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Molecular Genetic Analysis of Bacterial Antimicrobial Peptide Resistance Phenotypes

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

Morgan A. Pence

Committee in Charge:

Professor Victor Nizet, Chair
Professor Antonio De Maio
Professor Richard Gallo
Professor Theo Kirkland
Professor Joseph Vinetz

2012

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Chair

University of California, San Diego

2012

DEDICATION

To everyone who has ever believed in me, this is for you.

EPIGRAPH

"Our greatest glory is not in never falling but in rising every time we fall."

- **Confucius**

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LIST OF COMMON ABBREVIATIONS

1,10 PA	1, 10 phenanthroline
6-APA	6-aminopenicillanic acid
AMP	antimicrobial peptide
AP	animal-passaged
Aur	aureolysin
BlaI	β-lactamase inhibitor
CA-MRSA	community-associated methicillin-resistant *Staphylococcus aureus*
Cm	chloramphenicol
cfu/CFU	colony forming units
DPBS	Dulbecco's phosphate buffered saline
DNA	deoxyribonucleic acid
E-64	*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Em	erythromycin
FBS	fetal bovine serum
GAS	Group A *Streptococcus*
HaCaT	human keratinocyte cell line
HA-MRSA	hospital-associated methicillin-resistant *Staphylococcus aureus*
Km	kanamycin
kDa	kilodalton
LB	Luria-Burtani broth
MBC	minimum bactericidal concentration
MES	2-(N-morpholino)ethanesulfonic acid
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
MOPS	3-(N-morpholino)propanesulfonic acid
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar (micromoles/liter)
ml	milliliter
mM	millimolar (millimoles/liter)
MRSA	methicillin-resistant *Staphylococcus aureus*
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLL	poly-L-lysine
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RT	room temperature

S. aureus	*Staphylococcus aureus*
SIC	streptococcal inhibitor of complement
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
THA	Todd Hewitt agar
THB	Todd Hewitt broth
TLR	Toll-like receptor
TSA	tryptic soy agar
TSB	tryptic soy broth
WT	wild-type

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***

The work detailed in this thesis is a combination of works published, submitted or in preparation for submission. Permission was obtained from all co-authors of manuscripts presented herein prior to submission of the thesis.

Work described in Chapter 2 was modified from a published manuscript of which I was primary author: Pence M.A., Rooijakkers S.H., Cogen A.L., Cole J.N., Hollands A., Gallo R.L., Nizet V., 2010. Streptococcal inhibitor of complement (SIC) promotes innate immune resistance phenotypes of invasive M1T1 group A *Streptococcus*. J Innate Immun 2(6):587-595. This chapter was reproduced with permission from co-authors.

Work described in Chapter 3 was modified from a manuscript currently under revision of which I was primary author: Pence M.A., Malone C.L., van Sorge N.M., Ruyken M., Rooijakkers S.H.M, Horswill A.R., Nizet V. The metalloprotease aureolysin is not essential for cathelicidin resistance or virulence of methicillin-

resistant *Staphylococcus aureus*. This chapter was reproduced with permission from co-authors.

Work described in Chapter 4 was modified from a manuscript currently in preparation of which I was the primary author: Pence M.A., Haste N.M., Sibhatu H.M., Olson J., Gallo R.L., Nizet V.,Kristian S.A. The novel role of the β-lactamase repressor BlaI as a virulence factor that renders *Staphylococcus aureus* more susceptible to the innate immune defense. This chapter was reproduced with permission from co-authors.

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ABSTRACT OF THE DISSERTATION

Molecular Genetic Analysis of Bacterial Antimicrobial Peptide Resistance Phenotypes

by

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Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2012

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Group A *Streptococcus* (GAS) and *Staphylococcus aureus* (*S. aureus*) are pre-eminent pathogens capable of causing disease in otherwise healthy individuals. Both pathogens are associated with a wide spectrum of clinical disease ranging from relatively minor superficial infections, such as pharyngitis or impetigo, to more severe invasive disease such as bacteremia and sepsis. The increased frequency and severity of the M1T1 clone of GAS and increasing antibiotic resistance of methicillin-resistant *S. aureus* (MRSA) make these two pathogens excellent topics for research.

