaling mechanism. To date, there are four described quorum sensing signaling systems that are grouped according to the signaling molecules, receptors, and response regulators employed. A feature that is shared by all four types is the use of an autoinducer pheromone that is released into the environment by the bacteria to sense the density of the bacteria nearby. As the bacterial population increases, so does the presence of the autoinducer, which leads to the alteration of bacterial gene expression, resulting in changes including bacterial life cycle, metabolism, and phenotype (de Kievit and Iglewski, 2000). The first system to be described was found in *Vibrio fisheri*, a Gram-negative, luminescent, marine bacteria (Miller and Bassler, 2001). The luciferase operon of *V. fisheri* is regulated by two proteins, LuxI and LuxR. LuxI is responsible for the production of the *N*-acyl-homoserine lactone (AHL), termed autoinducer-1 (AI-1) and after synthesis, the AHL is free to diffuse across the membrane of the bacteria. As the density of bacteria increases, so does the extracellular concentration of AHL until the concentration is high enough that AHL freely diffuses back into the cell. The autoinducer

is bound by a transcription factor LuxR which controls gene expression in the presence of AHL (Devine et al., 1989). This system is used by numerous bacterial species including *Pseudomonas aeruginosa* and *Serratia marcescens* (Parker and Sperandio, 2009).

Another signaling system utilizes the bacterial transcription factor LuxS that modifies a compound, 4,5-dihydroxy-2,3-pentanedione (DPD), which cyclizes into furanones in the presence of water. The furanone serves as the autoinducer, termed AI-2, in this system and accumulates in the extracellular environment before binding to a transporter and entering the bacterial cell where it is phosphorylated (Reading and Sperandio, 2006). The phosphorylated AI-2 pheromone interacts with the transcriptional repressor to lift repression of the operon, *lsr* (Taga et al., 2001). This system has been observed in a wide range of Gram-positive and Gram-negative bacteria including *Escherichia coli*, *Salmonella typhimurium*, and *Streptococcus pyogenes*, suggesting that the AI-2 system represents a method of cross-species signaling (Xavier and Bassler, 2003).

A third mechanism of cell-to-cell signaling has thus far only been described in Gram-negative bacteria. Observed in bacterial species including *Shigella*, *Klebsiella*, and *Salmonella* as well as non-pathogenic *E. coli* (Walters et al., 2006), this system utilizes an autoinducer of unknown structure (AI-3) and a two-component system consisting of QseC, the sensor kinase, and QseB, the response regulator. The autoinducer is recognized by QseC which autophosphorylates and transfers the phosphate to QseB. Phosphorylated QseB then acts as a transcriptional regulator, activating downstream gene targets (Parker and Sperandio, 2009).

The fourth cell-to-cell signaling system, the AIP/*agr* system, is found exclusively in Gram-positive bacteria (Parker and Sperandio, 2009). It was first identified in *S. aureus* and is described in detail in the following section.

## Quorum Sensing in *S. aureus*

Quorum sensing in *S. aureus* is controlled in part by the accessory gene regulator (*agr*) which encodes a standard autoactivation circuit (Figure 1) (Novick and Geisinger, 2008). This type of quorum sensing is specific to Gram-positive organisms and has been identified in *Staphylococcus epidermidis* and *Enterococcus faecalis* after originally being characterized in *S. aureus* (Qin et al., 2001; Van Wamel et al., 1998; Yarwood and Schlievert, 2003). The components of this autoregulatory quorum sensing system include the signaling peptide AgrD, the secretory protein AgrB, responsible for the export and processing of AgrD to its active form, AgrC, a histidine kinase, and AgrA, a response regulator that detects AgrD levels and initiates the expression of the *agr*-dependent virulence factors (Koenig et al., 2004). The target for AgrA is the activation of two divergent promoters, P2 and P3. The activation of P2 drives the *agrA,B,C,D* operon while P3 activation promotes RNAIII production, a regulatory RNA. The significance of the *agr* locus in virulence has been illustrated in several experiments that demonstrate considerable attenuation of virulence in *S. aureus* strains that have a defect in the *agr* operon in multiple animal models of infection (Koenig et al., 2004). In this regard, RNAIII production is essential for acute invasive infection at the entry site of an infection (Rothfork et al., 2003b; Rothfork et al., 2004).

Upon activation of the P2 promoter, the AgrD peptide is secreted through AgrB, a transmembrane endopeptidase, where the peptide is cleaved, modified, and secreted through AgrB with a thiolactone ring (Ji et al., 1997). This secreted structure is termed an autoinducing peptide (AIP). There are four different types of AIP, which differ only in amino acid composition and length. All AIP types possess the thiolactone ring which is necessary for binding to and signaling through the AgrC sensor kinase receptor (Wright et al., 2004). The different AIP molecules, classified as AIP1, 2, 3, or 4 serves to further categorize the *S. aureus* strains as *agr* type 1, 2, 3, or 4 depending on which AIP the bacteria secrete (Jarraud et al., 2000; Ji et al., 1997). The AIPs selectively bind to their own cognate receptor and antagonize signaling when they bind to non-cognate receptors (Lyon et al., 2002; Wright et al., 2005). USA300 and USA400, the predominant CA-MRSA strains, are *agr* type 1 and *agr* type 3 respectively though all four types are represented in human disease (Klevens et al., 2007).

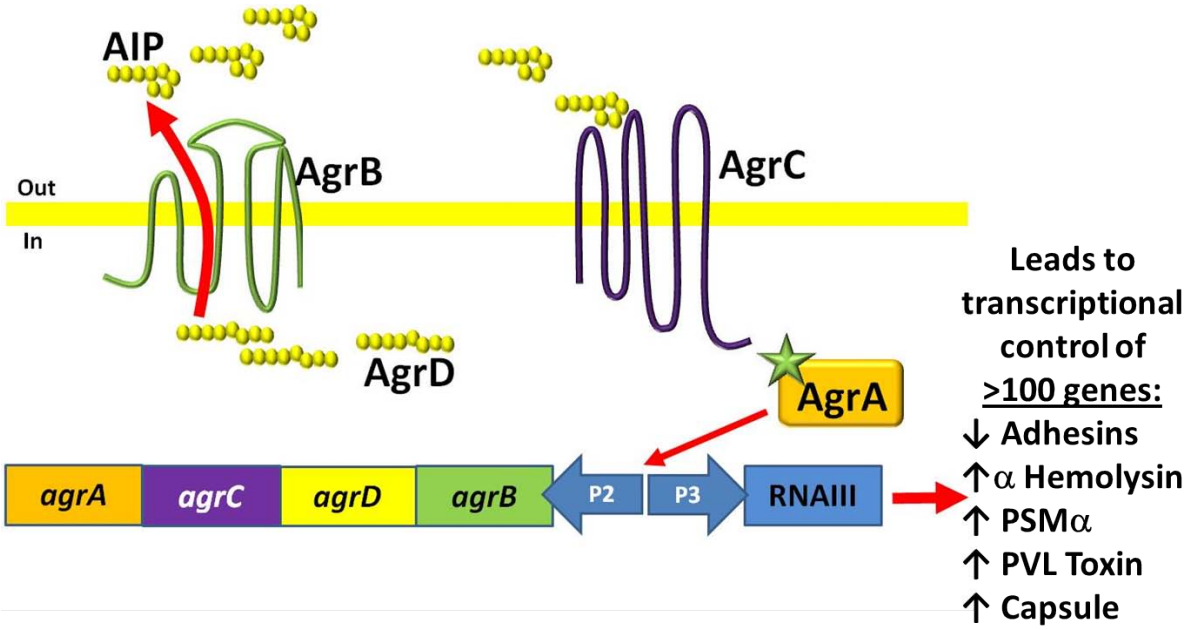


**Figure 1. The *agr* quorum sensing operon in *S. aureus***

Propeptide AgrD is exported through AgrB, modified to become the thiolactone AIP. The AIP signals through AgrC which phosphorylates the histidine kinase AgrA. AgrA acts on the divergent P2/P3 promoter as well as the promoter for the toxin psmα. The P2 promoter encodes the *agr* signaling and response regulator proteins. The P3 promoter induces transcription of the regulatory RNA, RNAIII, which acts on the promoters and translation initiation regions of numerous genes to up-regulate secreted virulence proteins important for invasion, and to down-regulate adhesins.

The AgrC sensor histidine kinase is the receptor for AIP and after AIP binding, AgrC dimerizes and autophosphorylates, transferring the phosphate to the response regulator AgrA (Novick, 2003). AgrA is required for the activation of the two *agr* promoters (P2 and P3) and completing the autoactivation circuit (Novick et al., 1993). The binding between AgrA and the P2 promoter is stronger than that between AgrA and the P3 promoter so autoactivation of the P2 operon precedes P3 activation (Cheung et al., 2004; Koenig et al., 2004). AgrA binding to the P2 promoter leads to autoinduction of the *agr* operon which is followed by the binding of AgrA to the adjacent and divergent promoter, P3, which activates the transcription of the RNAIII regulatory RNA (Novick and Geisinger, 2008).

*S. aureus agr* is unique in employing a regulatory RNA, RNAIII, as its effector as opposed to the response regulator AgrA (Novick and Geisinger, 2008). RNAIII is a 514-nt RNA molecule with a very complex secondary structure and regulates the expression of many genes encoding expoproteins and cell-wall-associated proteins (Benito et al., 2000). The structure of RNAIII is comprised of 14 hairpin stem loops, and the structure, but not its sequence, is well conserved across several staphylococcal species (Benito et al., 2000). The 3’ end of RNAIII acts to repress the transcription of the protein A gene (*SpA*) while the 5’ and 3’ non-overlapping subregions are independently active in stimulating the transcription of the alpha-hemolysin (*hla*) gene (Novick et al., 1993). RNAIII acts as antisense, directly upregulating translation of alpha-hemolysin by countering a secondary structure in the mRNA leader that blocks translation of the gene (Morfeldt et al., 1995). RNAIII also acts on Rot (repressor of toxins), another major transcription factor. Rot acts by upregulating translation of surface proteins while

downregulating translation of secreted proteins, counter to RNAIII (Said-Salim et al., 2003). RNAIII blocks translation of Rot by pairing with the translation-initiation region (Geisinger et al., 2006). This allows RNAIII to downregulate colonizing factors such as cell-surface protein genes and activate the transcription of virulence genes which code for secreted toxins and proteases (George and Muir, 2007).

Production of RNAIII leads to a phenotypic switch in the bacteria from one of colonization to an invasive phenotype (Figure 2). Colonization factors are molecules that *S. aureus* uses to adhere to host epithelial cells and the formation of biofilm. These factors include *S. aureus* surface protein A (SpA), a wall-anchored protein with four or five domains that bind to the Fc region of IgG (Uhlen et al., 1984), clumping factor A (clfA), the dominant fibrinogen-binding protein which is found on the surface of the bacteria during stationary phase (O'Brien et al., 2002a),clumping factor B (clfB), which also binds to fibrinogen but also cytokeratin 10, a major component of squamous cells (O'Brien et al., 2002b; Wertheim et al., 2008), and fibronection binding protein (FnBP), an important molecule in biofilm development (Houston et al., 2011). While these colonization factors are important for adherence to tissues, invasive factors serve to lyse cells causing damage to tissues and dissemination of the bacteria throughout the host.

While existing in the invasive phenotype, the bacteria produce molecules that include proteases, hemolysins, and toxins which together serve to lyse cells and destroy tissues which allow the bacteria to penetrate the tissues and enter the bloodstream. Enterotoxins and toxic shock syndrome toxin (TSST) are produced at this stage which contribute directly to the pathology seen in *S. aureus* infections (Yarwood and Schlievert,

2003). Alpha-hemolysin, a cytolytic toxin responsible for the destruction of a wide range of host cells with the exception of neutrophils (Valeva et al., 1997) is created as a result of the upregulation of virulence factors. Alpha-hemolysin is secreted as monomers which then associate into heptamers to form pores in the cytoplasmic membrane of target cells causing lysis (Bhakdi and Tranum-Jensen, 1991). Another virulence factor produced in response to quorum sensing is phenol soluble modulin α (PSMα), a cytolytic peptide that is expressed abundantly in CA-MRSA strains (Queck et al., 2008; Wang et al., 2007). The promoter of the *psmα* gene is directly activated by AgrA as opposed to other virulence factors which are activated by RNAIII (Queck et al., 2008; Wang et al., 2007). It has been postulated that the enhanced virulence of CA-MRSA strain USA300 may be due to amplified production of PSMα and alpha hemolysin (Li et al., 2009).

A bicomponent leukotoxin, Panton-Valentine leukocidin (PVL), is an *agr*-dependent leukocyte toxin unique to the earliest CA-MRSA strains. PVL may be responsible for the emergence, transmission, or enhanced virulence of these specific MRSA strains but presently, the exact target or mechanism is not clear (Gillet et al., 2002; Graves et al., 2010). CA-MRSA strains, notably USA300, produce an abundant amount of alpha-hemolysin, PSM α and PVL (Deleo et al., 2010) compared to HA-MRSA strains. While the presence of PVL has been a hallmark in USA300 strains causing human necrotizing pneumonia, mouse models of this disease using PVL deletion mutant strains demonstrated no protection compared to infection with a wild type strain (Bubeck Wardenburg et al., 2008; Gillet et al., 2002). Murine neutrophils are less sensitive to the lytic effects of PVL than human neutrophils and this may provide an

explanation for why PVL deletion mutants are not attenuated in mouse models of infection. In contrast, rabbit neutrophils are similarly susceptible to PVL-induced cytolysis as human and now experimental infections in rabbits are being used to determine if PVL, like alpha-hemolysin and PSM α, contribute to invasive USA300 infections (Diep et al., 2010). LukGH is a newly identified two-component leukotoxin that is secreted by USA300 (Ventura et al., 2010). LukGH synergizes with PVL to enhance human neutrophil lysis as well as contributing to the lysis of neutrophils after phagocytosis of the bacteria. The arginine catabolic mobile element (ACME) is another operon of interest that USA300 most likely obtained from *S. epidermidis*. ACME contains a gene for an arginine deiminase which allows the bacteria to better survive acidic conditions and have increased fitness for transmission (Diep et al., 2006; Goering et al., 2007). The role of ACME in pathogenesis is uncertain as comparison of an ACME deletion mutant to a wildtype USA300 strains is attenuated in some animal models of infection (Diep et al., 2008) but not in others (Montgomery et al., 2009). Therefore, the virulence factors most directly related to the pathogenesis of both experimental and human infections are alpha hemolysin and PSM α. The *agr*-regulated virulence factors described above are used by CA-MRSA strains for invasion into host tissues and for evasion of critical host defense elements. Because quorum sensing is essential for the phenotypic switch of S. *aureus* from colonizing to invasive, inhibition of quorum sensing, and thus the production of the virulence factors responsible for destructive pathogenesis, is an intriguing option to pursue for possible treatment of *S. aureus* infections.

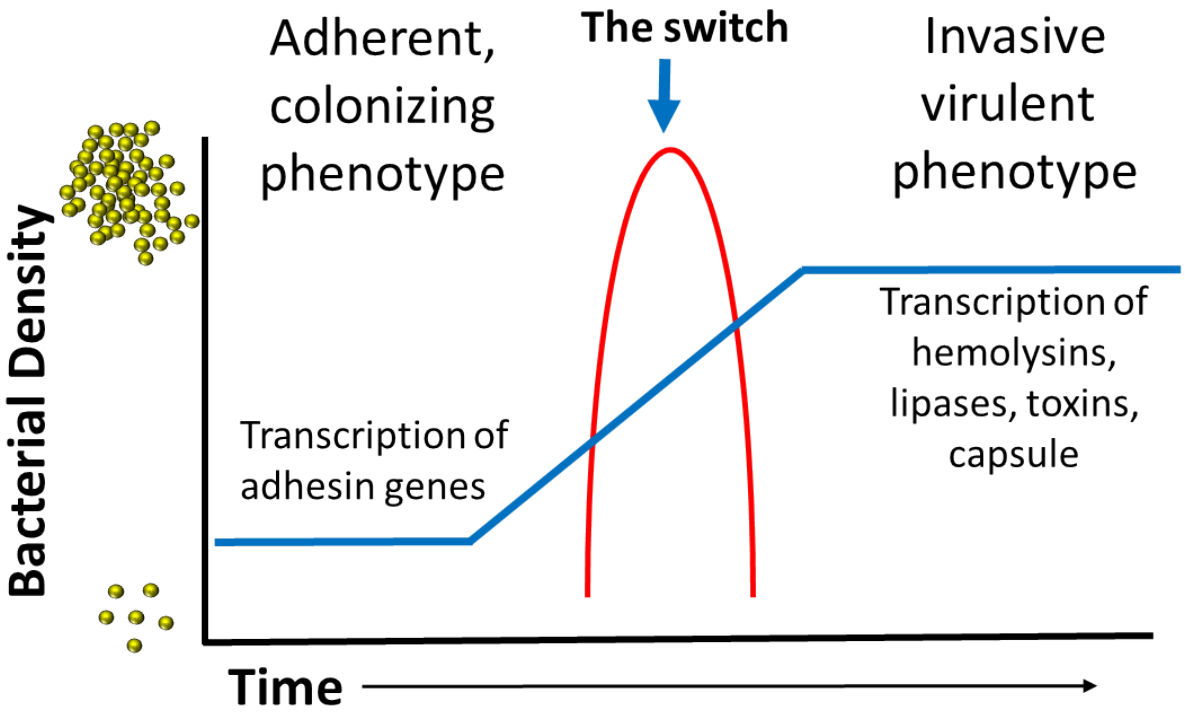


**Figure 2. The phenotypic switch of *S. aureus* is density dependent**

As the density of bacteria in an area increases, the higher concentration of extracellular AIPs increases the chances of signaling through the AgrC receptor. Upon signaling through AgrC, RNAIII expression will be activated which leads to a phenotypic switch from a colonizing phenotype where adhesion genes were expressed, to an invasive phenotype where virulence factors are being produced.

## Host Immunity to *S. aureus* Infection

The primary response of the host immune system to the presence of *S. aureus* is innate system dominated, recruiting neutrophils and other phagocytes to the site of the infection. Despite of the presence of *S. aureus* antibodies in most adults, the humoral adaptive immune system plays a limited role in the control and clearance of the bacteria and recovery from an *S. aureus* infection does not appear to confer protection against subsequent infections (Holtfreter et al., 2010; Schaffer and Lee, 2008).

Components of *S. aureus*, including lipoteichoic acid (LTA) and capsular polysaccharide, elicit the production of IL-8, a neutrophil recruiting cytokine, by peripheral blood monocytes and epithelial and endothelial cells (Soell et al., 1995; Standiford et al., 1994). Virulence factors such as toxic shock syndrome toxin-1 (TSST1) and enterotoxins A and B, also stimulate IL-8 production by human monocytes (Krakauer, 1998).

Host Toll-like Receptor 2 (TLR2) recognizes multiple pathogen-associated molecular patterns (PAMPs) on the bacteria including LTA, lipoproteins, and diacylglyceride (Kumar et al., 2011; Takeuchi et al., 2000)(Kumar et al., 2011; Kumar et al., 2011; Takeuchi et al., 2000; Takeuchi et al., 2000)(Kumar et al., 2011; Kumar et al., 2011; Takeuchi et al., 2000; Takeuchi et al., 2000). The host inflammasome, a set of cytosolic proteins responsible for the activation of caspase-1 and the secretion of IL-1 cytokines, also recognizes PAMPs of *S. aureus* (Craven et al., 2009). Caspase-1 activation by the inflammasome requires either all three hemolysins (α-, β-, and γ-hemolysin) or α- and β-hemolysin in combination with bacterial lipoprotein released by

*S. aureus* (Munoz-Planillo et al., 2009). The host system also has various ways to mark *S. aureus* for phagocytic uptake. Deposition of complement fragments and accumulation of fibrinogen and IgM antibodies on the surface of the bacteria efficiently opsonize *S. aureus*, leading to the uptake of the bacteria by phagocytes and the influx of inflammatory cells to the infection site (Laarman et al., 2010).

After phagocytosis of the bacteria, the neutrophils destroy the bacteria using oxygen-dependent and oxygen-independent mechanisms (DeLeo et al., 2009). Phagocytosis activates the NADPH oxidase which generates high levels of superoxide which quickly dismutates into hydrogen peroxide and then other secondary reactive oxygen intermediates including hypochlorous acid, singlet oxygen, and hydroxyl radicals (Klebanoff, 1975; Rosen and Klebanoff, 1977). The importance of the NADPH oxidase system and the production of reactive oxygen species is illustrated by the hereditary disorder chronic granulomatous disease (CGD). CGD patients have a defect in the NADPH oxidase which results in recurrent bacterial infections. This concept was also illustrated specifically for *S. aureus* where it was shown that mice deficient in the NADPH oxidase had increased susceptibility to invasive *S. aureus* infections indicating the vital role of NADPH in host defense (Rothfork et al., 2004). The oxygen-independent killing mechanism of the neutrophils is the fusion of the bacteria-containing phagosome with azurophilic granules which contain numerous antimicrobial peptides and proteins including α-defensins, cathepsins and azurocidin (Ganz et al., 1985; HIRSCH and COHN, 1960). Antibacterial proteins are also important in defense against *S. aureus* on the skin. The skin acts as a physical barrier against infection but keratinocytes possess

multiple antimicrobial peptides including β-defensins and lipocalin which help to protect against *S. aureus* on the skin and sterilization of wounded areas (Clarke et al., 2007; Komatsuzawa et al., 2006). Neutrophils also contribute to host defense by the creation of neutrophil extracellular traps (NETs) that are composed of chromatin threads with bound histones and azurophilic granule proteins (Brinkmann et al., 2004). NETs are formed following induction by chemokines, TLRs, and phagocytic receptors and results in the mixing of the NET components as the nuclear envelope and granule membranes disintegrate (Fuchs et al., 2007). NETs are released as the cell membrane breaks in a cell death process that is distinct from both necrosis and apoptosis and is dependent on the generation of reactive oxygen species by the NADPH oxidase to kill the bacteria (Fuchs et al., 2007). NET entrapment of the bacteria contributes to extracellular killing by antimicrobial peptides bound to the chromatin components.

The primary effectors of host defense against *S. aureus* include opsonization, phagocytosis, and killing by antimicrobial peptides, all components of the innate system. The adaptive system appears to primarily provide defense by antibody production utilized for opsonization leading to phagocytic uptake and by antigen-specific CD4 Th1 lymphocytes (Breuer et al., 2005). During an infection, anti-staphylococcal antibodies increase in the host serum, but this appears to provide limited control of the actual infection (Holtfreter et al., 2010). The antibodies seen in patient sera are produced in response to antigens involved in microbial adherence, the initiating event of the infectious process, and are specific to the strain colonizing the patient (Vernachio et al., 2003; Vernachio et al., 2006). Because of this specificity, the antibodies have little

activity against the more virulent strains of *S. aureus* that cause invasive disease and morbidity (Holtfreter et al., 2010). The Th17 subclass of lymphocytes enhance innate phagocytic killing of *S. aureus* due to the production of IFN-γ and IL-17 by the cells which are important for the activation of phagocytes and neutrophil migration (Lin et al., 2009). Given that Th17 cells are important in various other bacterial infections (Reiner, 2007), they may play a larger and more regulatory role during *S. aureus* infections.

In addition to direct cidal activity or promotion of bacterial clearance, host defense can also be provided by targeting virulence gene regulation. Importantly, both host hemoglobin and apolipoprotein B have been recently described to protect against *S. aureus* infection. The protective effect of hemoglobin (hemin) has been described as the possible inhibition of AIP secretion by AgrB (Attia et al., 2010; Schlievert et al., 2007). Apoliprotein B binds the secreted AIPs and prevents them from binding to the receptor, AgrC, inhibiting quorum sensing and subsequent production of downstream virulence factors (Peterson et al., 2008). These two host factors in addition to the inactivation of AIPs by reactive oxygen species (Rothfork et al., 2004) are three ways the host inhibits quorum sensing-dependent virulence factor production by *S. aureus* (Figure 3).

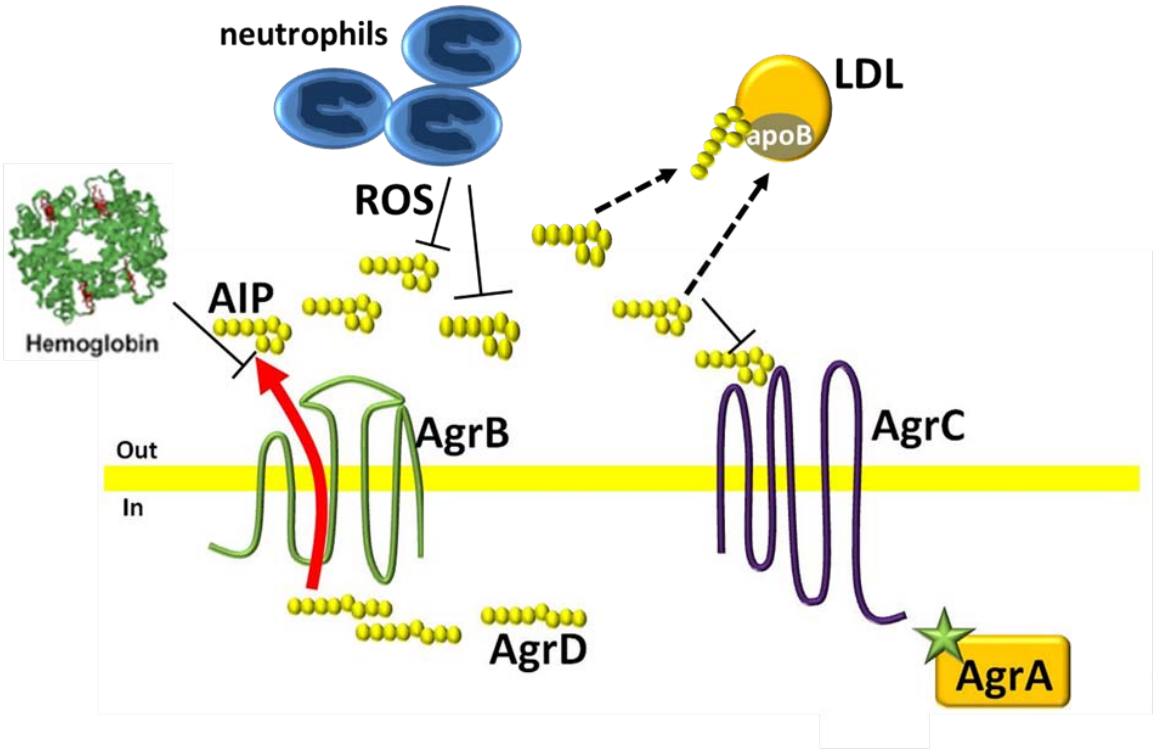


**Figure 3. Host control of quorum sensing**

Three host factors are known to prevent quorum sensing including hemoglobin (hemin), which inhibits AIP secretion through AgrB via membrane disruption, reactive oxygen species from neutrophils which inactivate AIPs, and apolipoprotein B which can sequester the AIP, inhibiting signaling through the AgrC receptor.

## Bacterial Escape of the Host Immune System

Although the host immune system possesses multiple and varied methods to quell the infection and kill the bacteria, *S. aureus* has equivalent amounts of escape mechanisms to evade the host immune system, avoid killing by phagocytes, and help ensure a successful infection.

*S. aureus* possess numerous factors that compromise the effectiveness of the first line of defense of the host, macrophages and neutrophils. The first evasion tactic is to avoid interaction with the phagocytes completely. At the onset of bacterial growth, host cells produce chemoattractants to recruit neutrophils to the site of infection. Neutrophils respond to the complement proteins C3a and C5a as well as bacteria-secreted formylated peptide, FMLP. Approximately 60% of *S. aureus* strains secrete the chemotaxis inhibitory protein of staphylococci (CHIPS) to inhibit the neutrophil response by binding to and blocking the formyl peptide receptor and the C5a receptor that are found on the neutrophil surface (de Haas et al., 2004; Foster, 2005b). The bacteria also express the extracellular adherence protein (Eap) which binds to ICAM-1, a ligand on the surface of endothelial cells. This binding, in turn, blocks the binding of ICAM-1 with LFA-1 found on the surface of neutrophils, preventing adhesion and extravasation of the neutrophils through the endothelial barrier (Chavakis et al., 2002; Sun, 2009).

*S. aureus* also has tactics to avoid phagocytosis through the use of varied surface-associated anti-opsonic proteins. The host complement system can be activated in three ways but all pathways converge at the central step of the activation of complement molecule C3. Activation of C3 results in the deposition of C3b molecules on the surface

of the bacteria which is essential for activation of complement. *Staphylococcus* complement inhibitor (SCIN), is secreted by *S. aureus*, and is capable of blocking all complement pathways (Rooijakkers et al., 2005a; Rooijakkers et al., 2005b). SCIN inhibits the production of C3b, the amplification loop which follows, and ultimately the formation of the neutrophil chemoattractant C5a (Jongerius et al., 2010; Rooijakkers et al., 2005a). SCIN also dimerizes the convertase enzymes, which leads to impaired binding of the convertase to the complement receptor resulting in the inhibition of phagocytosis (Jongerius et al., 2010). The extracellular fibrinogen-binding protein (EfB) and staphylococcal binding protein (Sbi) also inhibits complement activation by binding to C3 preventing deposition and accumulation of C3b on the bacteria (Burman et al., 2008; Lee et al., 2004). Clumping factor A (clfA), the dominant fibrinogen-binding protein found on the surface of the *S. aureus* during stationary phase, protects the bacteria against opsonization and phagocytosis by binding fibrinogen (O'Brien et al., 2002a). In addition, clfA increases the cleavage of C3b into the inactive form, iC3b, by complement factor 1 (Hair et al., 2010). Similar to clfA, clumping factor B (clfB) and fibronectin binding protein (FnBP) bind to fibrinogen providing the same protection as clfA but in the exponential phase of bacterial growth (Ni Eidhin et al., 1998; Wertheim et al., 2008).The capsule of the bacteria also serves to protect against opsonization and subsequent phagocytosis (Thakker et al., 1998). The excretion of the protease staphylokinase activates human plasminogen into plasmin which has the ability to cleave both surface-bound IgG and C3b (Bokarewa et al., 2006; Rooijakkers et al., 2005c). *S. aureus* surface protein A (SpA) is a wall-anchored protein with four or five domains and each of these domains bind to the Fc regions of IgG (Uhlen et al., 1984). This binding

results in the coating of the outside of the bacteria with IgG molecules in the incorrect orientation to be recognized by the Fc receptor of the neutrophils which protects against phagocytosis.

If the phagocyte is able to engulf the bacteria, the arsenal of *S. aureus* comprises more survival tactics to avoid killing by the neutrophil. All *S. aureus* species are catalase positive, another device the bacteria uses to thwart killing by neutrophils. Catalase inactivates hydrogen peroxide and free radicals that comprise the respiratory burst of the neutrophil following phagocytosis allowing the bacteria to persist inside the phagosome (Palazzolo-Ballance et al., 2008). *S. aureus* survival in the neutrophil is also facilitated by the ability of the bacteria to prevent fusion of the phagosome with azurophilic granules. *S. aureus* is able to escape the phagosome and multiple rapidly in the intracellular environment of the neutrophil (Gresham et al., 2000). The carotenoid pigment that gives *S. aureus* its hallmark gold color also plays an important role in protecting the bacteria from neutrophil killing by scavenging oxygen free radicals (Clauditz et al., 2006; Liu et al., 2005). *S. aureus* also secretes staphylokinase, which binds host defensins and stimulates the degradation of IgG and complement molecule C3, and aureolysin, a metalloprotease that cleaves and inactivates the defensin peptide cathelicidin LL37 (Jongerius et al., 2009; Sieprawska-Lupa et al., 2004)(Foster, 2005a; Jongerius et al., 2009; Jongerius et al., 2009; Jongerius et al., 2009; Jongerius et al., 2009; Sieprawska-Lupa et al., 2004; Sieprawska-Lupa et al., 2004).

*S. aureus* secretes many lytic factors which also help protect the bacteria from the host immune system by damaging or killing leukocytes. Alpha-hemolysin, a cytolytic

toxin, forms pores in the cytoplasmic membrane of target cells causing lysis, and Panton-Valentine leukocidin (PVL), a bicomponent leukotoxin, another pore-forming toxin, are two factors of note (Bhakdi and Tranum-Jensen, 1991; Gillet et al., 2002; Graves et al., 2010). Another important secreted lytic factor is α-type phenol soluble modulin (PSMα) which recruits and lyses neutrophils (Li et al., 2009). The multiple evasion mechanisms evolved by this pathogen illustrate why clearance by the host immune system alone is difficult and sometimes not sufficient.

## Current Therapeutics Against *S. aureus*

Antibiotic resistance is an increasing complication in the ability to combat *S. aureus* infections. As early as the 1940s, cases of penicillin resistance were identified in hospitals and by the 1950s, these strains were causing infections outside of the hospital setting (Chambers and Deleo, 2009). Methicillin resistant strains emerged in the hospital by 1961 and by the late 1990s appeared in the community, afflicting individuals who had no predisposing risk factors for infection and causing severe infections including necrotizing pneumonia and necrotizing fasciitis (Miller et al., 2005). With the exception of the narrow-spectrum drugs daptomycin and linezolid, there have been no new classes of clinically relevant antibiotics discovered in 40 years which illustrates a looming threat of a postantibiotic era (Clatworthy et al., 2007). Traditional antibiotics work by either killing the bacteria or inhibiting growth. These bacteriocidal or bacteriostatic agents inhibit bacterial functions such as cell wall synthesis, DNA replication, RNA transcription, and protein synthesis that are essential for *in vitro*, logarithmic growth (Clatworthy et al., 2007). Unfortunately, these strategies place a selective pressure on the

bacteria, leading to mutations and the development of antibiotic-resistant strains that are being seen.

A staphylococcal vaccine would be an ideal way to target the infection and protect the populations most at risk. However, attempts at vaccine creation have, thus far, been unsuccessful. Vaccine production against *S. aureus* has proven to be a challenge for many reasons. First, *S. aureus* has many membrane-bound surface molecules and secreted proteins with redundant functions which makes the targeting of one molecule by a vaccine easily overcome by the bacteria compensating with expression of a different protein with the same function (Shinefield and Black, 2006). Additionally, *S. aureus* has multiple strategies to escape from the host innate immune system (Foster, 2005a; Foster, 2009; Horswill and Nauseef, 2008), has the ability to persist insulated in biofilms (Yarwood et al., 2004), and can survive as small colony variants (Schaffer and Lee, 2008; Vaudaux et al., 2006).

Active immunization was attempted by Nabi Biopharmaceuticals which created StaphVAX, a bivalent vaccine that combined vaccination against capsular polysaccharide serotypes (CP) 5 and 8. This vaccine failed in phase III testing most likely due to capsule-negative, highly virulent clinical isolates and the circumstance that actively replicating staphylococci are do not express capsule (O'Riordan and Lee, 2004; Schaffer and Lee, 2008). Passive immunization has also been attempted by administering neutralizing antibodies against specific *S. aureus*-virulence factors. Clumping factor A was one of these products (Veronate, Inhibitex) where human immunoglobulin was collected from donors with high antibody titers against the *S. aureus* adhesin. The product failed in

phase III trials possibly due to low affinity or avidity to the target antigen (DeJonge et al., 2007). Another related product is a monoclonal antibody to clumping factor A (Aurexis/tefibazumab, Inhibitex) which has completed phase II trials. Other *S. aureus*-virulence factors that are currently in the pipeline as possible future targets for therapeutics include CP5 and CP8 (AltaStaph, Nabi), ABC transporter (Aurograb, NeuTec), and lipoteichoic acid (Pagibaximab, Biosynexus) (Schaffer and Lee, 2008).

Vaccines created from exotoxins have also been tried with some success. Alpha-hemolysin, a pore-forming toxin secreted by *S. aureus* is a major proponent of host tissue damage seen in infections. A mutant-alpha hemolysin (H35L) that was non-toxic and non-hemolytic was created by site-directed mutagenesis and antibodies against this mutant toxin had protective effects in mice that were passively immunized (Menzies and Kernodle, 1996). The H35L mutant was also protective in a lung infection model where the mice that were immunized with the protein prior to intranasal challenge with *S. aureus* exhibited reduced lethality (Bubeck Wardenburg and Schneewind, 2008). Immunization with mouse monoclonal antibodies to the H35L mutant were also shown to be protective (Ragle and Bubeck Wardenburg, 2009). These findings suggest that the inhibition of a single virulence factor that predominates in tissue injury may be sufficient to protect the host from a potentially lethal *S. aureus* infection.

This approach to antimicrobial therapies of targeting of bacterial virulence factors as opposed to growth has been gaining enthusiasm and promise. Removing the virulence properties of pathogens without altering growth would provide a reduced selective pressure environment for drug resistant mutant strains (Cegelski et al., 2008) as well as a

less virulent pathogen in which the host may be able to clear by way of its own immune effectors.

## High Throughput Screening for Identification of Small Molecule Inhibitors

One strategy emerging for modern drug discovery is high-throughput screening. It comprises the screening of large chemical libraries for activity against biological targets, various intracellular and extracellular molecules, and does so by the use of automation, miniaturized assays, and large-scale data analysis (Mayr and Bojanic, 2009)(Kool et al., 2010). Cell-based screening specifically has become an integral aspect of drug discovery, having the advantage of testing the effect of compounds against molecular targets within living cells (Black et al., 2011).

## Targeting Bacterial Virulence

Bacteria utilize a broad array of virulence factors to cause disease in the host. Adhesins facilitate colonization after binding to host cells, toxins can kill or alter signal transduction in host cells, and secretion systems deliver bacterial effectors (Rasko and Sperandio, 2010). All of these pathways represent areas that could be targeted for therapeutic intervention and inhibition. Targeting virulence in bacteria provides many advantages over traditional antibiotics. The most obvious being that when the targeted virulence factor is not essential for *in vivo* survival, selective pressure should not be an issue since growth is not being inhibited. If mutations develop that give rise to resistance, the fitness of the bacteria will not be affected. Also, alterations of the host microbiota that are associated with current antibiotic therapies would be avoided due to the specificity of the virulence-targeting drug. The preservation of the host flora would reduce the risk of

secondary infections and colonization with the drug-resistant organisms (Barczak and Hung, 2009; Clatworthy et al., 2007).

To effectively colonize the host and promote disease, bacterial adhesins are essential in the bacterial attachment to host cells. In both Gram-negative and Gram-positive bacteria, pili or fimbrae mediate this attachment. Adhesion can be blocked in an *E. coli* infection by saturating the exposed carbohydrate-binding sites on the pili with mannose derivatives and thus resulting in lower bacterial burden and invasion in a mouse model of cystitis (Wellens et al., 2008). In Gram-positive organisms, sortase, a necessary component for adhesion to host cells accomplished by catalyzing the assembly of surface proteins, also serves as a promising drug target (Maresso and Schneewind, 2008). Sortase A of *S. aureus* has been targeted in multiple studies where different small molecule inhibitors of the surface enzyme have been described (Chenna et al., 2008; Oh et al., 2004). Biofilm production, seen in bacteria such as *S. aureus* and *P. aeruginosa,* could also be inhibited by the targeting of bacterial adhesin molecules which would be beneficial given the notorious drug tolerance of bacteria in biofilms (Bayles, 2007).

Another popular target of these anti-virulence therapies is the production of the toxins themselves that have a direct role in causing the disease. Inhibiting toxin transcription, expression, and function have shown great promise as effective therapeutics (Barczak and Hung, 2009). Cholera toxin, in addition to toxin coregulated pilus, was inhibited by a small molecule in a mouse model of cholera infection. This compound, virstatin, directly inhibited ToxT dimerization, a transcription factor essential for virulence factor expression, which resulted in blocked intestinal colonization of mice

with *V. cholerae* (Hung et al., 2005; Shakhnovich et al., 2007). *Bacillus anthracis* secrets a zinc-dependent metalloprotease toxin known as anthrax lethal factor, one of the two toxins critical for anthrax pathogenesis, that contributes directly to cell death. Small molecule inhibitors were identified, specifically a hydroxamate derivative, to interact at the lethal factor active site and inhibit virulence of this lethal toxin (Panchal et al., 2004; Schepetkin et al., 2006; Shoop et al., 2005). Recently, five non-hydroxamic acid small molecules were identified as new lethal factor inhibitor scaffolds by a novel in silico high-throughput virtual screening assay (Chiu et al., 2009), furthering the interest and enthusiasm in this new direction of anti-virulence therapeutics.

The delivery system of these toxins is another area that has been investigated for its potential as a target. The type III secretion system (T3SS), used by many Gram-negative pathogens including *E. coli*, *Pseudomonas aeruginosa*, and species of *Yersinia* to inject the bacterial effectors into the cytosol of the host cell, is one such system that has been targeted (Cegelski et al., 2008). In *Yersinia pseudotuberculosis*, small molecule inhibitors of the T3SS prevented the effector proteins of the pathogen, *Yersinia* outer proteins, from being translocated into the host cells (Harmon et al., 2010; Kauppi et al., 2003; Nordfelth et al., 2005). This secretion system was again the target of inhibition in a species of *Chlamydia* because a small molecule inhibitor of T3SS inhibited intracellular replication and infectivity of *C. trachomatis* (Muschiol et al., 2006).

The quorum sensing systems of bacteria are also very attractive targets for therapeutic intervention owed, in part, to the fact that these systems are not usually found in eukaryotic hosts. Several strategies have been developed to target the signaling system

which is responsible for cell-to-cell signaling and synchronized downstream formation of virulence factors. An example of this has been demonstrated for *Pseudomonas aeruginosa*. *P. aeruginosa* utilizes a MvfR-dependent quorum-sensing regulatory pathway which controls the expression of key virulence genes. The introduction of anthranilic acid analogues to the pathogen inhibits 4-hydroxy-2-alkylquinolines (HAQ) biosynthesis and disrups MvfR-dependent gene expression and has shown great promise in a mouse model measured by improved mouse survival and decreased bacterial spread (Lesic et al., 2007). In enterohemorrhagic *E. coli*, *S. typhimurium*, and *F. tularensis*, QseC, is a membrane kinase that is essential for quorum sensing upon recognizing the bacterial autoinducer 3 signal or host adrenaline or noradrenaline hormones. Upon sensing, QseC increases its autophosphorylation and activates a virulence gene regulation cascade. This pathway was inhibited by a small molecule, identified by a high-throughput screening assay, that binds to QseC , preventing it from binding to its cognate signals and activating the expression of virulence genes (Rasko et al., 2008). Due to homology of QseC in many Gram-negative bacteria, this inhibitor demonstrated effectiveness in a wide range of pathogens lending credence to the possibility of a broad spectrum therapeutic for Gram-negative bacteria.

Two-component regulatory systems are necessary for quorum sensing in Gram-positive bacteria. In *S. aureus*, the global regulator, *agr*, responds to bacterial density and controls virulence gene expression when activated by autoinducing peptides (AIP) (Novick and Geisinger, 2008). The AIP binds to and activates a membrane-bound histidine kinase, AgrC, which dimerizes and results in the activation of target genes and the synthesis of additional AIP (George and Muir, 2007). The four AIPs identified in *S.*

*aureus* each bind selectively to their cognate AgrC receptor and quorum sensing is inhibited when AIPs bind to their non-cognate receptor (Wright et al., 2005). This suggests that *S. aureus* infections may be ameliorated by the synthesis and introduction of competing AIPs which would result in inhibited bacterial signaling (Wright et al., 2005). Another antagonist of *S. aureus* virulence is cyclic dipeptides produced from *Lactobacillus reuteri* (Li et al., 2011).The dipeptides inhibit *agr* dependent-quorum sensing and toxin production *in vitro* as well as inhibiting signaling through other sensor-regulators used by *S. aureus* to regulate gene expression, but whether this would provide protection *in vivo* has not been addressed.

Although not classified in one of the above groups, certain other small molecule inhibitors of virulence are of note due to their anti-staphylococcal activity. FtsZ is a bacterial cytoskeleton protein that is involved in cell division. A small synthetic molecule, PC190723, inhibits FtsZ and prevents cell division of the bacteria and by acting as a traditional antibiotic it cures mice infected with a lethal dose of *S. aureus* (Haydon et al., 2008; Singh and Panda, 2010). A different small molecule, 1835F03, was found to inhibit wall teichoic acid biosynthesis. The molecular target of the drug, TarG, is the transmembrane component of the ABC transporter that exports cell wall teichoic acids to the surface of the cell (Suzuki et al., 2011; Swoboda et al., 2009). The pigment that gives *S. aureus* its hallmark color has also served as a target for inhibition. Staphyloxanthin is a carotenoid pigment that protects the bacteria from reactive oxygen species by quenching free radicals (Clauditz et al., 2006; Liu et al., 2005). Synthase *CrtM* is essential in the biosynthesis pathway of staphyloxanthin. Treatment of *S. aureus* with phosphonosulfonate BPH-652, a cholesterol-lowering drug, inhibited *CrtM* which led to

decreased survival of the bacteria when exposed to hydrogen peroxide and to the phagocytic cells in whole blood (Liu et al., 2008). A small molecule inhibitor of *S. aureus* ribonuclease P (RnpA) a molecule essential in mRNA degradation, inhibits cellular mRNA turnover. The RnpA inhibitor effected the production of virulence factors due to the growth-phase dependent nature of virulence factor expression and exhibited protection in an acute lethal mouse model of infection (Olson et al., 2011). Thus, numerous targets have been developed for high throughput screening to advance drug discovery for the treatment of bacterial infections. Importantly for the work described here, the application of this strategy to quorum sensing regulated virulence by *S. aureus* has not been described.

## Summary and Hypothesis

The increasing emergence of methicillin resistant *Staphylococcus aureus* infections (Chambers and Deleo, 2009; Klevens et al., 2007), particularly those caused by a single clone (PFGE type USA300), coupled with the slowing of antibiotic discovery and challenges in effective vaccination makes research into novel therapies a priority ()(Garcia-Lara et al., 2005a; Garcia-Lara et al., 2005b; Garcia-Lara et al., 2005b; Lowy, 2007). One evolving strategy to treat these antibiotic resistant infections is to develop drugs that target bacterial virulence factors but not bacterial growth (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010). This approach is postulated to limit the development of resistance while enhancing host defense by permitting immune effectors to kill and clear the pathogenic bacteria rendered avirulent by the drug. While this approach has had success in animal models of primarily Gram-negative

bacterial infection (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010), it has not been pursued for treatment of MRSA infection.

The virulence factors identified to date as essential for invasive MRSA infection are globally regulated in part by a quorum sensing operon, *agr* (George and Muir, 2007; Novick and Geisinger, 2008; Yarwood and Schlievert, 2003). Our work identifying apolipoprotein B as an innate barrier that antagonizes *agr* signaling demonstrates that host defense against an invasive infection can be accomplished by blocking *agr* signaling (Peterson et al., 2008). Our work focuses on two small molecule inhibitors, CID# 2333 and CID# 3243271, identified in a screen of over 20,000 compounds for antagonism of *agr* signaling. We hypothesized that screening small molecules for inhibition of *agr* signaling could contribute to drug discovery by providing optimal host defense against quorum sensing dependent *S. aureus* infections.

# CHAPTER 2
# METHODS

## Reagents

Synthetic AIPs were generated as follows and stored in DMSO at -80°C: cyclic AIP1, AIP2, AIP3, and AIP4 were synthesized by Commonwealth Biotechnologies, Inc., Richmond, VA, and Biopeptide Co., Inc. San Diego, CA. The following reagents were obtained as indicated: glycine, bovine serum albumin (BSA) 3,3′-dipropylthiacarbocyanine iodide ($DiOC_2$), bovine catalase, clindamycin, Roswell Park Memorial Institute (RPMI), benzbromarone, 2-hydroxypropyl β-cyclodextrin, hydroxypropyl methylcellulose, 1M NaOH (Sigma-Aldrich, St. Louis, MO), $H_2O_2$ (EMD-Calbiochem, Gibbstown, NJ), Triton X-100 (Fischer Biotech), Hepes, Dulbecco's Modified Essential Media (DMEM) (Gibco), Casamino acids (Difco), Propidium iodide (Anaspec, Inc), savirin (Chem Div, San Diego, CA), BBL trypticase soy broth (TSB), BBL stacker plates with trypricase so agar with 5% sheep blood (Becton Dickinson and Company, Sparks, MD), rabbit erythrocytes (CS1081, Colorado Serum Company). Benzbromarone and savirin were solubilized in DMSO (1 mg/ml) for *in vitro* experiments.

## Bacterial Strains

*S. aureus* strains used in these studies were as follows: ALC 1743 (RN6390 containing reporter *agr*::P3-*gfp*) and Newman-GFP (*agr* group 1 Newman containing reporter *agr*::P3-*gfp*) were provided by Dr. Ambrose Cheung, Dartmouth School of Medicine. Strain 502A (*agr* group 2 clinical isolate) was purchased from ATCC. USA400 1560 strain was provided by Dr.Francoise Perdreau-Reminginton (UCSF). USA300 strain LAC, its Δ*agr* mutant, and USA400 strain MW2, were provided by Dr. Michael Otto

(NIAID). AH1677 (*agr* group 1 LAC containing reporter *agr*::P3-*gfp*), AH430 (*agr* group 2 502A containing reporter *agr*::P3-*gfp*), AH1747 (*agr* group 3 MW2 containing reporter *agr*::P3-*gfp*), and AH1872 (*agr* group 4 containing reporter *agr*::P3-*gfp*) were provided by Dr. Alexander Horswill (University of Iowa). Clinical isolate samples were obtained from the Veterans Affairs Hospital, Albuquerque, NM.