Using molecular genetics, we evaluated the roles of three genes, streptococcal inhibitor of complement (*sic*) in GAS and aureolysin (*aur*) and *blaI* in *S. aureus* in regards to antimicrobial peptide (AMP) resistance and virulence. Two of these genes, *sic* and *aur*, have previously been proposed to play a role in AMP resistance and virulence through analysis of purified protein. The third gene, *blaI*, is a novel AMP resistance factor found through screening of a transposon library in *S.*

aureus. In the following works, we demonstrate a contribution of SIC and BlaI to virulence in the context of the living pathogen but are unable to show a role for aureolysin.

CHAPTER 1.

Introduction

Group A *Streptococcus* (GAS, *S. pyogenes*) and *Staphylococcus aureus* (*S. aureus*) are two of the leading bacterial human pathogens, and both are capable of causing varying degrees of disease in patients, with infections ranging from mild skin infections to life-threatening invasive disease such as bacteremia and sepsis.

There has been an increase in the number of invasive GAS cases in the last three decades, mainly due to a single clonal lineage of the M1T1 serotype (Aziz and Kotb, 2008; Chatellier et al., 2000; Cleary et al., 1998; Cockerill et al., 1997). Each year, GAS is responsible for over 600 million cases of pharyngitis and 650,000 cases of invasive disease, resulting in 500,000 deaths worldwide (Carapetis et al., 2005).

S. aureus has become an increased burden in the last forty years with the emergence and spread of methicillin-resistant *S. aureus* (MRSA) (Boyce, 1992). The Centers for Disease Control estimated that there were approximately 95,000 invasive MRSA cases in 2005 resulting in 19,000 deaths, which is greater than the number of deaths due to HIV/AIDS in the United States (Klevens et al., 2007). Although the rates of hospital-associated MRSA cases fell between 2005 and 2008 (Kallen et al., 2010), both community-associated and hospital-associate MRSA continue to be a burden on the health system.

The human innate immune system is responsible for quickly identifying pathogenic organisms and defending against them. Bacteria are identified through the Toll-like receptors (TLRs), membrane receptors found on the surface of multiple

cell types, such as neutrophils, monocytes/macrophages, lymphocytes, natural killer (NK) cells and others (Hayashi et al., 2003; Muzio et al., 2000). TLRs recognize pathogen-associated molecular patterns (PAMPs) and subsequently initiate a signaling cascade to activate the immune system. Opsonins, such as complement and antibodies, can also aid in the fight against pathogens by contributing to phagocytosis.

One of the innate host defenses against pathogenic organisms includes antimicrobial peptides (AMPs). These peptides are a form of innate antibiotics, as they are bactericidal and non-specific in their targeting. Several classes of mammalian AMPs exist, including defensins and cathelicidins. In humans, only one form of cathelicidin, LL-37, exists, and it's murine counterpart, CRAMP, is the only form found in mice. Cathelicidins are found primarily in keratinocytes of the skin, neutrophil granules, mast cells and epithelial cells (Bardan et al., 2004) and are positively charged α-helical peptides that are attracted to the relative negative charge of bacterial membranes. In addition, cathelicidins are amphipathic molecules, with one hydrophobic and one hydrophilic side, which allows them to insert themselves into bacterial membranes and form pores. Pore formation causes membrane disruption, leading to leakage of cellular contents and resulting in death of the bacteria. In addition to their pore-forming ability, cathelicidins also have an immunostimulatory function (De et al., 2000). LL-37 has been shown to be involved in chemotaxis of neutrophils, mast cells and CD4 T cells (Agerberth et al., 2000; Niyonsaba et al., 2002), and this has been shown to occur through binding of the formyl peptide receptor-like 1 (FPRL1) (De et al., 2000). Thus, not only do

cathelicidins have a direct effect on the bacteria, they also have an indirect effect through the recruitment of additional components of the innate immune system.

Bacteria have developed ways to resist the action of AMPs, and multiple mechanisms have been demonstrated in Gram-positive pathogens. Regulating charge of the bacterial cell surface in order to repel the AMPs is a method utilized by both GAS and *S. aureus.* MprF in *S. aureus* adds lysines to phosphotidylglycerol (Peschel et al., 2001), and DltA in *S. aureus* and GAS adds alanines to lipotechoic acid (Kristian et al., 2005; Peschel et al., 1999); the addition of these molecules decreases the overall negative charge of the bacterial surface, making it a less attractive target for AMPs. A second mechanism used by Gram-positive pathogens is binding up free AMPs, thus inhibiting their activity at the cell surface. Streptococcal inhibitor of complement (SIC) and staphylokinase in GAS and *S. aureus*, respectively, are secreted factors that have been shown bind to AMPs (Frick et al., 2003; Jin et al., 2004). Proteolytic cleavage is a third mechanism by which Gram positive pathogens subvert the AMP defense. Bacteria secrete a variety of proteases into their surroundings, and a few of these are able to cleave AMPs into smaller, inactive compounds. Aureolysin in *S. aureus* and SpeB in GAS have been shown to do just that (Schmidtchen et al., 2002; Sieprawska-Lupa et al., 2004). Aureolysin and the SspA (V8) protease are the only two proteases in *S. aureus*, thus far, that have been shown to cleave AMPs. While aureolysin cleaves cathelicidin to smaller, inactive peptides, SspA cleaves cathelicidin to smaller peptides which retain activity (Sieprawska-Lupa et al., 2004), thus implicating aureolysin as the only *S. aureus* protease involved in resistance to AMPs.

In order to study AMP resistance factors in group A *Streptococcus* and *S. aureus*, we have taken advantage of molecular genetics and aimed to satisfy molecular Koch's postulates. Molecular Koch's postulates are the gold standard for demonstrating that a gene contributes to pathogenicity of an organism. They state:

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measureable loss in pathogenicity or virulence.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity. (Falkow, 1988)

As group A *Streptococcus* and *S. aureus* are pathogens, and the isolates in our lab originally came from patients infected with the specified bacterium, we have already met the requirement of the first postulate. However, according to the second postulate, a mutant strain must be created in which the gene of interest is inactivated, and this mutant must also be used to test the third postulate. Use of purified protein, while accepted by the scientific community, does not meet the requirements of molecular Koch's postulates; consequently, the following studies have been completed using molecular cloning methods.

Genetic manipulation of GAS and *S. aureus* is reasonably straightforward. Knock-out vectors exist for both strains, making it possible to create precise in-frame mutations (loss of function) in order to test the second molecular Koch's postulate. Additionally, we can express the gene of interest on a plasmid to restore the mutant's

activity (complementation) or alternatively to express the gene in a strain which normally does not contain the gene (gain of function); these tools will let us evaluate the third postulate.

Because of the medical significance of the M1T1 GAS clone and the antibiotic resistance of *S. aureus*, it is important to understand which genes are contributing to virulence. The hope is that one day these genes and their respective proteins may be used to develop targeted therapies against these pathogens in order to help fight disease. Three different genes have been examined in the following works, one in GAS and two in *S. aureus*. In GAS, the involvement of SIC in resistance to AMPs as well as in virulence is explored. Though SIC has been shown to bind and inactivate AMPs, this work was done using biochemical methods (Frick et al., 2003). Thus, we set out to evaluate the contribution of SIC using molecular genetics. We created an allelic exchange mutant in an invasive strain of M1T1 GAS and heterologously expressed the *sic* gene in another GAS serotype, M49, which normally does not express the gene. Using these tools, we determined that SIC is necessary and sufficient for AMP resistance and virulence.