## Preparation of Bacterial Cultures

Primary stocks of all bacterial strains were prepared as followed: 25 ml of Trypticase Soy Broth (TSB), (prepared with 4 μg/ml tetracycline for the LAC Δ*agr* strain) in a 50 ml tube was inoculated with a colony from an original culture or a 1:2500 dilution of original stock was loosely capped and incubated with shaking (200 rpm) overnight at 37°C. After overnight incubation, cultures were pelleted by centrifugation at 3000 rpm for 4 minutes at 4$^{o}$C and washed twice with 10 ml of saline. The bacteria were resuspended in 5 ml of TSB and two dilutions of 1:84 were made in two 50 ml Falcon tubes containing TSB and tetracycline if necessary (LAC Δ*agr*) The tubes were loosely capped and the cultures were incubated at 37°C with shaking (200 rpm) for 6 hours after which the cultures were centrifuged and each tube was washed with 5 ml saline. The cultures were washed again and combined with 10 ml of saline and centrifuged a final time. The bacterial pellet was resuspended with 2 ml of TSB/ 10% glycerol, and kept on ice while sonicating twice for 5 seconds each time. The bacterial stock was then aliquoted into cryovials at a volume of 100 μl and stored at -80°C.

To generate synchronized early exponential phase, nonfluorescent (where applicable) bacteria, frozen stocks were cultured in Trypticase soy broth (TSB) at a

1:2500 dilution in one 50 ml Falcon tube overnight at 37°C with shaking at 200 rpm. The overnight culture was washed twice with 20 ml volumes and once with a 10 ml volume of sterile saline and then diluted 1:1000 in TSB in a 50 ml Falcon tube. The tube was loosely capped and incubated for 2 hours at 37$^{o}$C with shaking at 200 rpm. After two hours, the culture was centrifuged as described, washed as before, and then diluted 1:84 into two tubes for 2 additional hours of incubation followed by a 1:125 dilution into four tubes for two final hours of incubation with washing in between. At the end of the three two hour growth periods, the cultures were washed with saline and combined into one tube. The bacteria were then resuspended in 2 ml of TSB/10% glycerol, placed on ice and sonicated for two intervals of five seconds each. The cultures were aliquoted into cryovials at a volume of 100 μl per vial and stored at -80$^{o}$C. The titer of each stock was determined after the vials had been frozen for at least 30 minutes. The bacterial pellet of four vials was resuspended by adding sterile saline to a volume of 1ml each and centrifuging in a microcentrifuge at 4$^{o}$C for 4 minutes at 12,500 rpm to wash out the glycerol. The supernatant was discarded and the pellets were resuspended in 1 ml of fresh saline. The vials were each sonicated for three intervals of two seconds and then serial dilutions of the bacteria were plated on sheep blood agar plates to determine the titer of the stock.

## Bacterial Promoter Activation Assay

Early exponential phase bacteria (2 x 10$^{7}$/ml TSB) containing plasmid *agr*P3 driving GFP expression were incubated in polystyrene tubes with or without 50 nM of the corresponding synthetic AIP, benzbromarone (5 μg/ml), savirin (5 μg/ml) or vehicle

control at 37$^{o}$C with shaking (200rpm) for a designated amount of time. After incubation, bacteria were centrifuged (3000 rpm, 4 minutes, 4$^{o}$C), decanted, washed with phosphate buffered saline (PBS) with 0.1% Triton X-100, fixed with 1% paraformaldehyde containing 25 mM $CaCl_2$, sonicated, and then evaluated for GFP expression by flow cytometry (Accuri C6, Accuri Cytometers, Inc., Ann Arbor, MI) and measured as the mean channel fluorescence (MCF).

An alternate bacterial promoter assay was performed during overnight culture in the absence of exogenous AIP and represented the bacteria/drug solution that was administered in the *in vivo* experiments. One milliliter solutions consisting of early exponential phase bacteria (~3 x $10^9$) containing the GFP plasmid on the P3 promoter (500 μl), 1 mg/ml savirin or vehicle control (100 μl), and TSB (400 μl) were made up and 50 μl aliquots of each solution was added to each well of a 96 well polystyrene micotiter plate. The plate was wrapped in foil and incubated with shaking (200 rpm, 37$^{o}$C) overnight (18 hr). After incubation, 100 μl of 1% paraformaldehyde containing 25 mM $CaCl_2$ was added to fix the cells and evaluation of GFP expression was determined using flow cytometry (Accuri C6, Accuri Cytometers, Inc. Ann Arbor, MI).

**Quantitative RT-PCR**

Early exponential phase bacteria (2 x $10^7$) were cultured in 1ml volumes at 37$^{o}$C with shaking (200 rpm) and with the indicated AIP and drug treatment for the designated amount of time. Bacterial RNA for various targets was quantified relative to 16S RNA using a probe-based assay as described with minor modifications (Sawires and Gresham, 2008). Bacterial RNA was isolated and purified using the Qiagen RNAprotect Bacteria

Reagent and RNeasy Mini Kit, Protocol 3 using mechanical (sterile 0.1 mm zirconia silica beads, Biospec, Bartlesville, OK) and enzymatic disruption by Proteinase K (Qiagen, Valencia, CA). The RNA was purified using a column-based purification. DNase treatment was used to remove contaminating DNA from the column. cDNA was generated from the purified RNA using a high capacity cDNA-reverse transcriptase kit with an accompanying RNase inhibitor (Applied Biosystems, Foster City, CA) and a PTC-100 thermocycles (MJ Research Inc, Watertown, MA). Thermal cycling conditions were described in the kit instructions were as follows: 10 minutes at 25$^{o}$C, 120 minutes at 37$^{o}$C, 5 minutes at 85$^{o}$C, hold at 4$^{o}$C. Quantitative PCR was performed using an ABI7500 Real-Time PCR system with Taqman Gene Expression master mix, ROX probe/quencher, and appropriate primer sequences (Applied Biosystems). Experiments were performed in duplicate or triplicate and samples were assayed in triplicate. Relative quantification of *S. aureus* targets was determined by the change in target gene expression relative to 16S gene expression. Cycling conditions were 2 minutes at 50$^{o}$C, 10 minutes at 95$^{o}$C, and 40 cycles of 15 seconds at 95$^{o}$C with 1 minute at 60$^{o}$C. The primer-probe sequences used for targets investigated were as follows:

| | |
|---|---|
| RNAIII forward primer | 5′-AATTAGCAAGTGAGTAACATTTGCTAGT-3′ |
| RNAIII reverse primer | 5′-GATGTTGTTTACGATAGCTTACATGC-3′ |
| RNAIII probe | 5′-6-FAMAGTTAGTTTCCTTGGACTCAGT-GCTATGTATTTTTCTT-BHQ-2-3′ |
| psmα forward primer | 5′-TATCAAAAG-CTTAATCGAACAATTC-3′ |
| psmα reverse primer | 5′-CCCCTTCAAATA-AGATGTTCATATC-3′ |

| | |
|---|---|
| psmα probe | 5′-6-FAMAAAGACCTCCTTTGTTTGTTA-TGAAATCTTATTTACCAGBHQ-2-3′ |
| hla forward primer | 5′ -ACAATTTTAGAGAGCCCAACTGAT-3′ |
| hla reverse primer | 5′-TCCCCAATTTTGATTCACCAT-3′ |
| hla probe | 5′-6-FAMAAAAAGTAGGCTGGAAAGTGATA-BHQ-2-3′ |
| PVL forward primer | 5′-CACAAAATGCCAGTGTTATCCA-3′ |
| PVL reverse primer | 5′-TTTGCAGCGTTTTGTTTTCG-3′ |
| PVL probe | 5′-6-FAMAGGTAACTTCAATCCAGAATT-TATTGGTGTCCTATC-BHQ-2-3′ |
| 16S forward primer | 5′-TGATCCTGGCTCAGGATGA-3′ |
| 16S reverse primer | 5′-TTC-GCTCGACTTGCATGTA-3′ |
| 16S probe | 5′-6-FAMCGCTGGCGGCGTGCCTA-BHQ-2-3′ |

## Microarray analysis

To compare the transcript levels of LAC USA300 and the Δ*agr* mutant in the presence or absence of savirin, the bacteria were grown for 5 hr with AIP1 (50 nM) or an equivalent amount of DMSO as the vehicle control and processed for microarray analysis as described (Voyich et al., 2005). *S. aureus* cDNA was hybridized to custom Affymetrix GeneChips, RMLChip7a520611F, containing 100% of the USA300 genome. Samples were scanned according to standard GeneChip protocols and each experiment was repeated in triplicate. The data were evaluated using GeneSpring.

## Viability Assays

**Benzbromarone:** LAC USA300 ($2 \times 10^7$) was incubated at 37°C with shaking (200 rpm) in the presence or absence of benzbromarone (5 μg/ml or 10 μg/ml) or vehicle control. Viability was determined by removing aliquots at desired timepoints of growth and dilutions of the cultures were plated on blood agar plates and enumerating the viable colony forming units (CFU).

**Savirin:** LAC USA300 ($1 \times 10^6$) was incubated in 5ml TSB in the presence or absence of savirin (5 μg/ml). The bacteria were centrifuged, decanted, and resuspended in 5ml fresh TSB with new addition of drug every 24 hours. This procedure was repeated for 3 days. Growth was determined by plating out dilutions of the culture on sheep blood agar every 24 hr and enumerating colony forming units.

## Alpha Hemolysin Assay

Alpha hemolysin was measured in 0.45 μm filtered cultured supernatant for bacterial strains grown overnight in TSB in the presence or absence of benzbromarone (5 μg/ml) or savirin (5 μg/ml). Alpha hemolysin was measured as described using rabbit erythrocyte lysis (Bernheimer, 1988)(Black et al., 2011)Black et al., 2011) as described (Peterson et al., 2008). Rabbit erythrocytes (CS1081, Colorado Serum Company) were washed in PBS twice by centrifugation at 3200 rpm for 10 minutes at 4°C to remove the Alsever's preservation buffer. Erythrocytes were then resuspended in PBS to a final concentration of 4% erythrocytes. Serial dilutions of the sample supernatants were mixed in equal volumes with the erythrocyte suspension in a 96 well round bottom plate and incubated for 1 hour at 37°C. After the incubation, the plate was centrifuged for 3

minutes at 3800 rpm at 4$^{o}$C to pellet the unlysed erythrocytes and the supernatants were transferred to a new 96 well plate then read on a spectrophotometer at a wavelength of 450 nm. The lysis by the individual supernatant samples was compared to the total lysis of erythrocytes that resulted from mixing equal volumes of the erythrocyte suspension and PBS/0.1% Triton. One unit of hemolytic activity was defined as the amount of bacterial supernatant able to liberate half of the total hemoglobin from the erythrocytes.

## Staphyloxanthin Production and Extraction Assay

Primary cultures of LAC USA300 or LAC USA300 Δ*agr* mutant (10 μl) were cultured in 25 ml TSB for 48 hr in the presence of benzbromarone (5 μg/ml). Aliquots of 3 ml were removed every 12-24 hr, centrifuged (3000 rpm, 4$^{o}$C, 4 minutes), decanted, and images of the pigmented pellets were taken. Quantification of staphyloxanthin production was measured as described (Resch et al., 2005). At timepoints of 24, 32, and 48 hr, 5 ml cultures were removed and equalized to an $OD_{600}$ to ~1.0. Bacteria were centrifuged, decanted, and treated with lysostaphin (3 μg/ml) diluted in PBS (total volume of 100 μl) for 20 minutes. Extraction of the pigment was performed by adding 2 ml of 100% methanol and incubating at 45$^{o}$C for 30 minutes. Cultures were centrifuged and the intensity of released pigment in the supernatants was measured by spectrophotometry at 460nm.

## Autolysis Assay

Triton X-100-stimulated autolysis of bacterial cultures was measured as described (Sieradzki and Tomasz, 1997). Primary cultures of LAC USA300 or LAC USA300 Δ*agr* mutant (10 μl) were cultured in 5 ml TSB for 18 hr in the presence of absence of

benzbromarone (5 μg/ml) or vehicle control. The cultures were then rapidly chilled in an ice/ethanol bath, pelleted by centrifugation (3000 rpm, 4$^{o}$C, 4 minutes) and washed once with ice-cold distilled water. The bacteria were resuspended to an optical density of 1.0 at 620nm ($OD_{620}$) in 50 mM glycine supplemented with Triton X-100 (0.01%) at pH 8.0. Cultures were incubated at 37$^{o}$C with shaking (200 rpm) and were removed at selected timepoints, sonicated, and autolysis measured as the decrease in $OD_{620}$.

## Acid Killing Assay

**Benzbromarone:** Early exponential phase LAC USA300 or LAC300 Δ*agr* mutant (1 x $10^{9}$) was incubated at 37$^{o}$C with shaking (200 rpm) in PBS for 1.5 hr in the presence of absence of benzbromarone (5 μg/ml). After the incubation, the cultures were centrifuged (3000 rpm, 4$^{o}$C, 4 minutes), resuspended in PBS acidifed with HCl to pH 2.5, and incubated for an additional 2 hr. Serial dilutions of the bacteria were plated on sheep blood agar plates to determine the surviving colony forming units.

**Savirin:** Early exponential phase LAC USA300 or LAC USA300 Δ*agr* mutant (1 x $10^{8}$/ml in phenol red-free Dulbecco's Modified Eagle Media, DMEM, containing 4.5g/L D-glucose/2%Hepes) were incubated for 5 hr at 37$^{o}$C with shaking (200 rpm) with 50 nM synthetic AIP1 (used only with LAC USA300) in the presence or absence of savirin (5 μg/ml) or vehicle control. Bacteria were centrifuged (3000 rpm, 4$^{o}$C, 4 minutes), washed once with PBS, resuspended in DMEM/2%Hepes acidified with HCl to pH 2.5, and incubated for an additional 1 hr. Dilutions were plated on sheep blood agar and viability determined by enumerating colony forming units.

## Oxidant Susceptibility Assay

Susceptibility of the bacteria to oxidants was measured as described (Liu et al., 2005).

**Benzbromarone:** LAC USA300 was cultured for 30 hr in the presence or absence of benzbromarone (5 μg/ml). Bacteria were centrifuged (3000 rpm, 4$^{o}$C, 4 minutes), washed twice with PBS, and resuspended in PBS to an $OD_{600}$ of 0.6. Hydrogen peroxide ($H_2O_2$) (30% solution) was added to a 1.5% or 3.0% final concentration and the cultures were incubated at 37$^{o}$C with shaking (200 rpm) for 2 hr. Catalase (from bovine liver) (1,000 U/ml) was added to the cultures to quench the residual $H_2O_2$. Dilutions were plated on sheep blood agar to determine surviving bacteria of each sample.

**Savirin:** Early exponential phase LAC USA300 or LAC USA300 Δ*agr* mutant (1 x $10^8$/ml DMEM/2%Hepes) were incubated for 5 hr with 50 nM synthetic AIP1 (used only with LAC USA300) in the presence or absence of savirin (5 μg/ml) or vehicle control. Bacteria were centrifuged (3000rpm, 4$^{o}$C, 4 minutes), washed once with PBS, and resuspended in DMEM/2%Hepes. Hydrogen peroxide ($H_2O_2$) was added to a 1.5% final concentration and the cultures were incubated at 37$^{o}$C with shaking (200 rpm) for 2 hr. Catalase (1,000 U/ml) was added to quench the residual $H_2O_2$. Dilutions were plated on blood agar to determine bacterial survival.

## Linoleic Killing Assay

Early exponential phase LAC USA300 were incubated at 37$^{o}$C with shaking overnight (18 hr) in TSB in the presence or absence of benzbromarone (5 μg/ml). The cultures were centrifuged, washed twice with PBS, and the cultures were resuspended in TSB to an $OD_{600}$ of 0.3. The cultures were aliquoted to a volume of 1ml in 3ml polystyrene tube and

linoleic acid (Sigma-Aldrich) was added to the cultures at various final concentrations from 0.2 mM to 1.0 mM making duplicates of each concentration. Cultures with linoleic acid were incubated as described above for 18 hr after which they were sonicated and dilutions of the cultures were plated out on blood agar plates to determine bacterial survival by enumerating CFUs.

## Whole Blood Killing Assay

Early exponential phase LAC USA300 (1 x $10^8$/ml DMEM/2%Hepes) were incubated for 5 hr with 50 nM synthetic AIP1 in the presence or absence of savirin (5 μg/ml) or vehicle control. Bacteria were centrifuged (3000rpm, 4$^o$C, 4 minutes), washed once with PBS, and resuspended in DMEM. Bacteria were diluted (1 x $10^5$/ml DMEM) and incubated in 10% normal human serum for 30 minutes at 37$^o$C. After a brief sonication, 50 μl of bacteria were mixed with 450 μl of heparinized whole mouse blood in 1.5 ml eppendorf tubes and rotated end-over-end at 37$^o$C for 2 hr. 100 μl of the solution was mixed with 100 μl PBS/1% Triton, sonicated, and plated on blood agar plates to determine bacterial survival by enumerating CFUs (Data not shown).

## Membrane Integrity Assay

Membrane integrity was measured as described using propidium iodide (Attia et al., 2010). Primary cultures of LAC USA300 were cultured overnight (18 hr) in Roswell Park Memorial Institute (RPMI) medium supplemented with 1% casamino acids (CAS) in the presence or absence of savirin (5 μg/ml). The cultures were then pelleted, washed once with PBS, and then resuspended in PBS supplemented with 1% bovine serum albumin

(BSA). The samples were then set to an $OD_{600}$ of 0.4 and then a select portion of the samples were heat killed (90$^{o}$C for 10 minutes) to serve as positive control. Aliquots of 50 µl of the samples were mixed with 1ml PBS/1% BSA containing 2 µg of propidium iodide. Membrane damage was determined by measuring mean channel fluorescence (MCF) using flow cytometry (Accuri C6)

**Membrane Potential Assay**

Membrane potential was measured as described (Gentry et al., 2010; Hu et al., 2010; Novo et al., 1999). LAC USA300 was cultured overnight (18 hr) in TSB in the presence or absence of savirin (5 µg/ml). The cultures were pelleted and washed twice with PBS. The cultures were resuspended in PBS, set to an $OD_{600}$ of 1.5, and a selection of the samples of LAC bacteria alone were heat killed (90$^{o}$C for 10 minutes) to serve as positive control. 3,3′-dipropylthiacarbocyanine iodide ($DiOC_2(3)$) was added to 500 µl samples of the bacteria to a final concentration of 50 µM and samples were incubated at room temperature in the dark for 5 minutes. 100 µl of each sample containing $DiOC_2(3)$ was diluted into 600 µl of a reaction buffer consisting of 60 mM $Na_2HPO_4$, 60 mM $NaH_2PO_4$, 130 mM NaCl, 5mM KCl, 0.5 mM $MgCl_2$, and 10 mM glucose. Flow cytometry was then used to measure membrane potential (Accuri, C6). Measurements from both the red and green channel were taken and data were presented as a ratio of red channel measurements divided by the green channel measurements to reflect the shift to greater change in membrane potential.

## Fibrinogen-Mediated Bacterial Promoter Activation Assay

Early exponential phase bacteria (2 x $10^7$/ml TSB) containing plasmid *agr*P3 driving GFP expression were incubated in polystyrene tubes with or without fibrinogen (American Diagnostica, Inc.) (100 μg/ml), in the presence or absence of savirin (5 μg/ml) or vehicle control. Cultures were incubated at 37$^o$C with shaking (200 rpm) for 4 hr. After incubation, bacteria were centrifuged (3000rpm, 4$^o$C, 4 minutes), decanted, washed with (PBS) with 0.1% Triton X-100, fixed with 1% paraformaldehyde containing 25 mM $CaCl_2$, sonicated, and then evaluated for GFP expression by flow cytometry (Accuri C6) measured as the mean channel fluorescence (MCF).

## *In Vitro* Development of Resistance

Early exponential phase LAC USA300 (1 x $10^6$/ml) were incubated for 10 days in 5 ml TSB with or without the addition of clindamycin (Sigma) (0.1μg/ml) or savirin (5 μg/ml) at 37$^o$C with shaking (200 rpm). The bacteria were centrifuged, decanted, and resuspended in 5 ml fresh TSB and new addition of drug was added to the respective tubes every 24 hr. On the tenth day, samples were centrifuged, washed once with PBS, and then plated onto sheep blood agar to determine the titer. The bacteria that had been repeatedly exposed for 10 days and unexposed bacteria (starting concentrations of 1 x $10^6$ in 5ml TSB) were then challenged in an overnight (18 hr) incubation with clindamycin (0.1μg/ml) or savirin (5μg/ml). After 18 hr, the bacteria were plated to determine viability. Bacteria repeatedly exposed to savirin for 10 days and unexposed bacteria (2 x $10^7$/ml TSB) were incubated in 1ml cultures with 50 nM synthetic AIP1 in the presence

or absence of savirin (5 μg/ml) for 2 hr at 37$^{o}$C with shaking (200 rpm). Expression of RNAIII was determined by qRT-PCR as described above.

## Mice

The appropriate institutional committees approved all experiments involving animals. SKH1-E mice (≈ 8 -10 wk,≈ 24 -32 g, male) were obtained from Charles River Labs (Wilmington, MA).

## Dermonecrotic Infection Model

Benzbromarone was solubilized in 12mM 2-hydroxypropyl β-cyclodextrin in endotoxin-free water. Savirin was solubilized in 0.5% hydroxypropyl methylcellulose in endotoxin-free water and 1M NaOH was added to a final concentration of 15mM. Sonication and incubation in a 37$^{o}$C water bath were necessary to ensure savirin was in solution. Solutions were filtered with a 0.22μM filter (Millex-GV) before injecting. Early exponential phase bacteria (4 x $10^{7}$) were injected simultaneously with either benzbromarone (5 μg), savirin (5 μg), or vehicle subcutaneously into the flank of SKH1-E mice as described (Wang et al., 2007) using a 3/10cc insulin syringe with a 28 ½ gauge needle (Becton Dickinson). Abscess area (white, puffy area) and ulcer area (necrotic area) were recorded daily in addition to weight loss as evidence of illness. The abscess area (mm$^{2}$) is calculated from the equation:

$$\{(\pi/6)[(\text{length of the ulcer}) \times (\text{width of the ulcer})]\}$$

The ulcer area (mm$^{2}$) is calculated from the equation:

{(length of the ulcer) x (width of the ulcer)}

On Day 3 or Day 7, the mice were sacrificed using isoflurane inhalation. The abscess/ulcer area was excised (1.5 $cm^2$) and the spleens were removed. Tissues and spleens were removed and placed in 1 ml of HBSS/HSA in a bead-beating tube containing sterile 2.3 mm beads (Biospec, Bartlesville, OK) and stored on ice. Tissue and spleen samples were processed for bacterial CFU by homogenizing the spleens in a bead beater, diluting all samples 1:10 in 1 ml 1X PBS/0.1%Triton, sonicating the sample, then plating serial dilutions on blood agar, as described (Rothfork et al., 2004).

### *In Vivo* Development of Resistance

Early exponential phase bacteria (4 x $10^7$/ml) were injected simultaneously with clindamycin (0.1μg/ml) or savirin (1 μg/ml) into the flank of SKH1-E mice as described (Wang et al., 2007) using a 3/10cc insulin syringe with a 28 ½ gauge needle (Becton Dickinson). At 24hr, weight loss was recorded and the mice were sacrificed using isoflurane inhalation. The abscess area was measured and spleens were removed and placed in 1 ml of HBSS/HSA in a bead-beating tube and processed as described above. Serial dilutions of the bacteria from the spleen were plated on blood agar plates and were incubated at 37$^o$C for 24 hr. Colonies were lifted from the blood agar plates and sonicated in saline until a desired density (as measured by $OD_{600}$ on the spectrophotometer) was reached. These bacteria were injected simultaneously with clindamycin (0.1μg/ml) or savirin (1μg/ml) into the flank of SKH1-E mice as before. At 24 hr, the mice were sacrificed and the spleens were processed as described and plated. The procedure was repeated twice more, carrying the bacteria taken from the spleen through four mice total

for each treatment. After the fourth mouse in each treatment group was sacrificed, the splenic bacteria taken from the plate were concentrated and stored in TSB/10% glycerol at -80$^{o}$C. The repeatedly exposed bacteria (from spleen) and unexposed bacteria were diluted to a starting concentration of 1 x $10^{6}$/ml in 5 ml TSB and challenged in an overnight (18 hr) incubation in the presence of clindamycin (0.1μg/ml) or savirin (1μg/ml). After 18 hr, bacteria were plated to determine viability. qRT-PCR assay was also performed using unexposed bacteria or bacteria repeatedly exposed to savirin (from spleen). 1ml cultures were incubated at a concentration of 2 x $10^{7}$/ml in TSB with 50 nM synthetic AIP1 in the presence or absence of savirin (1 μg/ml). Expression of RNAIII determined by qRT-PCR as described previously after 1 hr incubation.

## Statistical Evaluation

Data are displayed as the mean ± SEM. In vitro data were analyzed by the Student's t test using GraphPad Prism 4.0 and the in vivo data were analyzed by the Mann-Whitney U test for non-parametrics using Statview for MacIntosh.