Additionally, the contribution of aureolysin to AMP resistance and pathogenesis was explored in methicillin-resistant *S. aureus* (MRSA). Aureolysin has been touted to be a major AMP resistance and virulence factor (Beaufort et al., 2008; Laarman et al., 2011; Prokesova et al., 1991; Sieprawska-Lupa et al., 2004), though these studies were completed using purified protein. In Chapter 3, we created allelic exchange mutants in three different MRSA strains but were unable to demonstrate an AMP resistance or virulence role for aureolysin.

Lastly, we used transposon mutagenesis to look for novel AMP resistance factors in *S. aureus* and discovered that the β-lactamase inhibitor BlaI decreases susceptibility to cathelicidins. Through the construction of integrational mutants in two strains of *S. aureus* and complementation in one strain, we confirm the role of BlaI in AMP resistance as well as its role in virulence of MRSA.

As demonstrated in the following works, molecular genetics is a valuable tool for studying the contribution of genes to pathogenesis of an organism. Use of molecular genetics may validate experiments using purified protein, but it may disprove them as well. Studying the roles of SIC, Aur and BlaI will help determine if they would be appropriate to use for targeted therapies of GAS and *S. aureus*.

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CHAPTER 2.

Streptococcal inhibitor of complement promotes innate immune resistance phenotypes of invasive M1T1 group A *Streptococcus*

ABSTRACT

Streptococcal inhibitor of complement (SIC) is a highly polymorphic extracellular protein and putative virulence factor secreted by M1 and M57 strains of group A *Streptococcus* (GAS). The *sic* gene is highly upregulated in invasive M1T1 GAS isolates following selection of mutations in the *covR/S* regulatory locus *in vivo.* Previous work has shown that SIC (allelic form 1.01) binds to and inactivates complement C5b67 and human cathelicidin LL-37. We examined the contribution of SIC to innate immune resistance phenotypes of GAS in the intact organism, using (1) targeted deletion of *sic* in wild-type and animal-passaged (*covS* mutant) M1T1 GAS harboring the *sic 1.84* allele and (2) heterologous expression of *sic* in M49 GAS, which does not contain the *sic* gene in its genome. We find that M1T1 SIC production is strongly upregulated upon *covS* mutation but that the *sic* gene is not required for generation and selection of *covS* mutants *in vivo*. SIC 1.84 bound both human and murine cathelicidins and was necessary and sufficient to promote *covS* mutant M1T1 GAS resistance to LL-37, growth in human whole blood and virulence in a murine model of systemic infection. Finally, the *sic* knockout mutant M1T1 GAS

strain was deficient in growth in human serum and intracellular macrophage survival. We conclude that SIC contributes to M1T1 GAS immune resistance and virulence phenotypes.

INTRODUCTION

Group A *Streptococcus* (GAS) is a leading human bacterial pathogen producing illnesses ranging from mild pharyngitis or impetigo to severe necrotizing fasciitis and streptococcal toxic shock syndrome. Over the last 30 years, an increase in severe invasive GAS infections has been documented, largely attributable to the global dissemination and persistence of one clonal lineage of the M1T1 serotype (Aziz and Kotb, 2008; Chatellier et al., 2000; Cleary et al., 1998; Cockerill et al., 1997). In total, GAS is estimated to cause 650,000 invasive infections resulting in 150,000 deaths annually worldwide (Carapetis et al., 2005).