# CHAPTER 3

# SAVIRIN, A SMALL MOLECULE INHIBITOR, TARGETS *STAPHYLOCOCCUS AUREUS* VIRULENCE *IN VITRO* AND *IN VIVO*

## Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) has emerged as a major public health threat (Chambers and Deleo, 2009; Klevens et al., 2007) and the worldwide expansion of a single clone (USA300), associated with life threatening infections even in immunocompetent adults (Lowy, 2007), has re-focused interest in developing novel therapeutics to treat this infection (Garcia-Lara et al., 2005a). One strategy evolving to treat antibiotic resistant infections is to develop drugs that target virulence but not bacterial growth (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010). This approach is postulated to limit the development of resistance while enhancing host defense by permitting immune effectors to kill and clear the pathogenic bacteria rendered avirulent by the drug. While this approach has had success in animal models of primarily Gram-negative bacterial infection (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010), it has not been pursued for treatment of MRSA infection. The virulence factors identified to date as essential for MRSA infection, particularly those that contribute to the dermonecrotic skin infections typical of this pathogen, are globally regulated in part by a quorum sensing operon, *agr* (Li et al., 2009; Novick and Geisinger, 2008; Wang et al., 2007). Importantly, recent work from our laboratory identifying apolipoprotein B as an innate barrier that antagonizes *agr* signaling demonstrates that host defense against invasive infection can be accomplished by blocking *agr* signaling (Peterson et al., 2008). Therefore, we hypothesized that small molecule inhibitors (SMIs) identified by screening chemical libraries for antagonism of *agr* signaling could ameliorate experimental *agr*-dependent MRSA infections.

In this work, we describe a chemical compound identified in a high throughput screen that we have termed savirin, for *Staphylococcus aureus* virulence inhibitor. Savirin inhibited *agr* signaling in multiple *S. aureus* strains at a concentration that did not affect bacterial growth. Its inhibition of *agr* signaling and the ultimate production of virulence factors were verified by transcriptional analysis and assays for virulence factor function. It prevented the development of dermonecrotic ulcers following infection with *agr*+ bacteria in an experimental mouse model of skin and soft tissue infection. Moreover, repeated exposure of *S. aureus* to savirin either *in vivo* or *in vitro* did not induce resistance in those bacteria to the inhibition of *agr* signaling by savirin. These data demonstrate that chemical compounds can be identified that target quorum sensing dependent *S. aureus* virulence without inducing significant resistance upon repeated exposure and thus have promise as potential therapeutic adjuncts for the treatment of infections caused by this pathogen.

## Results

### *Savirin inhibits agr signaling without affecting bacterial viability*

The *agr* operon is activated by an autoinducing pheromone peptide (AIP) that binds to its cognate receptor and leads to activation of a promoter (*agr*:: P3) that produces a regulatory RNA, RNAIII, that is the effector of the operon. RNAIII alters the transcriptional profile of the bacteria to upregulate production of secreted virulence factors like capsule, toxins, and tissue damaging enzymes while downregulating surface adhesins (Novick et al., 1993).

A screen was performed by the New Mexico Molecular Libraries Screening Center targeting AIP-dependent bacterial quorum sensing utilizing the HyperCyt high throughput flow cytometry platform. The HyperCyt system interfaces a flow cytometer and autosampler (Edwards et al., 2004). Samples are sequentially aspirated from the wells of a 384-well microplate separated by a bubble of air. This delivery of samples to the flow cytometer makes the HyperCyt system 30 times faster than commercial alternatives (Edwards et al., 2004). A total of 24,087 compounds from the Molecular Libraries Small Molecule Repository (MLSMR) were screened to identify novel small molecule inhibitors of the quorum sensing pathway, specifically identification of a small molecule that inhibits the binding of AIP to cellular receptors.

A high-throughput fluorescence-based screening assay was used which examined the ability of the tested compounds to inhibit the activation of the RNAIII promoter encoded with a plasmid which drives GFP expression (Sawires and Gresham, 2008). When these reporter bacteria are in early exponential phase before quorum sensing has begun, they are non-fluorescent. When incubated with exogenous synthetic AIP1, the bacteria undergo quorum sensing and the RNAIII promoter is activated thus activating GFP production. In the same timeframe (3 hr), reporter bacteria incubated without exogenous synthetic AIP addition did not produce enough endogenous AIP to undergo quorum sensing so therefore these bacteria remained non-fluorescent. The test compounds were added at a final concentration of 2 μM and were measured by their ability to inhibit GFP fluorescence (RNAIII activation) in the presence of 0.1 μM AIP1 and a bacterial concentration of $2 \times 10^7$.

Savirin was one of ten compounds identified in a screen of over 24,000 compounds for inhibition of *agr*::P3 promoter activation by the Molecular Libraries *Screening* Centers Network (MLSCN) (University of New Mexico). Savirin (3-(4-propan-2-ylphenyl) sulfonyl-1H-triazolo [1,5-a] quinazolin-5-one (CID# 3243271) (Figure 1A) inhibited *agr*::P3 promoter activation with an $IC_{50}$ of < 1.0 μM. To be a useful therapeutic, ideally savirin would inhibit *agr* signaling without inhibiting bacterial growth. To determine if savirin affected either short term or long term growth, we cultured a USA300 strain, LAC, in trypticase soy broth (TSB) in the presence or absence of savirin (5 μg/ml) refreshing the broth and the drug every 24 hr. Viability was tested every 24 hr by enumerating viable colony forming units (CFU). Over the course of 72 hr, incubation in the presence of savirin had no effect on the viability of the bacteria as compared to bacteria grown in broth (Figure 1B).

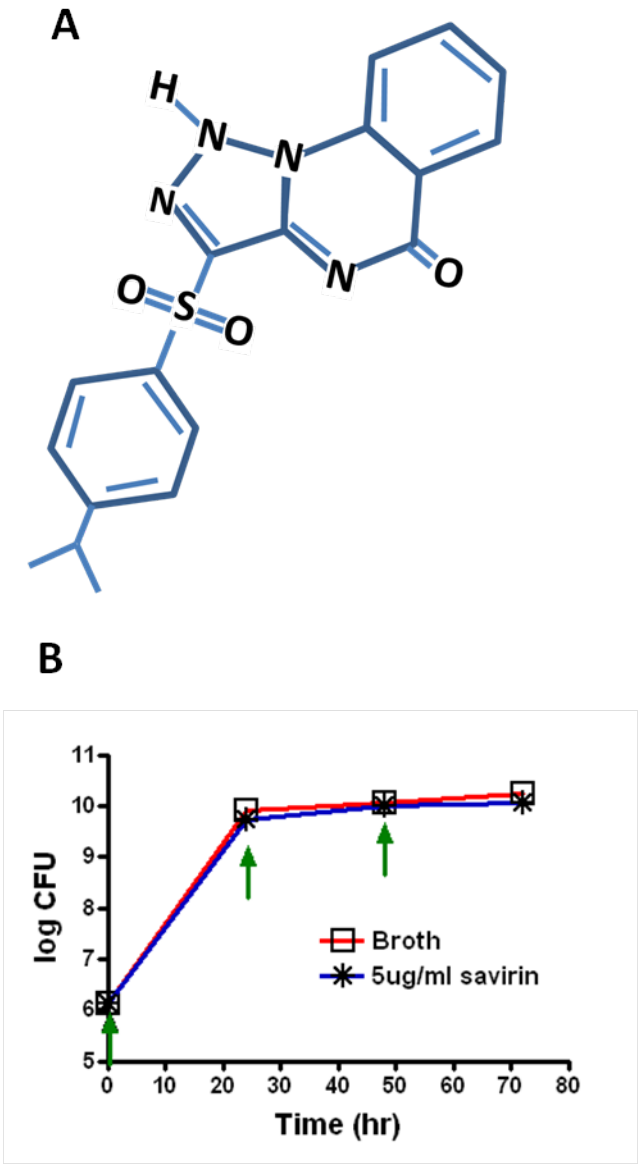


**Figure 1. Savirin does not affect bacterial viability in the LAC USA300 strain**

A) Chemical Structure of savirin (CID# 3243271) 1 of 10 compounds identified with an $IC_{50}$<1 μM for the inhibition of *agr*:: P3 promoter activation in a MLSCN screen of over 24,000 compounds

B) LAC USA300 WT was incubated in TSB in the presence or absence of savirin (5 μg/ml) and growth was determined every 24hr by enumerating colony forming units. Arrows indicate timing of savirin and fresh broth addition. Data are represented as the Mean ± SEM, n=3

All *S. aureus* strains can be classified into one of four groups based on the sequence of the AIP produced and its cognate receptor, AgrC. While the majority of clinical isolates are *agr* type 1, all types contribute to human disease. PFGE type strains USA300 and USA400, the most clinically problematic and abundant community acquired MRSA strains are *agr* type 1 and *agr* type 3 respectively (Lowy, 2007). For savirin to be a useful therapeutic against *S. aureus* infections, including those caused by antibiotic resistant strains, it should have actions against multiple, if not all, *agr* backgrounds.

To determine if savirin could be used to inhibit *agr::*P3 promoter activation in multiple genetic backgrounds, we used reporter strains of each agr type where activation of the *agr* P3 promoter drives expression of GFP (*agr1*: AH1677/LAC USA300, *agr*2: AH430/502A, *agr*3: AH1747/MW2, and *agr*4: AH1872/MNTG). The bacteria were incubated for 18 hr without addition of exogenous AIP, in the presence or absence of savirin (5 μg/ml) or an equal volume of vehicle control prior to evaluation of GFP expression by flow cytometry. Savirin significantly inhibited *agr* activation in the *agr*1 strain LAC (Figure 2A), indicating that savirin can antagonize AIP1 signaling without affecting bacterial growth (CFU) (Figure 1B). In addition, savirin inhibited *agr::*P3 activation in other *agr* backgrounds; *agr* type 2 strain 502A (Figure 2B), *agr* type 3 strain MW2 (Figure 2C), and *agr* type 4 strain MNTG (Figure 2D). These data demonstrate that the small molecule savirin significantly inhibits *agr* signaling of all four *agr* types at a concentration that does not inhibit bacterial growth *in vitro*.

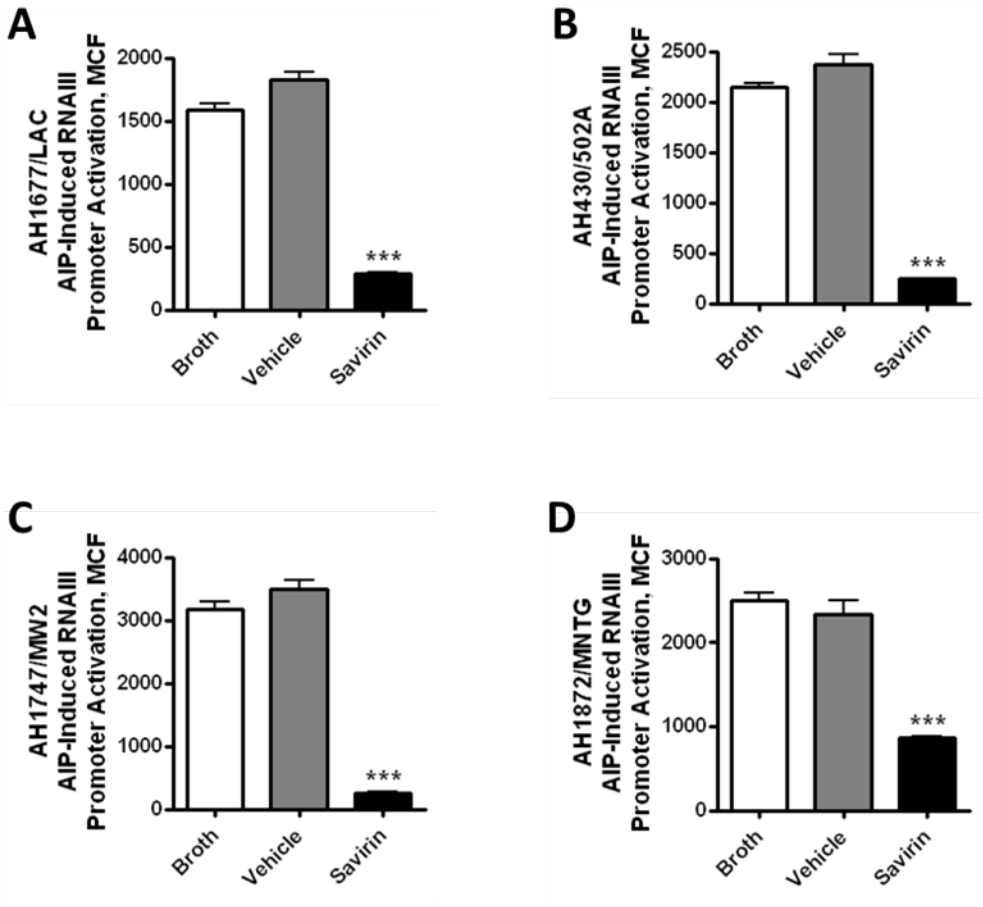


**Figure 2. Savirin inhibits *agr* signaling in multiple strain types**

*agr*::P3 promoter activation is inhibited in

(A) *S. aureus agr1* strain (AH1677/LAC with *agr*:: P3-*gfp*, $2\times10^7$/ml)

(B) *S. aureus agr2* strain (AH430/502A with *agr*:: P3-*gfp*, $2\times10^7$/ml)

(C) *S. aureus agr3* strain (AH1747/MW2 with *agr*:: P3-*gfp*, $2\times10^7$/ml)

(D) *S. aureus agr4* strain (AH1872/MNTG with *agr*:: P3-*gfp*, $2\times10^7$/ml)

during 18 hr of culture in the presence or absence savirin or equal volume of vehicle control. Reporter assay was performed in a microtiter plate assay in a final volume of 50 μl containing ~4 x $10^7$ bacteria and 5 μg savirin. Data are represented as the mean channel fluorescence (MCF) of total *S. aureus* by flow cytometry, ± SEM; ***p<0.0005, n=5

*The agr regulated virulence transcriptome is significantly affected by savirin*

Microarray analysis can provide valuable information as to the global transcriptional effects of savirin on LAC USA300 and its Δ*agr* mutant. A microarray analysis was performed in collaboration with Dr. Frank DeLeo at the Rocky Mountain Labs, NIAID, NIH. cDNA of bacteria incubated with exogenous AIP for 5 hr was hybridized to a custom Affymetrix GeneChip which contained 100% of the USA300 LAC genome. Three comparisons were performed in which the alterations of open reading frames were examined; LAC vs LAC+savirin, Δ*agr* vs Δ*agr*+savirin, and LAC vs Δagr. Figure 3A depicts a diagram of the effects of savirin on LAC USA300 and its Δ*agr* mutant. Savirin affected 211 open reading frames in the wild type strain representing 7.6% of the total LAC genome, up-regulating 59 and down-regulating 152. In the Δ*agr* mutant, 52 open reading frames were affected by savirin representing 1.8% of the total genome, 17 up-regulated and 35 down-regulated. Savirin affected 46 open reading frames similarly between the wild type and mutant, with 19 of those being up-regulated and 27 down-regulated. These data indicate that savirin primarily alters transcription in the *agr*+ strain while minimally altering transcription in the Δ*agr* mutant. An analysis of the actual genes altered revealed that 94 open reading frames significantly down-regulated by savirin in the *agr*+ strain (LAC vs LAC+savirin) were also down regulated in the absence of *agr* (LAC vs Δ*agr*), including all of the major virulence factors regulated by *agr*. These include genes for capsule production, multiple proteases, lipases, hemolysins, and cytolytic peptides (Table 1). In addition, genes involved in resistance to acid-mediated stress (potassium transporter and urease) were also down-

regulated by savirin (Table 1). Consistent with our observations made above (Figure 2), these data indicate that savirin has a significant inhibitory effect on *agr*-regulated gene transcription.

Of the genes affected in both the wild type and the Δ*agr* mutant, a subset includes genes encoding multidrug efflux pumps including the MATE efflux pump (increased 3.71 fold in wild type and 2.77 fold in the Δ*agr* mutant) and the less well understood multidrug resistance protein A (increased 10.84 fold in wild type and 8.45 in the Δ*agr* mutant) while transcription of the genes for the better understood drug transporters NorA and QacA were not affected, indicating that some stress response common to both strains is activated by savirin. The open reading frame most dramatically up-regulated in both strains by savirin is a hypothetical protein (increased 30.75 fold in wild type and 30.51 fold in the Δ*agr* mutant) suggesting that savirin may alter poorly understood stress responses.

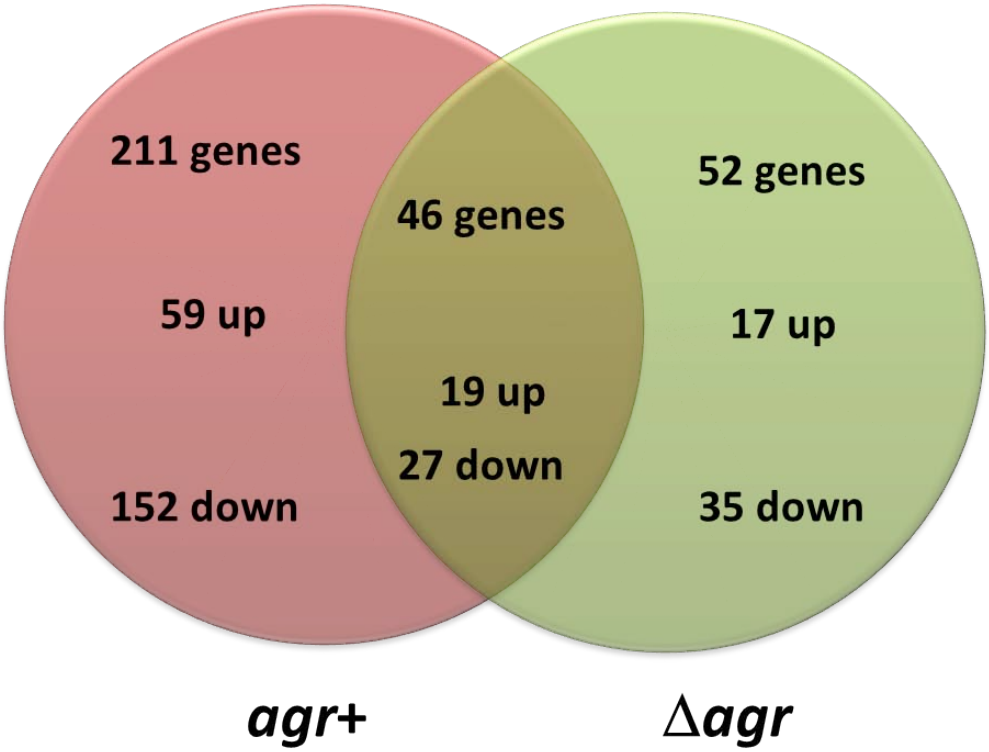


**Figure 3. Microarray analysis of the impact of savirin on gene transcription of *agr*+ and Δ*agr* CA-MRSA**

Effect of savirin on the transcriptome of LAC USA300 and its Δ*agr* mutant. Bacteria were cultured in the presence of exogenous AIP1 (50 nM) in the presence or absence of savirin (5 μg/ml). RNA was extracted, purified, reverse transcribed to cDNA, and hybridized to the Affymetrix GeneChip RML7a520611F containing the USA300 genome. n=3, significant effect > 2 fold change, $p<0.05$

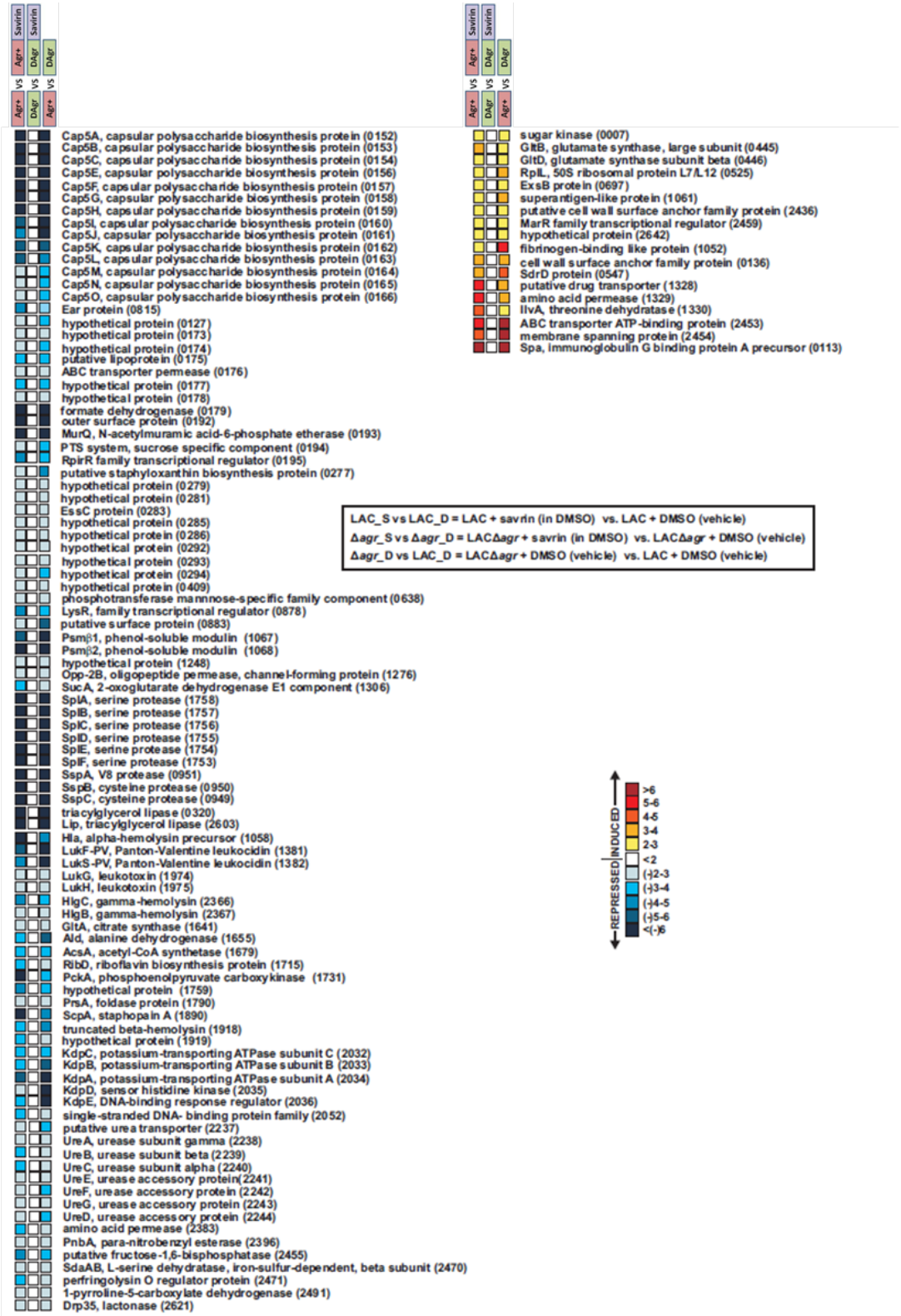


**Table 1. *agr*-regulated genes altered by savirin in LAC *agr*+**

Gene name, gene function, and fold change of gene altered by savirin in LAC *agr*+

*Savirin inhibits RNAIII synthesis and subsequent virulence factor production in multiple genetic backgrounds*

RNAIII synthesized downstream of *agr*::P3 activation is responsible for the transcriptional control of the production of numerous virulence factors including the cytolytic peptide psm α and the cytotoxin alpha hemolysin. Both of these are integral in causing the tissue destruction seen in skin and soft tissue infections caused by CA-MRSA strains (Li et al., 2009). Based on the results showing the ability of savirin to inhibit the *agr* P3 promoter and the results of our microarray analysis showing down-regulation of *agr*-dependent virulence factor gene expression, we predicted that savirin would be able to inhibit the synthesis of RNAIII in addition to the transcription of subsequent virulence factors that lie downstream of RNAIII.

To address this, we selected the *agr* type 1 USA300 strain LAC and utilized qRT-PCR to measure RNAIII expression either following stimulation by addition of exogenous synthetic AIP1 or during culture relying on endogenous production of AIP by the bacteria. Early exponential phase USA300 LAC were grown for 2 hr (with exogenous AIP1) or 5 hr (endogenous production of AIP1) at 37$^{o}$C in the presence or absence of savirin or vehicle control. As shown in Figure 4A, RNAIII production was significantly inhibited by the addition of savirin as compared to the vehicle control. Figure 4B shows a similar effect in the 5 hr assay where the AIP production was produced endogenously during culture. Savirin was also able to significantly inhibit the expression of RNAIII when added after the initiation of the bacterial culture (Figure 4C). Early exponential phase LAC were grown for 3 hr at 37$^{o}$C after which time savirin, vehicle, or broth were

added to the cultures and then incubated for an additional 2 hr. As Figure 4C shows, even when added to the cultures after 3 hr of incubation, savirin was still able to significantly inhibit the production of RNAIII transcripts.