The medical significance of the M1T1 GAS clone has stimulated research attempting to identify specific virulence factors which could explain the strain's propensity for severe invasive infections. In their global transcriptional analysis of clonal M1T1 GAS strains associated with mucosal vs. invasive human infections, Sumby *et al.* (Sumby et al.) made the critical discovery that characteristic frameshift mutations in the *covS* gene were present among the invasive isolates(Sumby et al., 2006). CovS is the sensor kinase of the two-component regulator CovR/S, which regulates the expression of 10-15% of genes in the GAS genome (Graham et al., 2002). Mutations in *covS* result in increased expression of hyaluronic capsule and several other known or hypothesized GAS virulence proteins (Engleberg et al., 2001; Sumby et al., 2006). Simultaneously, *covS* mutation downregulates expression of the broad-spectrum cysteine protease, SpeB, that would otherwise degrade many of the important GAS virulence determinants (Aziz et al., 2004; Raeder et al., 2000). The genetic and phenotypic switch to hypervirulence can be recapitulated in the murine

model, where M1T1 GAS *covS* mutations are selected *in vivo* and lead to neutrophil resistance and systemic dissemination (Cole et al., 2006; Sumby et al., 2006).

One of the most highly upregulated genes upon *covS* mutation and initiation of invasive M1T1 GAS infection is *sic*, encoding the **s**treptococcal **i**nhibitor of **c**omplement. SIC is a secreted 31 kDa protein first discovered because of its location in the Mga virulence regulon (Akesson et al., 1996). By biochemical assays, SIC was shown to bind C5b67 of the membrane attack complex, thus impairing terminal complement function (Akesson et al., 1996; Fernie-King et al., 2001). A screen of *emm* types revealed that *sic* is present only in M1 and M57 strains (Akesson et al., 1996). Later it was discovered that another protein with similarity to the C-terminal proline-rich region (PRR) of SIC, termed DRS (**d**istantly **r**elated to **S**IC), is expressed by M12 and M55 strains (Akesson et al., 1996; Hartas and Sriprakash, 1999). With nearly 300 alleles discovered, *sic* is the most polymorphic gene in the M1 GAS genome, suggesting it is under strong immune selective pressure (Hoe et al., 2001).

In more recent studies, purified SIC has been shown to bind several antimicrobial proteins important in host innate defense including lysozyme, secretory leukocyte protease inhibitor, human α-defensin-1, and human cathelicidin, LL-37 (Fernie-King et al., 2002; Fernie-King et al., 2004; Frick et al., 2003). SIC is also important for GAS epithelial cell adherence (Hoe et al., 2002) and mucosal colonization (Lukomski et al., 2000); however, the role of SIC in invasive M1T1 GAS infection has not been studied. The goal of the present study was to analyze the effect of SIC on M1T1 GAS resistance to cathelicidin antimicrobial peptides, growth in human serum and whole blood, and virulence and phenotype switching in a murine infection model. Work in our model invasive M1T1 GAS clone examines the

sic 1.84 allele, in contrast to the *sic 1.01* allele that has been the basis of most published studies using purified SIC protein. Our analysis is performed using intact organisms, pairing targeted mutagenesis of *sic* in GAS (loss of function) with heterologous expression of *sic* in a GAS strain that does not contain the *sic* gene (gain of function).

MATERIALS AND METHODS

Bacterial strains and generation of SIC mutants. The wild-type (WT) GAS M1T1 strain (5448) was originally isolated from a patient with NF and TSS (Chatellier et al., 2000). Polymerase chain reaction (PCR) was used to amplify DNA fragments directly upstream and downstream of *sic* from the M1T1 GAS genome. Primer sets used were: (1) upF (5'-cttcgtcgtgacttggacgc-3') + upR (5'-tagtattctctccttaata-3' with 30-bp 5' extension matching 5' end of the *cat* gene) and (2) downF (5'-agggatgcgataggaatag-3' with 30-bp 5' extension matching 3' end of the *cat* gene) + downR (5'-gcggattccggctataacg-3'). Fusion PCR was performed using the respective upstream and downstream fragments and an amplicon of the *cat* gene to create a new DNA fragment in which the *sic* gene was precisely replaced by *cat* in chromosomal context. The fusion construct was TA-cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into pHY304, a temperature-sensitive plasmid bearing erythromycin (Em) resistance. The knockout plasmid was transformed into the M1T1 GAS parent strain, and single recombination events were selected for by plating on Todd Hewitt Agar (THA, Difco), supplemented with 2 µg/ml Em, at 37°C. Single crossovers were relaxed at 30°C, and double crossovers were screened for sensitivity to Em. The knockout, herein referred to as M1T1 GAS Δ*sic*, was verified by PCR.