Host microenvironments that mediate bacterial clumping can enhance density-dependent virulence gene expression and this includes the clumping of this pathogen by the coagulation protein fibrinogen that creates constrained microenvironments around the bacteria (Rothfork et al., 2003a). To determine if savirin would inhibit *agr*::P3 promoter activation in an environment likely to be encountered in vivo, we included fibrinogen to promote clumping and quorum sensing. Figure 4D shows that even in the presence of fibrinogen, which mediates activation in the samples of bacteria alone or bacteria with vehicle, savirin is still able to significantly inhibit the activation of the promoter.

The virulence factors produced downstream of RNAIII are largely responsible for the pathogenesis of the disease. Because savirin inhibited expression of RNAIII, we predicted that the production of virulence factors controlled by RNAIII would be similarly impeded. We examined the effect of savirin on gene expression for alpha hemolysin. Early exponential phase LAC was grown for 5 hr (with exogenous AIP) or 8 hr (endogenous production) at 37$^{o}$C in the presence or absence of savirin. The addition of savirin to the culture significantly inhibited the production of alpha hemolysin mRNA both in assays where exogenous AIP was added (Figure 5A) and where the bacteria were allowed to spontaneously produce their own AIP (Figure 5B). qRT-PCR was used to determine the capacity of savirin to inhibit the expression of *psmα* mRNA which is a direct readout of AgrA activation (Queck et al., 2008). As expected, savirin inhibited

*psmα* mRNA in a 2 hr assay where exogenous AIP was added (Figure 5C). To confirm that savirin inhibited toxin production, we measured the function of alpha hemolysin by its ability to lyse rabbit erythrocyes. As shown in Figure 5D, LAC produced an abundant amount of alpha hemolysin toxin when cultured for 18 hr which was completely abrogated by the addition of savirin. These data demonstrate that savirin can inhibit the production of multiple AIP1 dependent virulence factors *in vitro*, both at the transcript and at the protein level, and confirms the results we obtained in the microarray analysis. We show that inhibition of quorum sensing by savirin can inhibit the phenotypic switch that prevents RNAIII expression and the downstream up-regulation of the virulence factors described above.

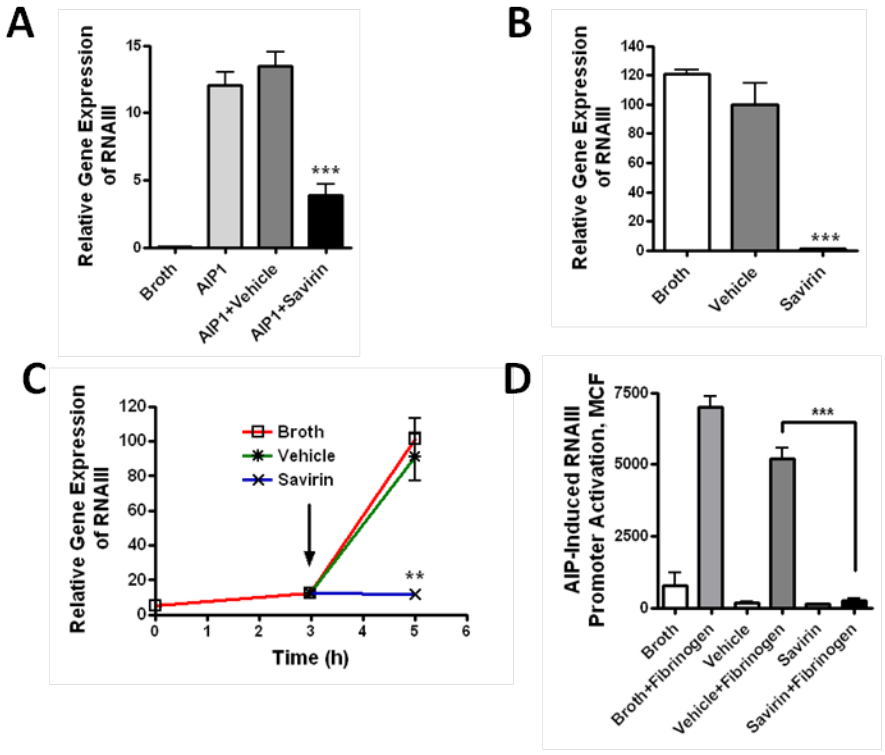


**Figure 4. Savirin antagonizes *agr*1 and RNAIII expression**

A) LAC USA300 (2 x $10^7$/ml TSB) was incubated with 50 nM synthetic AIP1 in the presence or absence of savirin (5 μg/ml). RNA was extracted, purified, reverse transcribed to cDNA, and then gene expression relative to endogenous control 16s was determined using a probe for RNAIII after 1 hr of culture. ***p<0.0005, n=4

B) LAC USA300 (2 x $10^7$/ml TSB) was incubated without synthetic AIP1 in the presence or absence of savirin (5 μg/ml). Expression of RNAIII determined by qRT-PCR as described above after 5 hr. ***p<0.0005, **p=0.0015, n=4

C) Effect of savirin (5 μg/ml) added to a culture of LAC USA300 (2 x $10^7$/ml TSB) at 3 hr and then incubated for an additional 2 hr. Expression of RNAIII determined by qRT-PCR as described above. **p=0.0012 n=3 Data are represented as mean ± SEM

D) LAC USA300 (2 x $10^7$/ml TSB) was incubated with fibrinogen (100 μg/ml) for 4 hr in the presence or absence of savirin (5 μg/ml). Data are represented as the mean channel fluorescence (MCF) of total *S. aureus* by flow cytometry, and as the mean ± SEM; ***p<0.0005, n=3

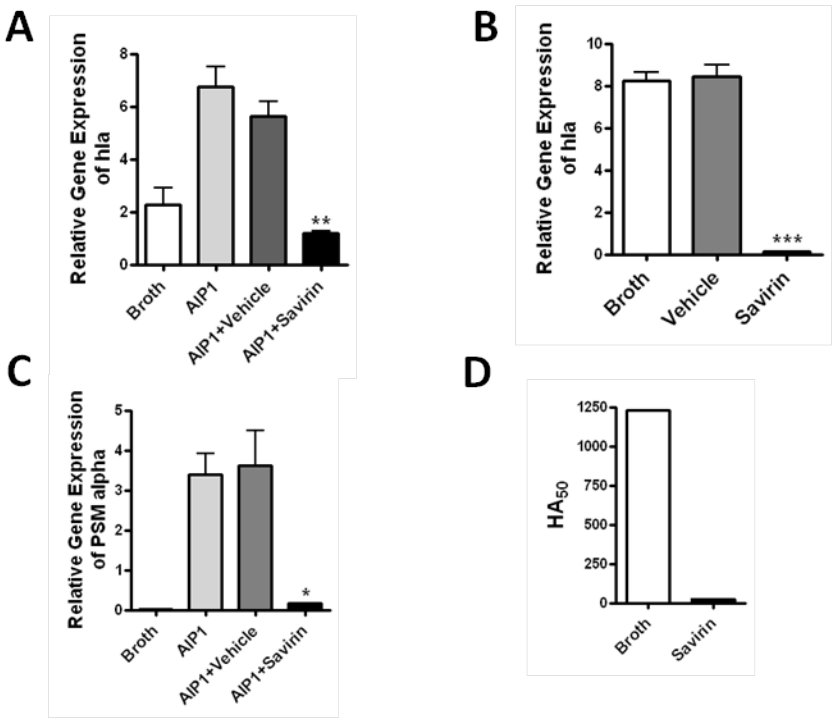


**Figure 5. Savirin antagonizes *agr*1 and virulence factor production**

A) LAC USA300 (2 x $10^7$/ml TSB) was incubated with 50 nM synthetic AIP1 in the presence or absence of savirin (5 μg/ml). RNA was extracted, purified, reverse transcribed to cDNA, and then gene expression relative to endogenous control 16s was determined using a probe for *hla* after 5 hr of culture. ***p<0.0005, n=4

B) LAC USA300 (2 x $10^7$/ml TSB) was incubated without synthetic AIP1 in the presence or absence of savirin (5 μg/ml). Expression of *hla* determined by qRT-PCR as described above after 8 hr. ***p<0.0005, **p=0.0015, n=4

C) LAC USA300 (2 x $10^7$/ml TSB) was incubated with 50 nM synthetic AIP1 in the presence or absence of savirin (5 μg/ml). Expression of PSMα determined by qRT-PCR as described above after 1 hr. *p=0.0164 n=3 Data are represented as mean ± SEM

F) Effect of savirin (5 μg/ml) added to an overnight culture of LAC in which alpha-hemolysin in the supernatant was measured by lysis of rabbit erythrocytes.

Although USA300 is responsible for the majority of MRSA infections, the other *agr* backgrounds also cause disease, most notably *agr* type 3 USA400. Therefore, it was important clinically to determine whether savirin would inhibit virulence factor production in strains other that *agr*1 type strains. Based on the observation that savirin inhibited *agr* signaling in multiple *agr* backgrounds (Figure 2), we hypothesized that the drug would have a similar inhibitory effect on the production of virulence factors of *agr2* strains and *agr3* strains. We examined alpha hemolysin production in an *agr2* strain (502A) and an *agr3* strain (1560). Both 502A and 1560 were cultured for 18 hr in the presence of absence of savirin and alpha hemolysin was measured by the supernatants ability to lyse rabbit erythrocytes. As predicted, Figure 6A and 6B show that savirin inhibited the production of alpha hemolysin in 502A and 1560 respectively. Next qRT-PCR was used to determine the effect of savirin on the production of *psm*α. As expected, savirin inhibited *psm*α expression in a 2 hr assay where exogenous AIP was added in both 502A and MW2 (Figure 6C, 6D). These data demonstrate that savirin can inhibit the production of virulence factors in *agr*2 and *agr*3 strains *in vitro*.

To confirm that savirin could inhibit alpha hemolysin production by current clinical isolates, we obtained clinical isolates from patients at the New Mexico VA Health Care System. A selection of these strains (both MRSA and MSSA) that produced a measurable amount of alpha hemolysin was incubated for 18 hr in the presence or absence of savirin. Figure 6E shows that regardless of infection location or methicillin susceptibility, savirin was able to inhibit alpha hemolysin production in all clinical

isolates tested. These data suggest that savirin is a small molecule inhibitor that selectively targets virulence in all *agr* strains as well as clinical isolates.

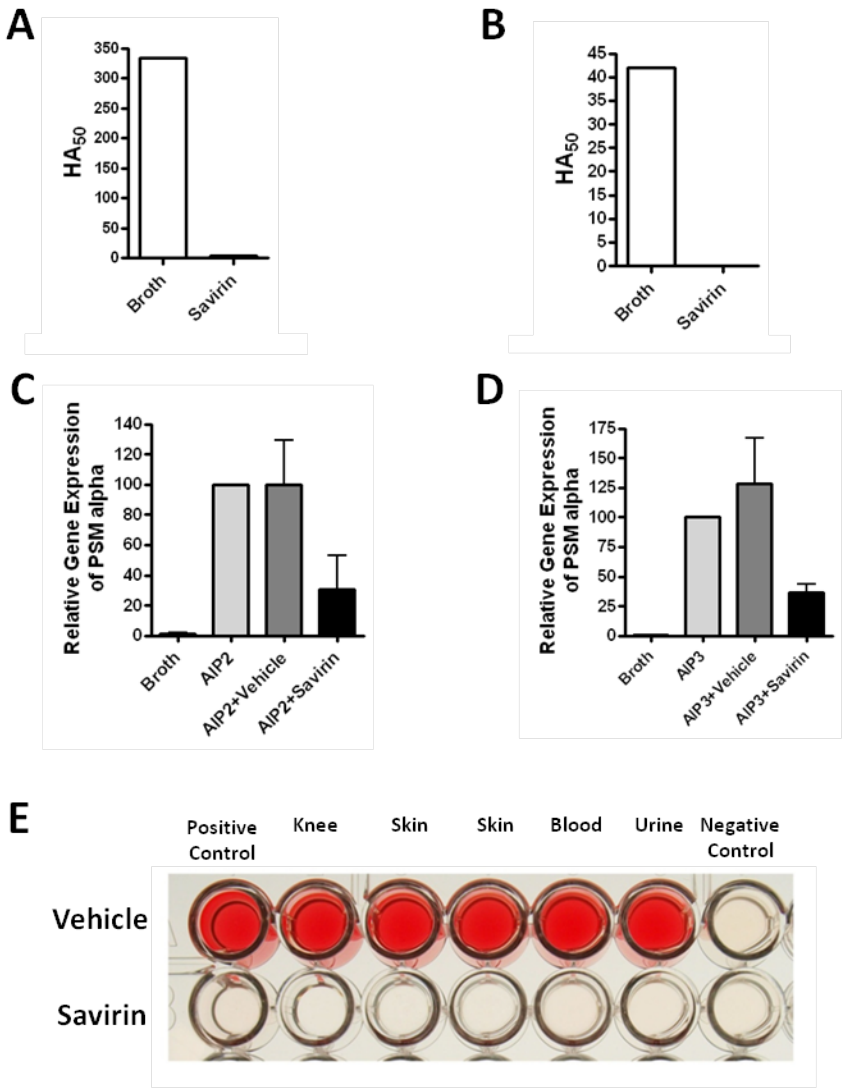


**Figure 6. Savirin Antagonizes *agr*2 and *agr*3 Signaling and Virulence Factor Production**

A.B) Effect of savirin (5 μg/ml) added to an overnight culture of *agr* 2 strain 502A (B) or *agr* 3 strain MW2 (B) in which alpha-hemolysin in the supernatant was measured by lysis of rabbit erythrocytes.

C.D) Gene expression of PSMα as measured by qRT-PCR as a percentage of AIP (100%) in *agr* 2 strain 502A (C) or *agr* 3 strain MW2 (D) ($2 \times 10^7$/ml) incubated with 50 nM synthetic AIP2 or AIP3 in the presence or absence of savirin (5 μg/ml) incubated at 37°C with shaking for 2 hours. n=2, n=3 respectively Data are represented as mean ± SEM

(E) Effect of savirin (100 μg/ml) added to overnight cultures of various clinical isolates in which alpha-hemolysin in the supernatant was measured. The upper row is treated with vehicle control and the lower row contains savirin.

*Savirin exhibits protection in an agr-dependent dermonecrotic S. aureus infection*

Skin and soft-tissue infections predominate as the most frequent form of disease caused by CA-MRSA and these vary in severity from impetigo, a painless rash, to life-threatening necrotizing fasciitis. Abscesses are the most common presentations, affecting 50% to 75% of patients that present with CA-MRSA (Stryjewski and Chambers, 2008). Both PSMα and alpha hemolysin are integral factors in causing the tissue destruction that constitute skin and soft tissue infections (Li et al., 2009). Because savirin inhibited the production of these toxins *in vitro,* we predicted that savirin would inhibit production of *in vivo* as well and be protective in a dermonecrotic mouse model of infection.

To test whether treatment with savirin could confer protection in an invasive *S. aureus* infection, we used a mouse model of dermonecrosis (Wang et al., 2007). Early exponential phase LAC *agr*+ bacteria were injected subcutaneously into the flank of SKH1-E hairless mice with the addition of either savirin or vehicle alone. The infections were monitored for three days and abscess area, ulcer area, and weight loss were recorded. At the termination of the experiment, the ulcerated areas were excised (1.5 $cm^2$) and homogenized to determine the number of bacteria. As shown in Figure 7A-7C, infection of the mice with LAC in the presence for savirin resulted in smaller abscesses and ulcer area as compared to those given vehicle. Mice given savirin also experienced decreased weight loss compared to the mice treated with vehicle (Figure 7D), while the number of viable bacteria in the ulcerated tissue indicate that savirin can have a therapeutic effect in a dermonecrotic infection and protects without altering the viability

of the bacteria at the 3 day time point. This suggests that the drug is not killing or restricting growth of the bacteria but rather affecting what the bacteria are producing.

CA-MRSA skin infections are transmitted from person to person with interfamilial outbreaks being reported (Ippolito et al., 2010). CA-MRSA infections are also associated with increased expense and duration of hospital stays so resolution time of the infection is an important factor to take into consideration. Because savirin exhibited protection in an *in vivo* dermonecrosis model, we predicted this initial protection would lead to faster resolution of the ulcer and enhanced clearance of the bacteria by the host immune system. Figure 8A shows the decreased necrotic area of the savirin-treated mice on Day 7 in contrast to the significantly larger necrotic area of the mice treated only with vehicle. Figure 8B and 8C show the course of the infection over the seven days measuring ulcer area (Figure 8B) and abscess area (Figure 8C) with the peak of the infection area at Day 3 and then subsequent resolution. After seven days post infection, the mice treated with savirin had a lower bacterial burden in the necrotic area (Figure 8D) and in the spleen (Figure 8E). These data illustrate that bacteria treated with savirin are cleared more readily than bacteria treated with vehicle thus leading to quicker resolution of the infection.

Due to the overwhelming percentage of abscess manifestations that MRSA patients present with, the *in vivo* model of dermonecrosis we utilize for these experiments is clinically relevant. Our results showing that savirin is protective in an *in vivo* dermonecrotic infection suggests that savirin may be a useful therapeutic against skin and soft tissue infections caused by CA-MRSA.

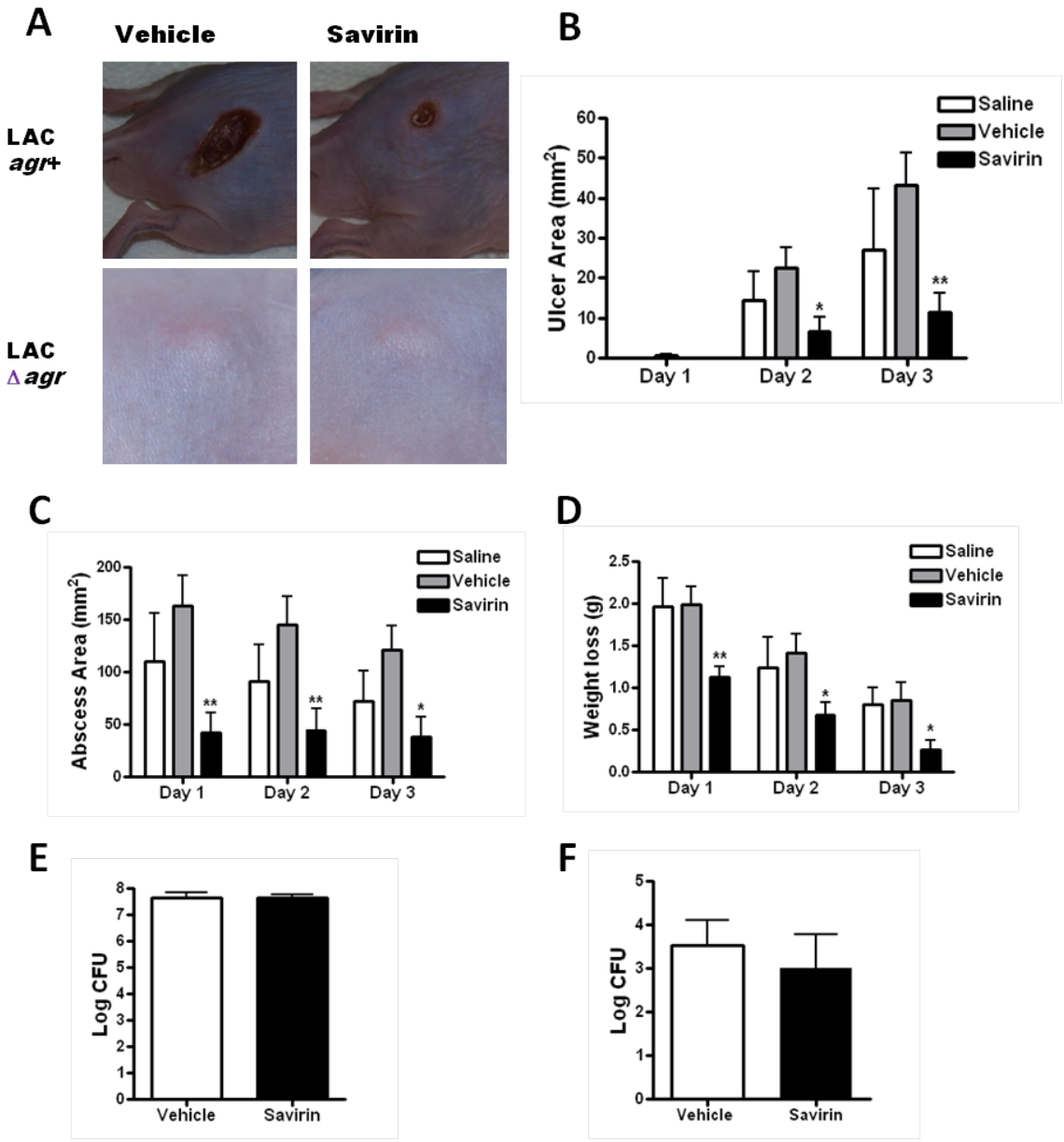


**Figure 7. Effect of savirin on an invasive dermonecrotic *S. aureus* infection with LAC USA300 over three days**

A) LAC USA300 WT (5 x $10^7$/ml saline at early exponential phase) were injected subcutaneously on the flank (n=11 mice for each group, n=4 for saline) with either 5 µg savirin or equal volume of vehicle. Pictures of the injection sites were taken at three days

B.C.D.E.F) Measurements of dermonecrosis (ulcer area (B) and abscess area (C)), weight loss (D), CFUs from the lesion (n=5) (E), and CFUs from the spleen (n=5) (F) were taken on Day 3 of the infection. Savirin compared to vehicle: **p=0.0027, *p<0.026. Data represented as the mean ± SEM.

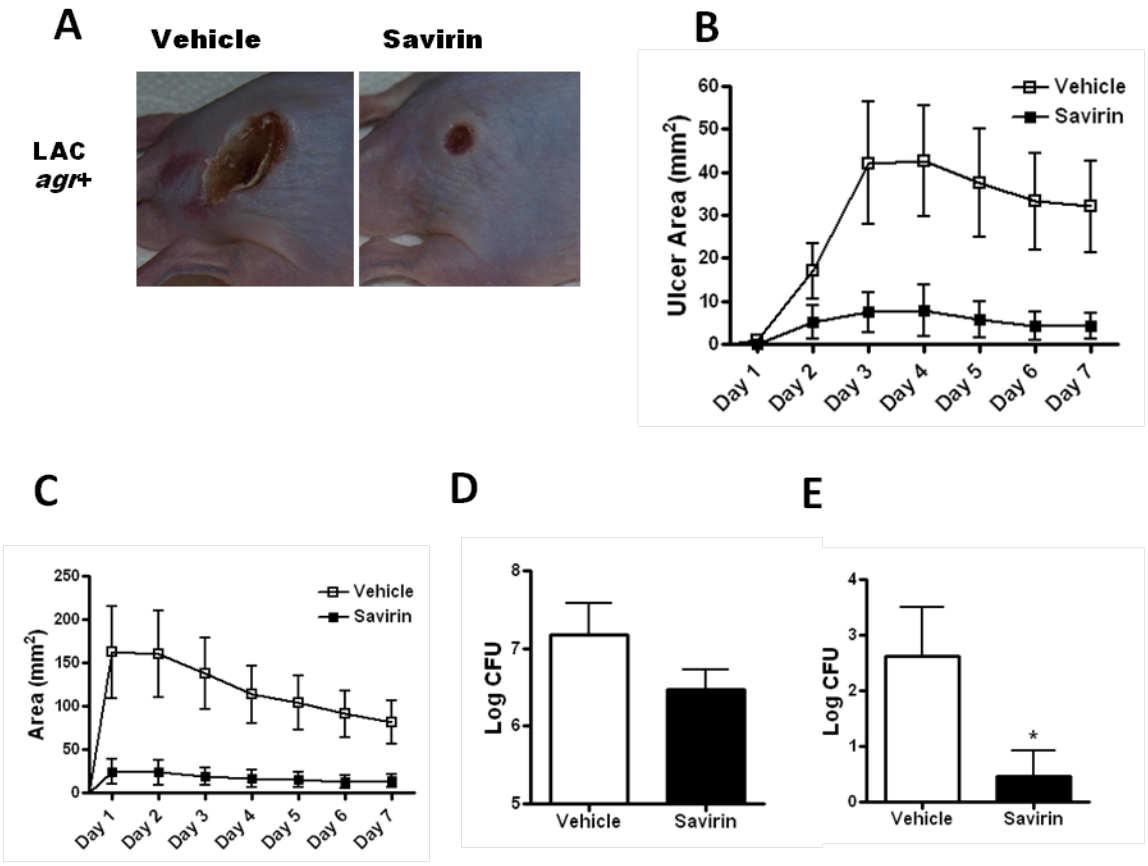


**Figure 8. Effect of savirin on the resolution of an invasive dermonecrotic *S. aureus* infection with LAC USA300**

A) LAC USA300 WT (5 x $10^7$/ml saline at early exponential phase) were injected subcutaneously on the flank (n=6 mice for each group) with either 5 μg savirin or equal volume of vehicle. Pictures of the injection sites were taken at seven days

B.C.D.E) Measurements of dermonecrosis (ulcer area (B) and abscess area (C)), CFUs from the lesion (p=0.16) (D), and CFUs from the spleen (p<0.05) (E) were taken on Day 7 of the infection. Data represented as the mean ± SEM

*S. aureus remains sensitive to inhibition of quorum sensing by savirin after repeated exposure both in vitro and in vivo*

When bacterial growth is targeted through the use of bacteriocidal or bacteriostatic agents such as antibiotics, selective pressure is generated in which mutations arise which can lead to drug resistance. In order for savirin to have therapeutic potential, the bacteria must remain sensitive to the drug over repeated exposure. To address whether bacterial sensitivity to savirin was retained through successive exposures, we chose to repeatedly challenge the bacteria *in vivo* with the traditional antibiotic clindamycin to serve as a positive control. We injected SKH1-E mice subcutaneously on the flank with early exponential phase LAC USA300 and a sub-inhibitory dose of clindamycin. After 24 hr, the spleen was removed and bacteria cultured from that spleen were combined with clindamycin and used to infect another mouse. This procedure was carried through four mice with the final splenic bacteria being challenged in an 18 hr growth assay side by side with unexposed bacteria. As shown in Figure 9A, the growth inhibitory effects of clindamycin can be overcome by four passages through mice. We used this procedure to then test whether the action of savirin on the bacteria can be overcome by repeated exposure *in vivo*. We injected SKH1-E mice subcutaneously on the flank with early exponential phase LAC USA300 and a sub-optimal dose of savirin and followed the identical procedure. No change was seen in bacterial viability between drug treated and control or between the unexposed and the repeatedly exposed bacteria (Figure 9B). qRT-PCR was used to measure RNAIII expression to compare the sensitivity to savirin between the unexposed bacteria and the bacteria repeatedly exposed *in vivo*. Bacteria from the spleen of the fourth mouse were incubated for 2 hr in the

presence or absence of savirin and the bacteria that had been repeatedly exposed to savirin *in vivo* still retain sensitivity to RNAIII inhibition by the drug (Figure 9C). These data suggest that within the time frame that *S. aureus* can develop resistance to the growth inhibitory effects of clindamycin *in vivo*, they still retain sensitivity to the effects of savirin.