An animal-passaged (AP) version of the M1T1 GAS parent strain (5448AP) has been previously published and has been shown to contain a single inactivating adenine insertion at the 877 base pair position of *covS*. (Aziz et al., 2004; Walker et al., 2007). The animal-passaged version of the M1T1 GAS Δ*sic* was created by injecting 12-week-old female C57BL/6J mice (Charles River Laboratories)

subcutaneously with 2 x 10^8 colony forming units (CFU) of mid-log phase M1T1 GAS Δ*sic* in 100 µl DPBS. After 72 h, mice were sacrificed, lesions excised and homogenized and bacteria were plated. Bacteria were subsequently screened using a cysteine protease assay to detect SpeB activity, and the *covR/S* genes of SpeB negative colonies were sequenced directly. A single base pair mutation (C→T) in *covS* was discovered at the 1261 base pair position, producing a premature stop codon. Hereforeward, the animal-passaged SIC mutant strain is designated: M1T1 GAS (AP) Δ*sic*.

Heterologous expression of SIC. The *sic* gene was amplified from the M1T1 WT genome by PCR using primers *sic*F (5'-ggagagaatactaatg-3') and *sic*R (5'-ttacgttgctgatggtgt-3'). The PCR product was TA cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into shuttle expression vector pDCerm (Jeng et al., 2003). The resulting plasmid was introduced via electroporation into M49 GAS strain NZ131 (Simon and Ferretti, 1991), resulting in heterologous expression of SIC.

Western immunoblot analysis. Bacterial supernatants from late-log phase (SIC expression) or overnight cultures (SpeB expression, SIC cleavage) were collected after centrifugation at 3,220 x g for 10 min. For SIC cleavage, cultures were grown with or without 10 µg/ml of E-64, a cysteine protease inhibitor. Supernatants were filtered using a 0.22 µm filter (Millipore) and then left unconcentrated (for SpeB blot) or concentrated 50-fold (for SIC blots) using a Vacufuge (Eppendorf). Samples were mixed with 4X sample buffer and 10X reducing agent (Invitrogen), boiled for 10 min, loaded onto a 10% Bis-Tris gel (Invitrogen) and run in MOPS running buffer at 140V. The gels were then transferred onto a nitrocellulose membrane using a semi-dry apparatus (Bio-Rad) at 12V for 45 min. After transfer, the membranes were

blocked for 1 h at RT in 0.03% phosphate buffered saline (PBS)-Tween + 5% nonfat milk. For SIC western blots, primary anti-SIC antibody was incubated at RT for 1 h at a 1:200 dilution in blocking buffer. For the SpeB blot, primary anti-SpeB antibody (Toxin Technology, Inc.) was incubated at RT for 1 h at a 1:1,000 dilution in blocking buffer. After three washes with PBS-Tween, the membranes were incubated with a goat anti-rabbit HRP secondary antibody (Bio-Rad) at a 1:20,000 dilution for 1 h at RT. The membranes were subsequently washed 3X with PBS-Tween and developed using chemiluminescence.

Minimum inhibitory concentration testing. Bacterial cultures were grown to mid-log phase in Dulbecco's phosphate buffered saline (DPBS) + 20% Todd-Hewitt Broth (THB, Difco). Bacteria were resuspended to 10^5 CFU/ml in the same buffer, and 90 µl of bacteria was added to 10 µl of decreasing concentrations of LL-37 or CRAMP in a 96-well plate. After incubation at 37°C for 24 h, 25 µl was removed from each well, diluted and plated for enumeration of bacteria to determine the minimum inhibitory concentration (MIC, inhibited bacterial growth) and minimum bactericidal concentration (MBC, no bacterial growth).