We next determined whether that sensitivity to savirin was retained through successive exposures *in vitro* in order to confirm our *in vivo* findings. We incubated LAC USA300 for ten days in the presence of clindamycin, refreshing the broth and drug every 24 hr. The repeatedly exposed bacteria were challenged in an 18 hr viability assay side by side with unexposed bacteria. As shown in Figure 10A, the growth inhibitory effect that clindamycin has on unexposed bacteria could be almost abolished by ten days of repeated exposure. We then followed a similar procedure, exposing bacteria to savirin for ten days, refreshing the broth and drug every 24 hr. We first tested for effects on bacterial viability and found no change in growth between drug treated and control or between the unexposed and the repeatedly exposed bacteria (Figure 10B). We used qRT-PCR to measure RNAIII expression to compare the sensitivity to savirin between the unexposed bacteria and repeatedly exposed bacteria. Figure 10C shows that the repeatedly exposed bacteria retained sensitivity to savirin for RNAIII inhibition equal to that of unexposed bacteria. Our data demonstrate no induction of resistance at the time points used and that the bacteria remain sensitive to the effects of savirin while during the same time frame the bacteria did develop resistance to a currently prescribed and commonly utilized antibiotic.

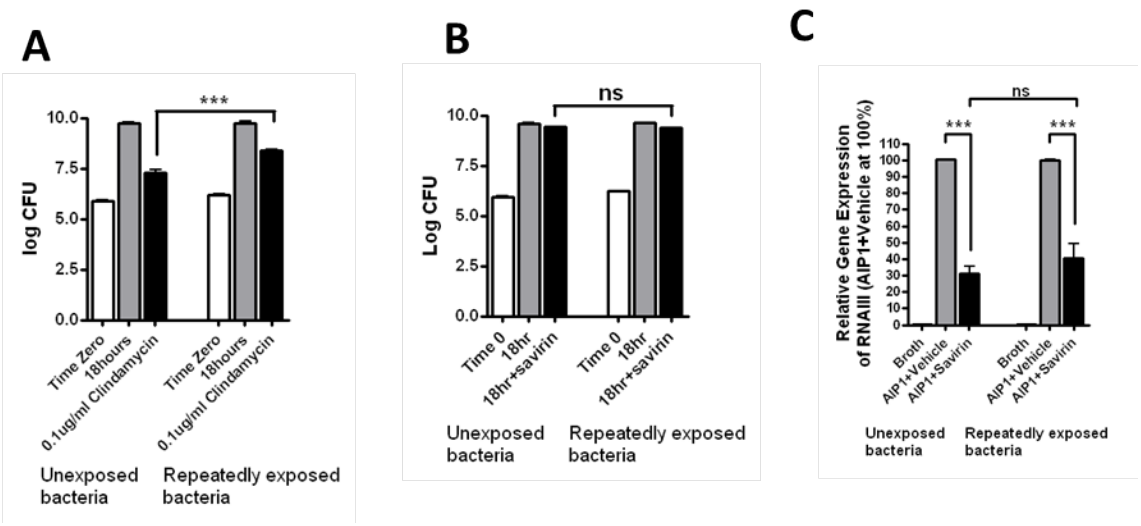


**Figure 9. Bacteria remain sensitive to inhibition of *agr* signaling by savirin after repeated exposure *in vivo***

A.B) LAC USA300 (4 x $10^7$/ml) was injected simultaneously with clindamycin (0.1μg) (A) or savirin (1μg) (B). After 24 hr, mice were sacrificed and spleens were homogenized and plated on blood agar. Colonies were lifted and injected simultaneously with clindamycin (0.1μg) (A) or savirin (1μg) (B) into the flank of mice. Process was repeated for a total of four mice in each treatment group. Repeatedly exposed bacteria (*in vivo*) and unexposed bacteria were challenged in an 18 hr incubation with clindamycin (0.1μg/ml) (A) or savirin (5 μg/ml) (B). Data are depicted as the mean +/- SEM of 3 replicates of two representative experiments. ***p<0.0001.

C) Unexposed bacteria or bacteria repeatedly exposed to savirin *in vivo* (2x$10^7$/ml TSB) incubated with 50 nM synthetic AIP1 in the presence or absence of savirin (5μg/ml). Expression of RNAIII determined by qRT-PCR as described previously after 1 hr. Data are depicted as the mean +/- SEM. n=4-6. AIP1+vehicle compared to AIP1+savirin: ***p<0.0001.

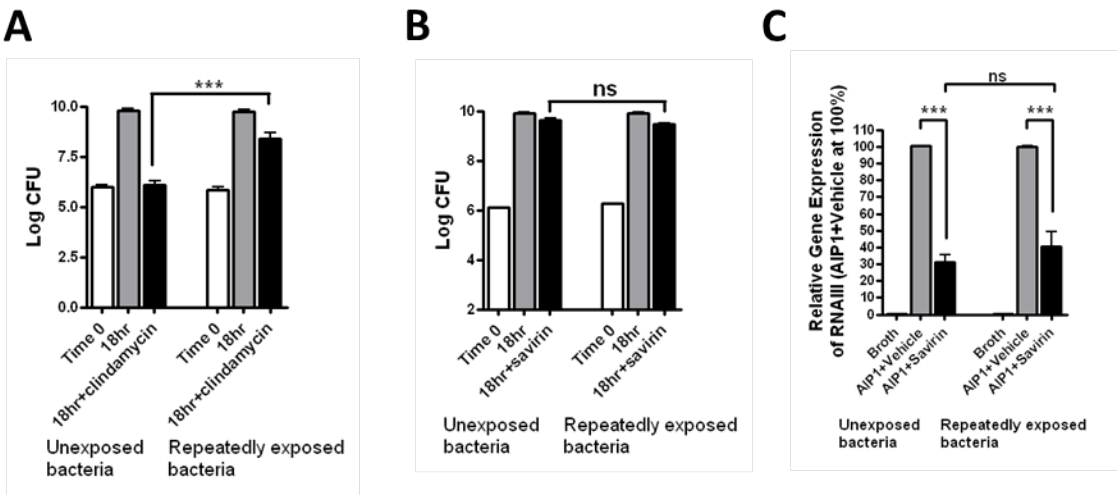


**Figure 10. Bacteria remain sensitive to inhibition of *agr* signaling by savirin after repeated exposure *in vitro***

A.B) LAC USA300 is incubated for 10 days in TSB with or without clindamycin (0.1 μg/ml (A) or savirin (5 μg/ml) (B) with repeated addition every 24 hr. Repeatedly exposed bacteria and unexposed bacteria were challenged in an 18 hr incubation with clindamycin (0.1 μg/ml) (A) or savirin (5 μg/ml) (B). Data are depicted as the mean +/- SEM of 3 replicates of two representative experiments. ***$p<0.0001$.

C) Unexposed bacteria or bacteria repeatedly exposed to savirin (2 x $10^7$/ml TSB) incubated with 50 nM synthetic AIP1 in the presence or absence of savirin (5 μg/ml). Expression of RNAIII determined by qRT-PCR as described previously after 1 hr. Data are depicted as the mean +/- SEM. n=4. AIP1+vehicle compared to AIP1+savirin: ***$p<0.0001$.

*Savirin-induced alterations in agr-regulated genes predict enhanced innate immune killing and potential membrane damage*

The strategy behind inhibiting bacterial virulence through the use of savirin is to attenuate the bacteria, ameliorate the infection, and allow the host immune effectors to clear the pathogen. We postulated that to be an effective therapeutic, savirin treatment would enhance bacterial killing by the host innate immune system. One such immune effector is low pH that is integral in host defense. Acidic conditions are key to host defense of the skin as well as the phagolysosome of macrophages that acidifies to destroy engulfed bacteria. The microarray analysis demonstrated that some of the bacterial genes down-regulated in response to savirin treatment were *agr*-dependent genes responsible for the generation of ammonia, an essential component for bacterial survival in acidic environments. This led us to predict that treatment with savirin would decrease bacterial survival in acidic conditions. We used *in vitro* tests with acidic buffer to replicate the host innate effector to determine if exposure to savirin made the bacteria more susceptible to killing by acid. Specifically we cultured LAC USA300 or Δ*agr* in DMEM/2%Hepes for 5 hr in the presence of absence of savirin. After washing, the bacteria were incubated in acidic conditions for 1 hr. As shown in Figure 11A, the wild type bacteria were significantly more susceptible to killing by acid when they had been exposed to savirin while the Δ*agr* strain (Figure 11B) showed no difference but had decreased survival in all the groups tested. These data illustrate that exposure to savirin makes the LAC wild type bacteria more susceptible to killing by acid, an innate effector.

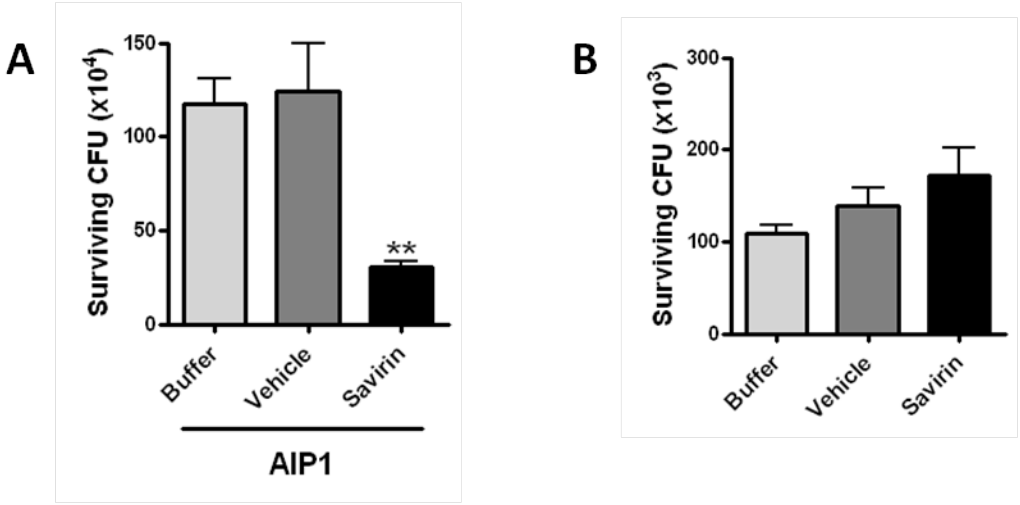


**Figure 11. Exposure to savirin results in increased killing of the wild type bacteria to acid**

A.B) LAC USA300 (A) or LAC USA300 Δ*agr* mutant (B) ($1x10^8$/ml DMEM/Hepes) incubated for 5hr with 50 nM synthetic AIP1 (used in LAC USA300 only) in the presence or absence of savirin (5 μg/ml). Bacteria were washed and resuspended in DMEM/Hepes, pH 2.5 for 1hr. Dilutions were plated on sheep blood agar and viability determined by enumerating colony forming units. **p=0.004, n=6-12, Data represented as the mean ± SEM.

To determine if the inhibition of capsule production by savirin led to increased killing by phagocytes, we performed a whole blood killing assay in which LAC USA300 were cultured in DMEM/2%Hepes for 5 hr in the presence of absence of savirin. After washing and diluting, the bacteria were incubated with whole mouse blood for 2 hr. Under the conditions of this assay, we failed to see any killing of either group of bacteria and instead saw 1 log of growth after the 2 hr incubation (Data not shown).

The microarray analysis demonstrated that transcripts for membrane transport proteins (eg; multidrug resistance transporter) were modestly up-regulated by exposure to savirin in both *agr*+ and Δ*agr*. These increased transcript levels may be indicative of a membrane stress response and can be induced following exposure to antibiotics. This led us to postulate that savirin may be acting on the membrane of cell. To address this, we first determined if savirin affected membrane potential which is a mechanism employed by many antibiotics (Gentry et al., 2010; Hu et al., 2010; Novo et al., 1999). LAC USA300 was incubated for 18 hr in the presence of absence of savirin, washed, and heat-killed LAC was used as a positive control. The bacteria were then loaded with $DiOC_2$ a fluorophore which exhibits green fluorescence in all bacterial cells but shifts toward red emission as the dye molecules self-associate at higher cytosolic concentrations following disruption of membrane potential. As Figure 12A shows, savirin-treated bacteria had no increase in fluorescence indicating limited membrane disruption as compared to heat killed LAC. These data suggest that the mechanism of action of savirin is unique from these membrane disruption events.

Antibiotics that create pores in the bacterial membrane can also induce a membrane stress response that leads to reduced membrane integrity. We used propidium iodide to detect membrane damage that may be caused by the addition of savirin. (Attia et al., 2010). Specifically, we incubated LAC USA300 cultures for 18 hr in RPMI/1% CAS in the presence or absence of savirin (5 μg/ml) and used heat-killed bacteria as a positive control. Propidium iodide was added to each sample at a final concentration of 2 μg/ml. Mean channelfluorescence (MCF) was measured by flow cytometry as a readout of membrane damage. Figure 12B shows that there is no increase in membrane damage (MCF amount) upon treatment with savirin compared to heat killed bacteria. These data suggest that savirin-induced alterations in membrane integrity are unlikely to contribute to the effects of savirin on *agr* signaling.

If the savirin was inhibiting quorum sensing by blocking the secretion of AIP by AgrB, we hypothesized that if we added excess exogenous AIP, this inhibition of RNAIII gene expression could be overcome. To test this hypothesis, we added excess exogenous AIP into the bacterial cultures in the presence or absence of savirin to determine if the inhibition of RNAIII gene expression by the inhibitor could be overcome. As shown in Figure 12C, even when 200 nM exogenous AIP was added, savirin was still able to significantly inhibit RNAIII gene expression. These data suggest that since the effects of savirin cannot be overcome with excess exogenous AIP, that savirin is not exerting its primary effect on *agr* signaling by inhibiting AIP secretion by AgrB. On aggregate, our data indicate that savirin does not inhibit *agr* signaling by inducing changes in membrane potential or damaging membrane integrity.

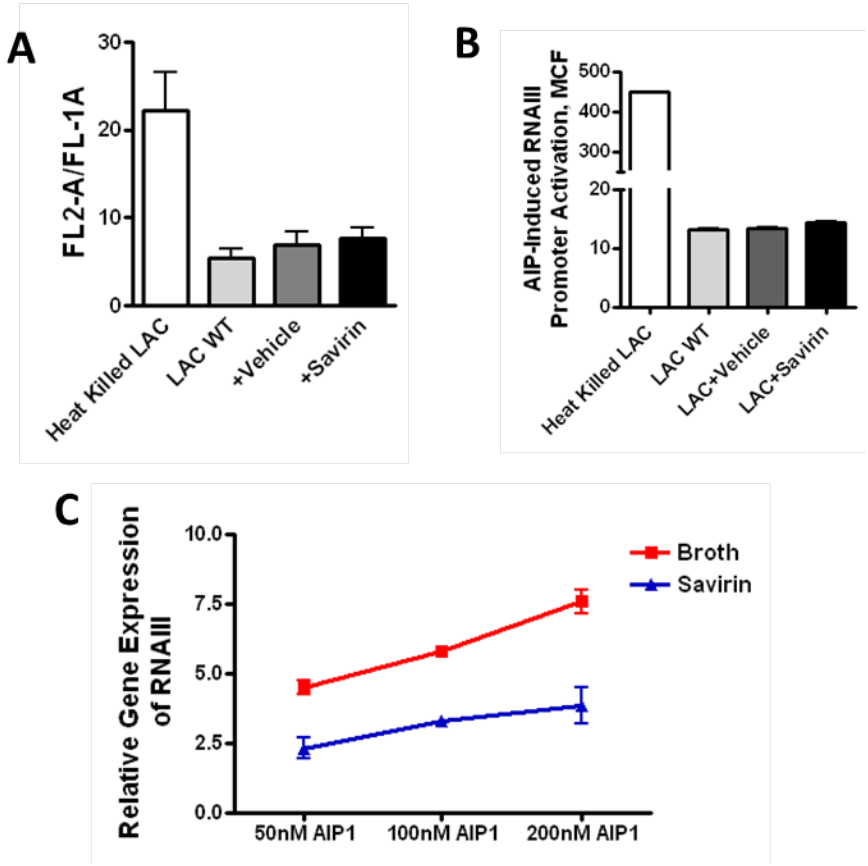


**Figure 12. Effect of savirin on bacterial membrane charge and integrity**

A) LAC USA300 was cultured 18 hr in TSB in the presence or absence of savirin (5 μg/ml). Bacteria were washed, resuspended in PBS, and equalized to $OD_{600}$ of 1.5. $DiOC_2$ was added to samples at a final concentration of 50 μM, incubated in the dark for 5 minutes, then diluted 1:6 in reaction buffer described previously. Data are presented as a ratio of the red channel value divided by the green channel value. n=4

B) LAC USA300 was cultured 18 hr in RPMI/1% CAS in the presence or absence of savirin (5 μg/ml). Bacteria were washed, resuspended in PBS/1% BSA, and equalized to $OD_{600}$ of 0.4. 50 μl of the samples were mixed with 1ml PBS/1% BSA containing 2 μg of propidium iodide. Data are represented as the mean channel fluorescence (MCF) of total *S. aureus* by by flow cytometry. n=4

C) LAC USA300 ($2 \times 10^7$/ml TSB) was incubated with 50 nM, 100 nM, or 200 nM synthetic AIP1 in the presence or absence of savirin (5 μg/ml). Expression of RNAIII determined by qRT-PCR as described previously after 1 hr of culture. n=2

## Discussion

New therapies are required for treatment and prevention of invasive *S. aureus* infections. A new therapeutic strategy is the development of drugs that target virulence factor production but not growth. In this work, we demonstrate that the small molecule, savirin, inhibits the production of *agr*-dependent virulence factors that render the bacteria less virulent. This allows the host to clear the infection by immune effectors and limits the likelihood of the bacteria developing resistance.

The data presented here directly address four criteria we had deemed essential for a successful small molecule inhibitor of *S. aureus* quorum sensing-dependent virulence. We show that savirin treatment inhibits *agr* signaling and subsequent downstream virulence factor production in an *agr*1 strain without affecting bacterial viability. We also show that savirin can inhibit *agr* signaling and virulence factor production in various *agr* backgrounds and in numerous clinical isolates, indicating that savirin may have clinical therapeutic relevance. Importantly, we demonstrate that multiple re-exposures of this pathogen to the drug either *in vivo* or *in vitro* did not induce resistance of the bacteria to subsequent savirin-mediated inhibition of *agr* signaling timepoints we investigated.

We illustrate that savirin inhibits *S. aureus* virulence *in vivo*. We show that mice treated with savirin are significantly protected from the dermonecrotic ulcers resulting from the production of the cytolytic proteins alpha hemolysin and PSM $\alpha$ in an infection with a CA-MRSA USA300 strain. Despite significant differences in abscess formation and ulcer area, there was no difference in the bacterial burden at the site of infection after 3 days suggesting that savirin is inhibiting the production of the bacterial virulence

factors that contribute to tissue injury as opposed to affecting bacterial viability. However, after the abscess and the ulcer begin to resolve, the bacterial burden in the necrotic area and spleen after 7 days is decreased in the savirin treated mice indicating that savirin enhances bacterial clearance by host innate effectors which shortens the time of infection. Because of the timing of this effect, we suspect that macrophage-dependent resolution of tissue injury is enhanced by savirin inhibition of several virulence factors that impede macrophage function including capsule production and acid resistance provided by urease and the KdpABC potassium transport operon.

Although we have yet to determine a specific mechanism of action of savirin, there are possible mechanisms that can be eliminated. We know that the actions of savirin are independent of the transcriptional regulator SigB because it inhibits *agr* signaling in SigB–deficient strains like RN6390. Also the microarray results indicate that savirin treatment does not lead to alterations in genes that are SigB dependent, including asp23 (Gertz et al., 1999). In addition, savirin treatment does not lead to the production of small colony variants (data not shown) and transcripts including hemB that are induced by compounds which lead to the development of small colony variants (Senn et al., 2005; von Eiff et al., 1997) are not up-regulated by savirin. Because savirin induced transcripts in both the *agr*+ and the Δ*agr* strains that promote toxic compound efflux, we addressed whether induction of a membrane stress response would lead to either alterations in membrane potential or in membrane integrity as demonstrated for many classical antibiotics like daptomycin (Muthaiyan et al., 2008) and membrane disrupting agents like hemin (Attia et al., 2010). However, savirin treatment did not alter either of these

properties. We speculate that savirin may be altering signal transduction leading directly to RNAIII production. The majority of transcripts altered by savirin are directly regulated by *agr*, of the 152 genes downregulated by savirin, 94 are also downregulated by deletion of the *agr* operon including transcripts for the major virulence factors. Whereas other genes not directly regulated by *agr* were significantly altered by savirin treatment, the effect was more modest and their impact on the phenotype of the pathogen is uncertain.

Targeting virulence as compared to using bactericidal agents or vaccines that induce opsonic antibodies that promote killing is a relatively new concept being applied to *S. aureus* infections (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010). While our work is the first to describe a small molecule inhibitor for targeting virulence rather than growth, this approach has been applied to experimental vaccines for prevention of *S. aureus* infection. The inhibition of a single virulence factor is protective in an *in vivo* infection where mice were immunized with a mutated form of alpha hemolysin (Bubeck Wardenburg and Schneewind, 2008). Mice that were exposed to H35L (a non-hemolytic mutant-alpha hemolysin) before being infected with a CA-MRSA strain displayed decreased mortality in a lung infection model. In addition, antibody neutralization of alpha hemolysin protects against dermonecrotic skin infection (Kennedy et al., 2010) indicating that for some kinds of tissue injury, neutralization of this single virulence factor can be protective. Savirin may have an advantage over vaccination against a single virulence factor because it targets the production of numerous *agr*-dependent virulence factors that may be essential for tissue injury at different sites of infection. Moreover, some *S. aureus* strains produce little or no alpha hemolysin like the CA-MRSA USA400 strains (Bubeck Wardenburg et al., 2007; Bubeck

Wardenburg and Schneewind, 2008). Our work demonstrates that savirin inhibits *agr* signaling in multiple *agr* types indicating potential for targeting virulence independent of the genetic background of the strain and the virulence factors used to mediate the infection.

Compounds that target virulence factor production rather than promoting bacterial death could have therapeutic potential in clinical situations where critical host defense factors are intact and reducing exposure to cidal antibiotics is desirable. For example, the majority of skin and soft tissue infections, including those caused by CA-MRSA, occur in immunocompetent individuals. In these individuals, surgical drainage alone without additional administration of antibiotics is sufficient for clearance of the infection (Stryjewski and Chambers, 2008). Therefore, topical administration of a compound like savirin could be used as an adjunct to limit tissue injury and assist in promoting host dependent clearance of the bacteria and resolution of the infection. Ideally, savirin use would speed resolution of the infection and limit the exposure of both the patient and bacteria to antibiotics. In addition, savirin administration could be particularly effective in treating CA-MRSA skin infections in patients who have conditions that lead to impaired defensins in the skin such as dermatitis. This would prevent the induction of antibiotic resistance in this pathogen and the transmission of resistant bacteria between hosts and throughout the environment (Deleo et al., 2010; Stryjewski and Chambers, 2008). Moreover, it would diminish the effect of antibiotic administration on normal microbiota that help to maintain normal host mucosal barriers for the prevention of invasive *S. aureus* infection. In contrast, infections in immunocompromised hosts with impaired host defense especially those that invade beyond the blood stream will always

require treatment with bactericidal agents. It is intriguing to speculate that compounds like savirin could complement traditional bactericidal therapy by limiting direct tissue injury caused by secreted virulence factors. However, further work is required to determine if savirin would have efficacy in experimental models of these clinical settings.

# CHAPTER 4

# RE-PURPOSING A GOUT DRUG, BENZBROMARONE, TO TARGET *STAPHYLOCOCCUS AUREUS* VIRULENCE AND GOLDEN PIGMENT PRODUCTION

## Introduction

The rise of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections, particularly those caused by a single clone (PFGE type USA300), coupled with the slowing of antibiotic discovery makes research into novel therapies a priority (Lowy, 2007). One strategy emerging is to develop drugs that target bacterial virulence factors as these are largely responsible for tissue injury and resultant morbidity and often work by inhibiting host immune defense (Cegelski et al., 2008). The inhibition of virulence factors would attenuate the infection and thus allow the bacteria to be cleared by the innate immune factors of the host. Moreover, re-purposing existing drugs to target bacterial virulence would additionally speed the pace of drug discovery as safety in human hosts has been established. The virulence factors identified to date as essential for CA-MRSA infection are globally regulated in part by the *agr* quorum sensing operon (Novick and Geisinger, 2008; Yarwood and Schlievert, 2003). Our work identifying apolipoprotein B as an innate barrier that antagonizes *agr* signaling demonstrates that host defense against CA-MRSA infection could be accomplished by blocking *agr* signaling (Peterson et al., 2008). We hypothesized that screening libraries of existing drugs for inhibition of *agr* signaling could identify compounds capable of re-purposing for targeting *S. aureus* virulence.

Our work in this chapter focuses on the use of a small molecule inhibitor, benzbromarone, identified in a screen of the Prestwick library of off-patent drugs for antagonism of *agr*. Benzbromarone, a drug formerly used to treat gout, inhibits both *agr*-mediated virulence factor regulation *in vitro* and ameliorates a murine model of dermonecrotic infection *in vivo*. Bacteria exposed to benzbromarone were more

susceptible to killing by several, but not all, innate immune host factors. In addition, benzbromarone inhibits bacterial viability over a 20 hr growth curve and also inhibits staphyloxanthin pigment production and autolysis, two *agr*-independent processes. These data indicate that while benzbromarone does inhibit *agr*-dependent virulence, it also has more global effects on the bacteria than originally hypothesized.

## Results

### *Benzbromarone inhibits agr signaling and subsequent virulence factor production*

Benzbromarone (Figure 1A) was one of ten compounds with an $IC_{50}$<1μM identified in a high throughput screening assay that examined compounds for possible antagonism of *agr* performed by the Molecular Libraries *Screening* Centers Network (MLSCN) (University of New Mexico) as well as in a screen of the Prestwick library of off patent drugs. We used a reporter strain where activation of the *agr*::P3 promoter drives expression of GFP as a preliminary test to determine whether the small molecule benzbromarone could inhibit *S. aureus agr* signaling. As shown in Figure 1B, benzbromarone inhibited AIP-dependent *agr* signaling in a 2 hr assay while the vehicle alone used to solubilize the drug had no effect. We measured the viability of the bacteria incubated with benzbromarone during both exponential and stationary phase growth. We cultured the CA-MRSA strain LAC USA300 in TSB in the presence or absence of benzbromarone (5 μg/ml and 10 μg/ml) and viability was evaluated by enumerating viable colony forming units (CFU). As seen in Figure 1C, over the course of 20 hr, benzbromarone did not affect exponential growth but significantly inhibited stationary phase growth.

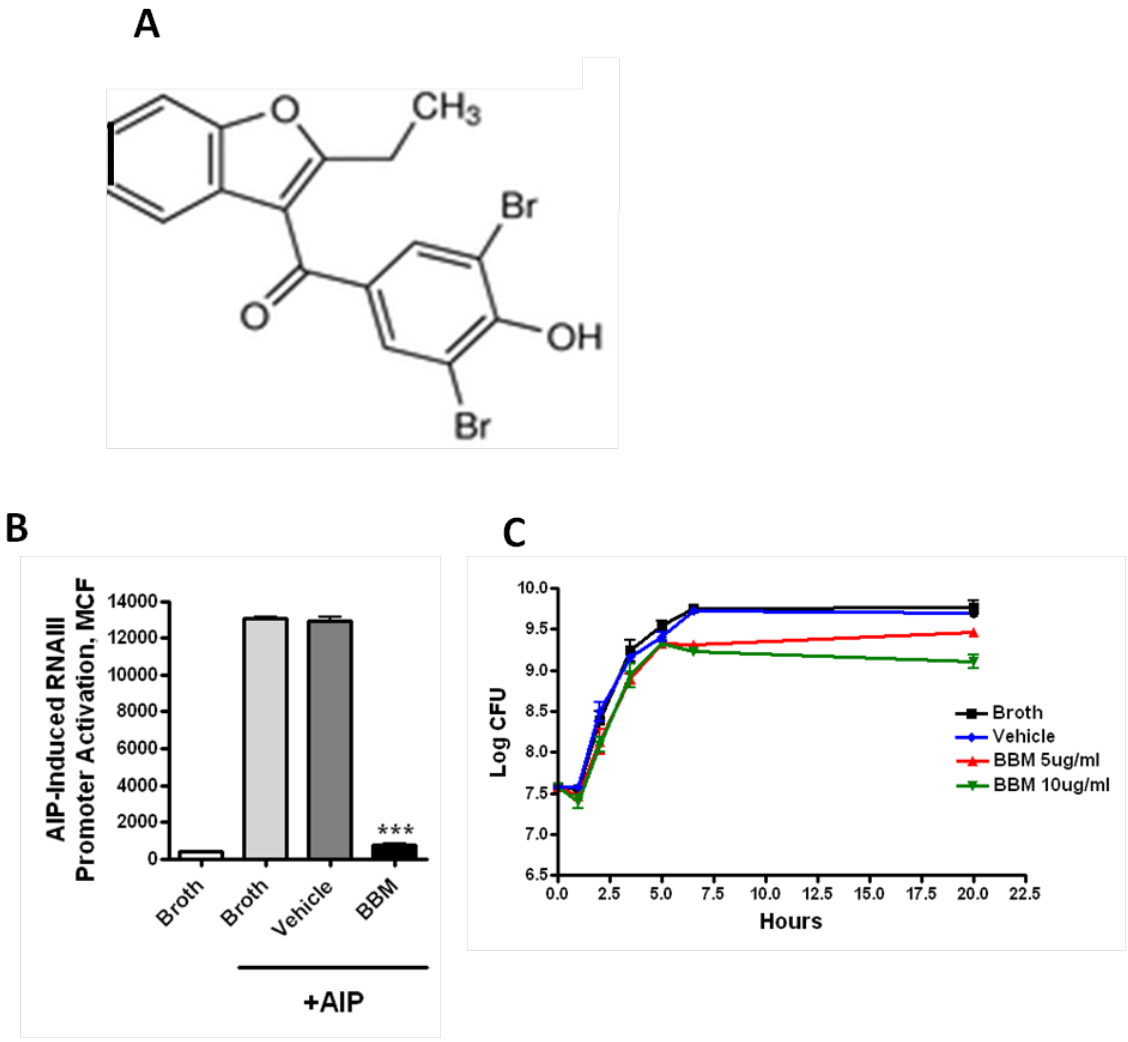


**Figure 1. Benzbromarone antagonizes *agr* signaling and inhibits stationary phase growth but not exponential growth**

(A) Chemical Structure of benzbromarone (CID# 2333) 1 of 10 compounds identified with an $IC_{50}$<1μM in a MLSCN screen of over 24,000 compounds

(B) Newman (2 x $10^7$) was incubated with 50nM synthetic AIP1 in the presence or absence of benzbromarone (5 μg/ml). After washing, fixing, and sonicating, the bacteria were evaluated for GFP expression by flow cytometry. Data are represented as the mean channel fluorescence (MCF) and as the mean ± SEM; ***p<0.0001, n=3

(C) LAC USA300 (2 x $10^7$/ml TSB) was incubated in the presence or absence of benzbromarone (5 μg/ml or 10 μg/ml) or vehicle control. Growth was determined by plating out dilutions of the cultures on sheep blood agar and enumerating colony forming units. Data are represented as mean ± SEM; n=3

In addition, benzbromarone inhibited stationary phase growth of an Δ*agr* mutant of LAC indicating that its effect on stationary phase metabolism was independent of *agr* signaling.

Based on the results of the fluorescent-based reporter assay performed with the lab strain Newman, we asked whether benzbromarone had a similar inhibitory effect on quorum sensing in a more clinically relevant CA-MRSA strain. To address this, we used qRT-PCR to measure RNAIII expression in the *agr*1 strain, USA300 LAC. The assay was performed both by adding exogenous synthetically derived AIP and by allowing the bacteria to create its own AIP endogenously. Early exponential phase USA300 LAC were grown for 2 hr (with exogenous AIP1) or 5 hr (endogenous production of AIP1) at 37$^{o}$C in the presence of absence of benzbromarone or vehicle control added at an equal volume as the drug. As shown in Figure 2A, RNAIII expression was significantly inhibited by the addition of benzbromarone while no inhibition was seen with the vehicle control. Figure 2B shows a similar effect in the 5 hr assay where the AIP production was endogenous.

We then assessed the ability of benzbromarone to inhibit virulence factors dependent on AIP-induced quorum sensing. We postulated that since the drug was able to inhibit RNAIII production, benzbromarone would be able to inhibit the production of virulence factors dependent on RNAIII. We first used qRT-PCR to evaluate the ability of benzbromarone to inhibit expression of *psmα*, a gene target of activated AgrA (Queck et al., 2008). As hypothesized, benzbromarone was able to inhibit *psmα* gene expression in a 2 hr assay with the addition of exogenous AIP (Figure 2C). A similar procedure was used to measure gene expression of PVL, Panton-Valentine Leukocidin, which lies

downstream of RNAIII, in response to benzbromarone treatment. As expected, gene expression of PVL was also significantly inhibited due to drug treatment as compared to treatment with the vehicle control (Figure 2D). Next, we measured the ability of benzbromarone to inhibit the production of the alpha-hemolysin toxin produced by the USA300 strain LAC. CA-MRSA USA300 strains make substantial amounts of alpha-hemolysin and it is a major virulence factors for these strains (Deleo et al., 2010). Benzbromarone inhibited the production of alpha-hemolysin to an undetectable level (Figure 2E). These data suggest that benzbromarone is able to inhibit RNAIII activation and the subsequent production of the downstream virulence factors of the *S. aureus* LAC strain *in vitro*.

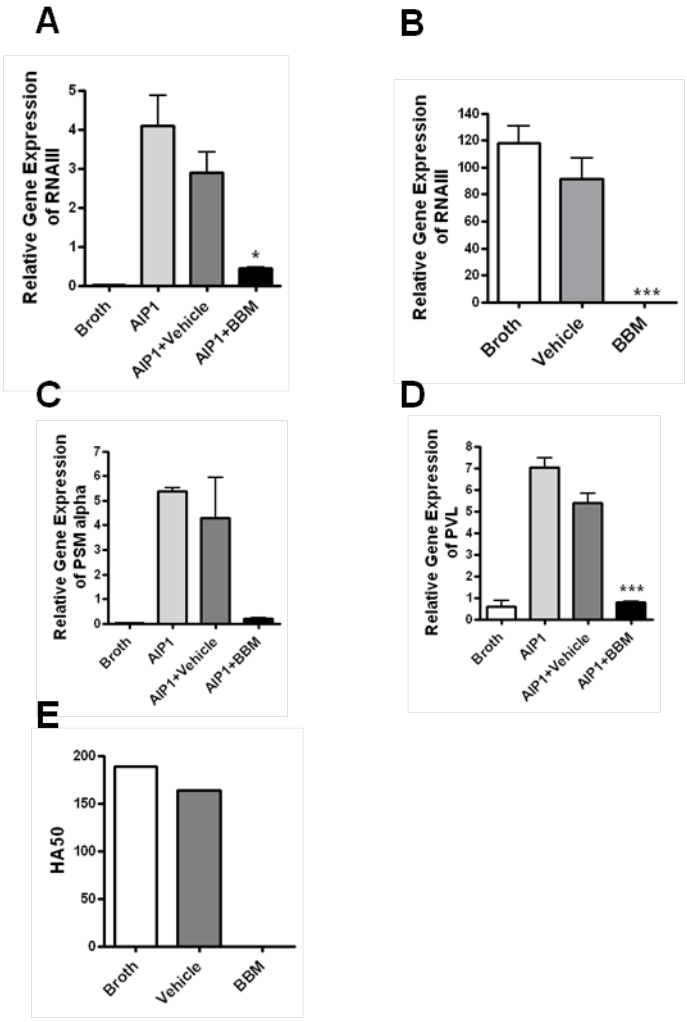


**Figure 2. Benzbromarone inhibits *agr*-dependent virulence factor production**

A.C.D) LAC USA300 (2 x $10^7$/ml TSB) was incubated with 50 nM synthetic AIP1 in the presence or absence of benzbromarone (5 μg/ml). RNA was extracted, purified, reverse transcribed to cDNA, and then gene expression relative to endogenous control 16s was determined using a probe for either RNAIII after 1 hr of culture (A) n=3, PSM alpha after 2 hr of culture (B) n=2, or PVL after 2 hr of culture (C) n=3. *p<0.01, ***p<0.0005

(B) LAC USA300 (2 x $10^7$/ml TSB) was incubated without synthetic AIP1 in the presence or absence of benzbromarone (5 μg/ml). Expression of targets determined by qRT-PCR as described above for RNAIII after 5 hr. ***p=0.0009, n=4

E) Effect of benzbromarone (5 μg/ml) added to an overnight culture of LAC in which alpha-hemolysin in the supernatant was measured by lysis of rabbit erythrocytes

Data are represented as mean ± SEM

*Benzbromarone affects metabolism in both LAC USA300 agr+ and Δagr*

Staphyloxanthin is a carotenoid pigment that gives *S. aureus* its hallmark gold color. It is a product of carotenoid biosynthesis pathway and is the end product of multiple dehydrogenase reactions. Staphyloxanthin plays a role in the protection of the bacteria from reactive oxygen species and a carotenoid-deficient mutant strain exhibited decreased survival in a neutrophil killing assay in (Liu et al., 2005). Because benzbromarone inhibited stationary phase growth and staphyloxanthin is produced in late stationary phase, we determined the effect of benzbromarone on staphyloxanthin production. We documented the production of staphyloxanthin over time by photographs and quantified the amount of pigment by methanol extraction in LAC USA300 (Figure 3A) and LAC USA300 Δ*agr* mutant (Figure 3B). The pictures of Figure 3A-B illustrate that as staphyloxanthin was produced, the addition of benzbromarone in the culture inhibited the production of the pigment in both the LAC wild type and the Δ*agr* mutant strain. This was confirmed by quantification of the pigment. These data indicate that staphyloxanthin production is independent of *agr* activation and that benzbromarone has broader effects on *S. aureus* than suppression of *agr*-dependent genes.

Autolysis is an integral part of the bacterial life cycle and, like staphyloxanthin production, is also *agr*-independent. Autolysis has been proposed in bacteria to fulfill the same function as programmed cell death observed in eukaryotic cells, that is to eliminate damaged cells preventing these bacteria from becoming a burden to the population (Bayles, 2007). Upon lysis, the dead bacteria release genomic DNA, termed eDNA (extracellular DNA). This eDNA is an essential matrix molecule that provides

intracellular adhesion and biofilm stability (Bayles, 2007; Rice and Bayles, 2008; Sharma-Kuinkel et al., 2009). We examined the effect of benzbromarone on the physiologic process of autolysis using the procedure described (Sieradzki and Tomasz, 1997). We predicted since benzbromarone had an effect on staphyloxanthin production, that it might also affect another *agr*-independent process that occurs late in stationary phase growth. Specifically, we cultured LAC or Δ*agr* mutant for 18 hr in the presence or absence of benzbromarone before washing and resuspending in the autolysis buffer (50mM glycine supplemented with Triton X-100). As shown in Figure 3C and 3D, the addition of benzbromarone decreased the rate of autolysis over the course of a 28 hr assay in both the LAC wild type and the Δ*agr* mutant. These data suggest that benzbromarone prevents the bacterial cells from lysing and the resulting production of eDNA. If eDNA production is limited, bacterial biofilm stability may be reduced limiting the tolerance of this pathogen to commonly used antibiotics (Lewis, 2001).

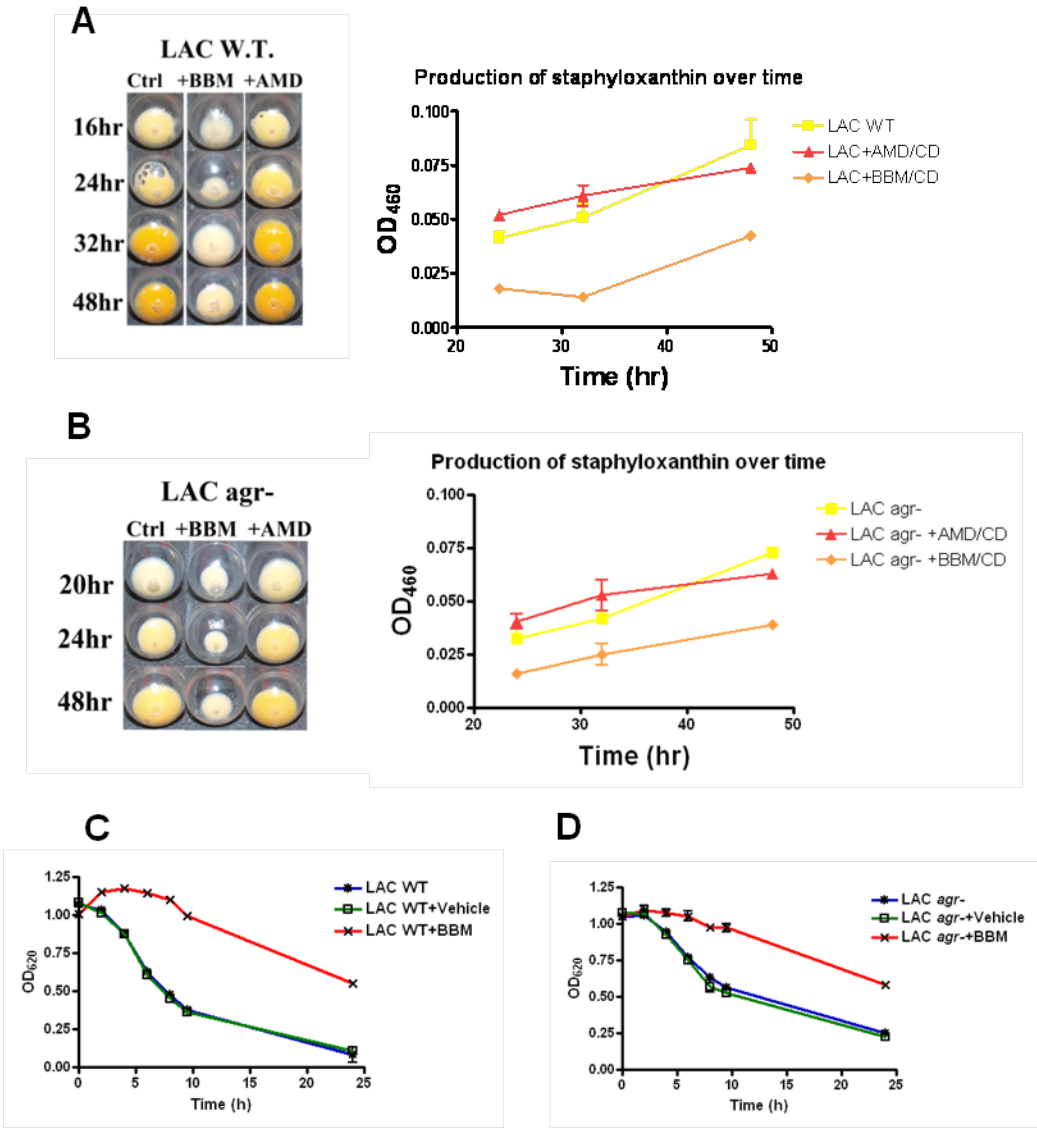


**Figure 3. Effect of benzbromarone on staphyloxanthin production and bacterial autolysis in LAC USA300 and LAC Δ*agr* mutant**

A.B) LAC USA300 (A) or LAC USA300 Δ*agr* mutant (B) was incubated in TSB in the presence or absence of benzbromarone (5 μg/ml) and staphyloxanthin production was determined at the indicated time by removing aliquots of the culture, equalizing the samples to an $OD_{600}$, extracting the pigment with methanol, and determining the optical density of the sample by spectrophotometer at a wavelength of 460nm.

C.D) LAC USA300 (C) or LAC USA300 Δ*agr* mutant (D) were cultured for 18 hr in the presence or absence of benzbromarone (5 μg/ml). Bacteria were then chilled, washed, and equalized to $OD_{620}$ of 1.0 in 50mM glycine/0.01% Triton X-100. Cultures were incubated at 37°C, aliquots were removed at certain timepoints, and autolysis was measured as a decrease in $OD_{620}$.

*Benzbromarone ameliorates an agr-dependent dermonecrotic S. aureus infection*

Skin and soft-tissue infections predominate as the most frequent forms of disease caused by CA-MRSA which vary in severity from painless to life-threatening with abscesses being the most common presentations, affecting 50% to 75% of patients with CA-MRSA (Stryjewski and Chambers, 2008). Both PSMα and alpha hemolysin are integral factors in causing the tissue destruction of these skin and soft tissue infections (Li et al., 2009). Because we have shown that benzbromarone inhibits the production of these elements *in vitro,* we predicted that it would inhibit these factors *in vivo* as well and be protective in a dermonecrotic mouse model of infection.

To address whether benzbromarone administration would be protective in an invasive *S. aureus* infection, we used a mouse model of dermonecrosis (Wang et al., 2007). Early exponential phase bacteria were injected subcutaneously into the flank of SKH1-E mice with the addition of either benzbromarone or vehicle alone. The infections were monitored for two days and abscess volume, ulcer area, and weight loss recorded. At the termination of the experiment, the ulcerated areas were excised and homogenized to determine the number of bacteria in the area. As shown in Figure 4A and 4B, infection of the mice with LAC in the presence of benzbromarone resulted in smaller abscesses and ulcer size as compared to those given vehicle while the LAC Δ*agr* bacteria were unable to produce a necrotic ulcer. These data show that benzbromarone is protective in a dermonecrotic infection with LAC USA300 and suggests that benzbromarone may be able to be utilized as a therapeutic. Next, in an effort to make the experiment more clinically relevant, we tested whether benzbromarone administration could protect against

dermonecrosis from other *agr* types *in vivo*. We infected mice as before using *agr*2 and *agr*3 strains. Our results show that benzbromarone exhibited protection in an *agr2* (502A strain) (Figure 4C) and an *agr3* (1560 strain) (Figure 4D) *S. aureus* infection. These data indicate that benzbromarone is protective in an *in vivo* dermonecrotic infection and may be a useful therapeutic against skin and soft tissue infections caused by CA-MRSA.

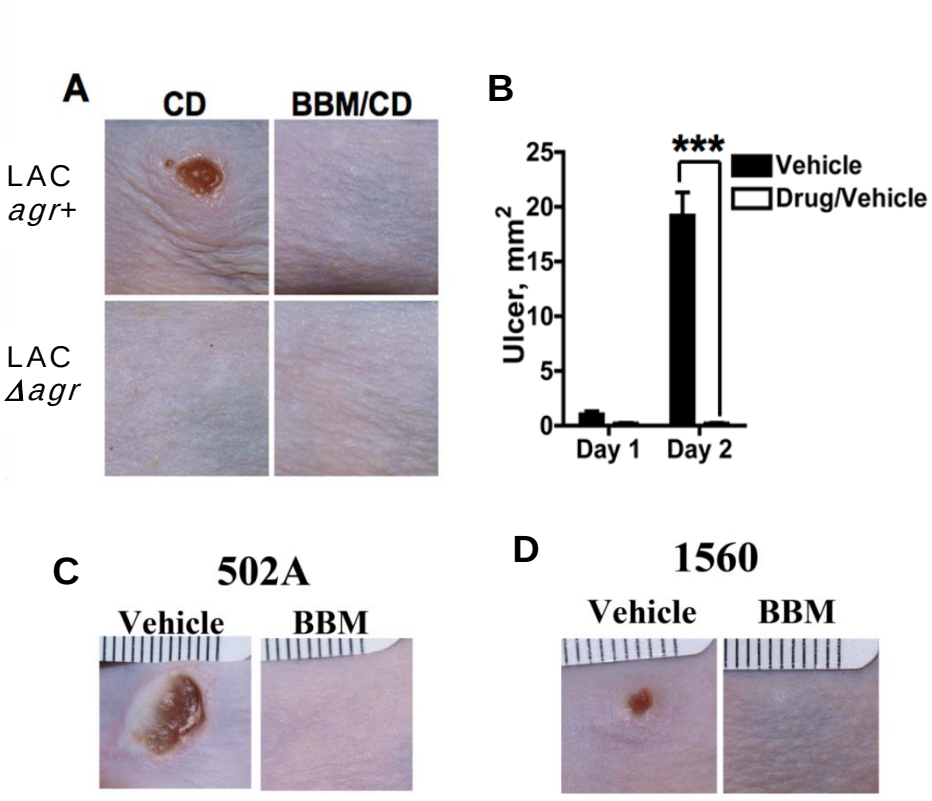


**Figure 4. Effect of benzbromarone on an invasive dermonecrotic *S. aureus* infection of multiple *agr* types**

A) LAC USA300 WT or LAC USA300 Δ*agr* mutant (2 x $10^7$/ml saline at early exponential phase) were injected subcutaneously on the flank (n=8 mice for each group) with either 5 μg benzbromarone or equal volume of vehicle. Pictures of the injection sites were taken at two days

B) Measurements of dermonecrosis (ulcer area) were taken on Day 2 of the infection. ***p=0.0005, Data represented as the mean ± SEM

C.D) 502A (C) or 1560 (D) (2 x $10^7$/ml saline at early exponential phase) were injected subcutaneously on the flank (n=4 mice for each group) with either 5 μg benzbromarone or equal volume of vehicle. Pictures of the injection sites were taken at two days.

*Benzbromarone treatment results in increased susceptibility of the bacteria to killing by some, but not all, innate immune host factors*

Targeting virulence using benzbromarone should attenuate the bacteria thus allowing the host innate immune system to clear the bacteria. We postulated that to be an effective therapeutic, benzbromarone treatment would enhance bacterial killing by the host innate immune system, including low pH. Acidic conditions exist in niches like the skin as well as phagolysosome of macrophages to combat pathogens. *S. aureus* expresses several factors to resist these acidic environments. We used *in vitro* tests with acidic buffer to replicate the host innate effector to determine if exposure to benzbromarone made the bacteria more susceptible to killing by acid. Following treatment with benzbromarone or vehicle, the bacteria were incubated in PBS (pH 2.5) for 2 hr. As shown in Figure 5A and 5B, both the wild type bacteria and the Δ*agr* strain were significantly more susceptible to killing by acid following treatment by benzbromarone (Figure 5A). These data indicate that benzbromarone impairs an acid resistance mechanism that is independent of *agr* signaling.

Because benzbromarone inhibited pigment production and staphyloxanthin provides defense against reactive oxygen intermediates (Liu et al., 2005), we predicted that benzbromarone treatment would make the bacteria more susceptible to killing by hydrogen peroxide. LAC USA300 was incubated for 30 hr in the presence or absence of benzbromarone and then subjected to hydrogen peroxide at final concentrations of 1.5% and 3.0% for 2 hr. As shown in Figure 5C, the wild type bacteria that had been cultured in the presence of benzbromarone had significantly more killing than bacteria alone at

two different percentages of hydrogen peroxide as compared to the control indicating that benzbromarone does confer increased susceptibility to at least one oxidant.

Linoleic acid is a free fatty acid that is bacterial lytic lipid constituent of the skin and provides host defense at mucosal barriers (Kenny et al., 2009). LAC USA300 was incubated for 18 hr in the presence or absence of benzbromarone after which were challenged with increasing concentrations of linoleic acid and incubated an additional 18 hr. Shown in Figure 5D, benzbromarone treatment had no effect on the survival of the bacteria when incubated with linoleic acid. These data suggest that exposure to benzbromarone makes the bacteria more susceptible to killing by some but not all immune effectors we tested.

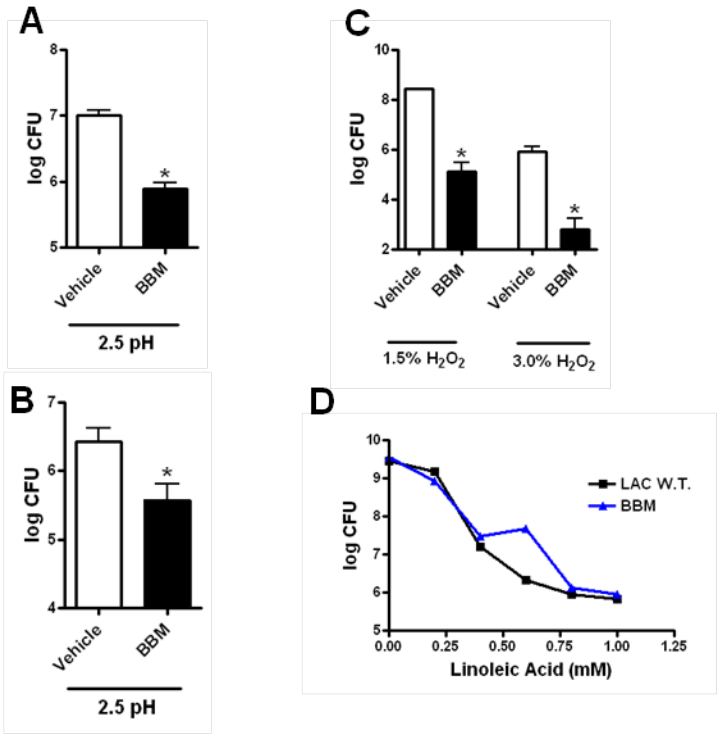


**Figure 5. Treatment of LAC USA300 with benzbromarone results in increased killing by some, but not all, tested host immune factors**

A.B) LAC USA300 or LAC USA300 Δ*agr* mutant (1 x $10^9$/ml TSB) were incubated for 1.5hr in the presence or absence of benzbromarone (5 μg/ml). Bacteria were centrifuged and resuspended in PBS, pH 2.5 for 2hr. Dilutions were plated on sheep blood agar and viability determined by enumerating colony forming units. *p<0.1, n=6, Data represented as the mean ± SEM.

C) Early exponential phase LAC USA300 was incubated in TSB for 30 hr in the presence or absence of benzbromarone (5 μg/ml). Bacteria were centrifuged, washed twice, and resuspended in PBS to an $OD_{600}$ of 0.6. $H_2O_2$ was added to a final concentration of 1.5% or 3% and incubated for 2 hr. Catalase was added (1000 U/ml) to quench the reaction. Dilutions were plated on sheep blood agar and viability determined by enumerating colony forming units. *p<0.1, n=6.

D) Early exponential phase LAC USA300 were incubated in TSB for 18 hr in the presence or absence of benzbromarone (5 μg/ml). Bacteria were centrifuged, washed twice, and resuspended in TSB to an $OD_{600}$ of 0.3. Linoleic acid was added to 1ml volumes of the cultures to final concentrations ranging from 0.2 mM to 1.0mM. Bacteria with linoleic acid were incubated an additional 18 hr then sonicated and dilutions were plated on blood agar and surviving bacteria were determined by enumerating colony forming units. n=3.

## Discussion

CA-MRSA is resistant to most commonly used antibiotics and also has mechanisms to escape host immune capture and killing (Graves et al., 2010). The emergence of hypervirulent, antibiotic-resistant strains emphasizes the need to develop effective therapeutics to which the bacteria will develop limited resistance to. One strategy illustrated here and by others is the targeting of bacterial virulence factors which contribute to disease.(Alksne and Dunman, 2008; Garcia-Lara et al., 2005a). Host defense mechanisms inhibit quorum sensing dependent virulence factor production such as hemoglobin (Schlievert et al., 2007), neutrophils (Rothfork et al., 2004), and apolipoprotein B (Peterson et al., 2008) and therefore providing a therapeutic that targets virulence regulation during some types of *S. aureus* infections is one mechanism for re-storing host defense. Here we have shown results which indicate that bacterial virulence production can be inhibited by the small molecule, benzbromarone. Moreover, because benzbromarone is an off patent drug formerly used for the treatment of gout, re-purposing it to target *S. aureus* virulence is yet another mechanism for speeding drug discovery.

The data presented here show the ability of a compound, benzbromarone, to target the production of *agr*-dependent virulence factors. Our results show that it is capable of inhibiting RNAIII production and subsequent downstream virulence factor production seen at both the transcript and protein level. We confirm in our *in vivo* dermonecrosis model of infection that *agr*-dependent quorum sensing is required for the formation of dermonecrotic ulcer, an observation made by others (Wang et al., 2007) and we also illustrate that benzbromarone is protective in an infection with the CA-MRSA strain LAC USA300. This *in vivo* protection also extends to other *agr* backgrounds. While

administration at the time of infection demonstrates efficacy, whether it can be given later during the course of the infection is yet to be determined. Our data indicate that ongoing quorum sensing is required to achieve maximal necrosis and therefore we would predict that administration of benzbromarone after the initiation of the infection should be efficacious.

In addition to its effect on *agr* signaling, benzbromarone inhibited stationary phase growth but not exponential phase growth of both *agr*+ and Δ*agr* strains indicating that it has more broad effects on metabolism. However, this pattern of growth inhibition is unique and true antibiotics that target metabolic pathways like protein synthesis, cell wall production, or nucleotide synthesis inhibit exponential phase growth. While unexpected, stationary phase growth inhibition could have a therapeutic benefit for biofilm infections or stages of infection where stationary phase growth is achieved. Consistent with this late stage effect on metabolism, benzbromrone also inhibits production of staphyloxanthin, a carotenoid pigment used by the bacteria as protection against oxidants that is optimally produced during late stationary phase. Because cholesterol lowering drugs that inhibit staphyloxanthin production have therapeutic benefit against experimental *S. aureus* infection (Liu et al., 2008), one mechanism by which benzbromarone could provide a therapeutic benefit *in vivo* is through staphyloxanthin inhibition.

The mechanism of action of benzbromarone alters signaling and metabolic pathways in the bacteria that make them more susceptible to killing by some but not all host immune effectors. Because an increased susceptibility to acid killing was observed

at a time prior to growth or staphyloxanthin inhibition, and for both *agr*+ and Δ*agr* strains, we suspect that this involves overcoming both *agr*-dependent and *agr*-independent gene regulation. Gene products that are suspected to provide defense against acid include urease-mediated production of ammonia, the function of the osmotic sensor and potassium transporter *KdpABC,* and arginine deiminase production of ammonia. Microarray analysis comparing LAC USA300 wild type to its *agr* deletion mutant demonstrated that both the urease and KdpABC operons are *agr* regulated while arginine deiminase is not. Thus, the use of benzbromarone as an adjunct for treating skin infection could assist in acid killing at mucosal barriers and within phagolysosomes. In addition to increased susceptibility to acid killing, benzbromarone also increased susceptibility to hydrogen peroxide killing. Because this increased susceptibility occurred only at a time by which staphyloxanthin is inhibited, we suspect that this increased killing is mediated by a failure of the bacteria to scavenge the reactive oxidants permitting their action on critical pathways like DNA synthesis.

Due to its more global effects on processes such as stationary phase growth, staphyloxanthin production, and autolysis, benzbromarone is not sufficiently selective for *agr*-dependent virulence factors to function exclusively as an anti-virulence compound. The inhibitory actions of benzbromarone on growth and metabolic pathways suggest that bacterial resistance to its effects could be developed. However, even with this limitation, the mechanism by which it selectively inhibits stationary phase growth could have therapeutic benefits in particular infections where that pattern of growth predominates, such as in biofilm infections.

# CHAPTER 5
# DISCUSSION AND CONCLUSIONS

## Summary and Future Directions

Mammalian hosts have evolved numerous mechanisms that target and inhibit *S. aureus* quorum sensing-dependent virulence. Upon infection, neutrophils are recruited to the site where the reactive oxygen species they create inactivate the peptide pheromones that drive *agr* signaling (Rothfork et al., 2004), host hemoglobin inhibits the secretion of the peptide pheromone through AgrB (Attia et al., 2010; Schlievert et al., 2007), and apolipoprotein B of LDL and VLDL particles binds to and sequesters the pheromone, inhibiting the signaling of AgrC. The ability of the chemical compounds described here to antagonize quorum sensing could act as additional inhibitors to pheromone signaling and virulence factor production that could attenuate the infection and thus facilitate clearance by the immune system of the host.

The work presented here describes a role for small molecular compounds as inhibitors of *S. aureus* virulence. Although anti-virulence compounds have been developed for Gram-negative infections (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010), this is the first description of small molecule inhibitors utilized in a Gram-positive organism to target quorum sensing-dependent virulence both *in vitro* and *in vivo*. The compound savirin met all of the necessary criteria we established to be a potential relevant therapeutic for the treatment of CA-MRSA infections. Savirin inhibits bacterial virulence without affecting viability, works in multiple *agr* backgrounds, does not induce resistance to savirin during the time frame we utilized, and enhances the susceptibility of the bacteria to killing by low pH. In contrast, benzbromarone, which has the advantage of already being an FDA approved drug,

appears to have a much more global effect on *S. aureus*, affecting not only RNAIII expression and downstream virulence factors, but also staphyloxanthin production, autolysis, and stationary phase growth. While these actions of benzbromarone on metabolism components of *S. aureus* may create a selective pressure and lead to the loss of sensitivity of the bacteria to the drug, these additional effects could also be advantageous. For example, conventional antibiotics target exponential growth. The ability of benzbromarone to inhibit stationary phase growth could have utility during infections where the bacteria have reached stationary phase and are relatively resistant to killing by conventional antibiotics.

Other known antagonists of *S. aureus* virulence include competing AIPs (Lyon et al., 2002; Wright et al., 2005) and *Lactobacillus reuteri*-produced cyclic dipeptides (Li et al., 2011). Type 2 AIPs exhibited inhibition of quorum sensing in an *agr*1 infection by binding to the AgrC receptor, blocking the binding of the type 1 AIPs being released by the bacteria. This binding of AIP2 blocked signaling through AgrC and inhibited the expression of RNAIII and the production of virulence factors. In addition, administration of AIP2 prior to an *agr*1 experimental skin infection conferred protection in an animal model (Wright et al., 2005). The cyclic dipeptides produced from *Lactobacillus reuteri* inhibit *agr* dependent-quorum sensing and toxin production *in vitro* but whether this would provide protection *in vivo* has not been addressed (Li et al., 2011). Intriguingly, these peptides also inhibit signaling through other sensor-regulators used by *S. aureus* to regulate gene expression in addition to *agr*. Thus, these could have more broad effects on global gene regulation and could represent a mechanism by which normal microbiota provide defense against invasive *S. aureus* infection. Another mechanism for targeting

virulence is vaccination with modified virulence factors that induce neutralizing antibodies but are not themselves capable of significant tissue injury. The inhibition of a single virulence factor provided protection in an *in vivo* infection where mice were immunized with a mutated form of alpha hemolysin (Bubeck Wardenburg and Schneewind, 2008). Mice that were vaccinated with a variant of alpha hemolysin at a single amino acid site (H35L) before being infected with a CA-MRSA strain had significantly decreased mortality in a lung infection model. Moreover, antibody neutralization of alpha hemolysin was sufficient to protect mice from an experimental dermonecrotic skin infection (Kennedy et al., 2010). In comparison to these approaches, the use of a compound like savirin has several advantages. First, as a non-peptide antagonist it would have greater stability in blood and tissues where proteases can actively cleave peptides. Secondly, it targets the production of numerous *agr*-dependent virulence factors as opposed to only one and there are numerous clinical isolates that produce minimal or no alpha hemolysin but still rely on *agr* signaling to regulate virulence.

The microarray analysis of stationary phase, savirin-treated *S. aureus* demonstrated the extent of the specificity of the savirin to *agr* signaling. The analysis of the actual genes altered revealed that 94 open reading frames significantly down-regulated by savirin in the *agr*+ strain were also down regulated in the absence of *agr*, including all of the major virulence factors regulated by *agr*. This shows great selectivity of the small molecule to the targeting of *agr*-dependent genes. To look at the effect of savirin from a more global view, we looked at the savirin-altered genes in relation to the entire *S. aureus* genome. The USA300 genome has a total of 2770 open reading frames

and savirin affects 211 of them. These data show that the effect of savirin is relatively minor, leaving almost 93% of the open reading frames of the bacteria unaffected. When *S. aureus* strain ATCC 29213 was exposed to daptomycin, the antibiotic effected the transcription of 869 open reading frames, over four times the number of open reading frames affected by savirin (Muthaiyan et al., 2008). This study, as well as others, demonstrate that conventional antibiotics used at sub-inhibitory concentrations alter gene transcription in a much more global fashion.

Several aspects of the action of both small molecules described here remain to be elucidated, most importantly being a precise mechanism of action. From the microarray analysis, we postulate that savirin passes through the bacterial membrane becoming intracellular due to the creation of a membrane stress response, seen as an up-regulation of efflux pumps and transporter genes. This stress response is characteristic of the actions of traditional antibiotics which alter either the membrane potential or membrane integrity. However, the stress response induced by savirin was not sufficient to alter either one of these membrane events. We believe the up-regulation is in response to the bacteria sensing something foreign or toxic and attempting to transport savirin out of the cell. This may be due to the quinolone structure that comprises a portion of the savirin molecule. Fluoroquinolones, a family of broad-spectrum antibiotics, are the antibiotics most associated with the up-regulation of these efflux pumps. These drugs act by interfering with DNA replication and transcription and they share a quinolone ring system (Kleinkauf et al., 2001), which is also seen in savirin. Thus, we postulate that the quinolone ring structure of savirin is responsible for the up-regulation of the efflux pump but in the absence of a required halide, it is not sufficient for targeting DNA replication.

Similar to the results of our microarrarray analysis, others have shown similar up-regulation of membrane transport genes upon antibiotic exposure. For example, upon treatment with berberine chloride, a protoberberine alkaloid with antimicrobial activity, a large number (98) of putative transporter genes were differentially regulated and transcripts for several multidrug resistance transporters were up-regulated 10-20 fold (Wang et al., 2008). In contrast, while savirin up-regulated transcripts involved in membrane transport, the fold increase and the number of genes up-regulated were modest compared to the actions of conventional antimicrobials. Due to the selectivity of the effects of savirin on *agr*-dependent genes, we postulate that savirin is having a direct interaction with *agr*, possibly by binding to a RNA polymerase directly preceding the P3 promoter.

Another aspect of savirin treatment that requires more investigation is the effect the molecule has on biofilm formation. From the microarray analysis, the up-regulation of adhesive factors such as *SpA* leads us to predict that savirin treatment may result in a phenotypic shift towards biofilm formation. However, a potassium-specific transport system that is up-regulated in biofilms is inhibited upon exposure to savirin. Three genes of the *kdp* operon, *kdpABC* are induced in biofilms as compared to planktonic growth conditions (Beenken et al., 2004). The *kdp* operon contributes to pH homeostasis through exchange of K+ to H+ which is accomplished by kdpD, a membrane spanning sensor kinase that responds to osmotic pressure, membrane stretch, or potassium level, and thus transphosphorylating KdpE, a cytosolic transcriptional activator, which then up-regulates transcription of the *kdpFABC* operon. Our microarray analysis shows that *kdpDE* is also down-regulated in response to savirin while others have shown the two genes to be up-

regulated in biofilms compared to the exponential growth phase (Beenken et al., 2004). Two other significant biofilm-related genes affected by savirin encode surfactant like proteins and proteases. Production of these components is essential for the release of clumps of biofilm and dispersal of the bacteria (Boles and Horswill, 2008; Wang et al., 2011). If savirin exposure did lead to biofilm production, the dispersal of clumps of the biofilm would most likely be inhibited. An animal model that could be useful to determine the effect of savirin on biofilm formation is an air pouch model of *S. aureus* infection (Peterson et al., 2008). The epithelial lining of the pouch could be excised and treated with an anti-staphylococcal antibody so bacteria and biofilm production could be visualized and quantified using confocal microscopy. In this manner, whether savirin administration enhances biofilm growth on the damaged tissue could be determined.

Another result from the microarray analysis that should be examined is the contribution that the alteration of TCA genes contributes to the mechanism of action of savirin. Citrate synthase and acetyl coenzyme A are two genes of the TCA cycle that are down-regulated by savirin. This suppression may cause concern since affecting bacterial metabolism may lead to the development of resistance however the effect is a relatively modest 2-3-fold inhibition. The TCA cycle is one of three metabolic pathways of *S. aureus* and is largely repressed in nutrient abundant media (Somerville and Proctor, 2009). This could explain why we do not see inhibited growth in our *in vitro* assays that are performed in trypticase soy broth. It is unclear thus far whether these effects on the TCA cycle are linked to *agr* signaling or if they are a separate effect. If the alterations of the TCA cycle are a not an essential component of the mechanism for inhibition of *agr* signaling, then even if the bacteria develop resistance to the inhibitory actions on the

metabolic pathways, the bacteria will still remain sensitive to the anti-virulence effects of savirin and the infection will still be attenuated upon administration of the small molecule.

Therapeutic application of savirin is an area with many avenues for investigation as well. An obvious question is whether the anti-virulence actions of savirin are specific to *S. aureus* or if savirin could be a useful therapeutic for other Gram-positive infections. A related pathogen we would investigate first would be *S. epidermis*. Since savirin appears to act on *agr* signaling, we would predict that savirin would have an equally inhibitory effect on *S. epidermis* given that this bacteria also has an *agr* operon (Otto, 2009). Clinically, it would be of interest whether savirin would work in concert with traditional antibiotics. We could utilize our *in vivo* model of dermonecrosis to see if infections treated with antibiotics in addition to savirin would create smaller ulcers initially, due to the inhibition of virulence by savirin, and then exhibit decreased time to resolution and bacterial clearance due to the actions of the antibiotic. Whether or not there would be an additive effect between the two treatments is unknown. The action of savirin on systemic *S. aureus* infections has also yet to be investigated. The air pouch model of *S. aureus* infection could be utilized again to determine the extent of bacterial invasion. The pouch tissue and underlying muscle could be excised and stained with anti-staphylococcal antibody to determine if savirin treatment kept the bacteria from invading through the tissues into the dermis or muscle as described (Peterson et al., 2008). Other clinically relevant questions include whether savirin could be effective after the infection has been established as well as if savirin treatment could be administered as a prophylactic for incoming hospital patients to prevent acquisition of a CA-MRSA

infection. We can use our *in vivo* dermonecrosis model to determine if these treatments are protective and feasible before they can be proposed for clinically useful therapeutics in humans.

The current therapy for skin and soft tissue infections is surgical drainage, required by ~80% of the patients that present to an emergency department with purulent infections (Stryjewski and Chambers, 2008). These patients are frequently cured by surgical drainage alone and are postulated to not require antibacterial therapy. In fact a clinical trial reported cure rates of >85% for patients who underwent surgical drainage and received a placebo and the response was equivalent to patients who underwent surgical drainage and were given cephalexin (Stryjewski and Chambers, 2008). These results indicate that there are clinical scenarios where antibiotics are not absolutely required. In cases where surgical drainage alone may be appropriate (the patient is healthy and the lesion is small) the small molecule inhibitors described here, savirin and benzbromarone, could be used as adjunct drug therapies to speed the resolution of the infection. Administration of savirin may be useful in the treatment of CA-MRSA infections in patients with dermatitis or other similar skin conditions. The inhibition of virulence factor production by the bacteria may limit the amount of tissue damage in these people who have impaired skin defensins as well as limit the exposure of the patient and bacteria to antibiotics, thus preventing their negative consequences.

## Conclusions

The results presented here describe the ability of two small molecular compounds to target quorum sensing-dependent virulence production of *S. aureus*. While this approach

of targeting virulence has shown promise in Gram negative *in vivo* infections (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Sperandio, 2010), it has yet to be applied to Gram-positive pathogens. This work demonstrates that benzbromarone and savirin, two molecules identified in a high throughput screening assay examining compounds for possible antagonism of *agr*, are able to inhibit *S. aureus* virulence *in vitro* and *in vivo*. One molecule addressed here, savirin, has the ability to inhibit bacterial virulence without affecting viability, has the ability to inhibit virulence in multiple *agr* backgrounds, remains effective over the timecourse tested without induction of bacterial resistance, and also makes the bacteria more susceptible to killing by acidic conditions, a host immune effector. The demonstration of the protective effects of savirin and benzbromarone in an *in vivo* dermonecrotic infection shows the potential of these compounds as a clinically relevant therapeutic for immunocompetent patients. Savirin- and benzbromarone-treated bacteria create smaller areas of dermonecrosis in addition to a faster ulcer resolution and bacterial clearance over time.

With the emergence of the hypervirulent CA-MRSA strains which produce abundant amounts of tissue-destroying virulence factors and an ability to develop resistance to most antibiotics, identifying novel therapeutics has renewed importance. The results presented here show that both savirin and benzbromarone are effective inhibitors of CA-MRSA virulence factor production and confers protection in an animal model of CA-MRSA infection.

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