Ll-37 killing kinetics. Bacterial cultures were grown to mid-log phase in DPBS + 20% THB. Bacteria were resuspended to 10^5 CFU/ml in the same buffer, and 450 µl of bacteria was added to 50 µl of 320 µM LL-37 (for a final concentration of 32 µM) in siliconized tubes. Tubes were placed on a rotisserie at 37°C, and 25 µl was removed at each time point to be diluted and plated for enumeration of CFU.

Co-immunoprecipitation. One milliliter of supernatant from an overnight culture of M1T1 GAS (AP) was incubated with either 1 µM LL-37 or CRAMP at room temperature for 8 h. Twenty microliters of Protein A/G PLUS (Santa Cruz

Biotechnology) beads were added to each tube and incubated while rotating at 4°C for 1 h to allow preclearing. Beads were centrifuged at 5,000 x *g* for 5 min, and the supernatant was transferred to new tubes in 500 µl aliquots. For immunoprecipitation of LL-37, 1 µg of chicken anti-LL-37 was added while 1 µg IgY was added to a second tube as a negative control. Tubes were incubated, rotating, at 4°C overnight. The next day, goat anti-IgY was added to each tube and incubated, rotating, at 4°C for 2 h. Twenty microliters of Protein A/G PLUS beads were added to each tube and incubated at 4°C for 4 h. Beads were pelleted by centrifugation at 5,000 x g for 5 min and subsequently washed four times with 500 µl ice-cold PBS. Each tube of beads was resuspended in 20 µl of PBS. Samples were then analyzed by for western blotting exactly as in the protocol above, except that subsequent to boiling, samples were spun down at 5,000 x *g* for 5 min to remove beads. For immunoprecipitation of CRAMP, 1 µg of rabbit anti-CRAMP was added while 1 µg rabbit IgG was added to a second tube as a negative control. Tubes were incubated, rotating, at 4°C overnight. The next day Protein A/G PLUS beads were added, and the assay was carried out exactly as for LL-37.

Growth in human whole blood. Blood was drawn from healthy donors after informed consent, and 2 x 10^3 CFU (M1T1) or 2 x 10^7 CFU (M49) of late-log phase bacteria in 100 µl PBS was added to 300 µl heparinized whole blood in siliconized tubes. Tubes were placed on a rotisserie at 37°C, and a 25 µl aliquot was removed at each time point to be diluted and plated for enumeration of CFU. Growth index was calculated as the ratio of surviving CFU after incubation versus the initial inoculum.

Growth in human serum. Blood was drawn from healthy human volunteers after informed consent. Normal human serum was prepared by pooling sera of 6 different donors and stored at -80°C. Overnight bacterial cultures grown in THB were diluted 1:100,000 in RPMI-1640 tissue culture medium (Invitrogen). Fifty microliters of bacteria were mixed with 50 µl of serum in RPMI-1640 in 96-wells plates. Plates were incubated for 6 h at 37°C, and surviving bacteria were enumerated by plating serial dilutions on THA. Growth index was calculated as the ratio of surviving CFU after incubation versus the initial inoculum.

Macrophage intracellular survival. Eight-week-old CD-1 male mice (Charles River Laboratories) were injected intraperitoneally with 3 ml of 3% thioglycolate to stimulate macrophage induction. Three days post-infection, mice were sacrificed and macrophages were harvested by rinsing the peritoneal cavity twice with 8 ml DPBS. Macrophages were counted, resuspended in RPMI + 10% FBS, seeded at 7 x 10^5 cells/well in a 24-well plate and allowed to adhere overnight. The next day, bacteria were grown to late-log phase and resuspended in RPMI + 2% FBS. An aliquot of 500 µl bacteria, representing a multiplicity of infection (MOI) of 10:1 (GAS:macrophage), was added to wells after media was removed, and plates were centrifuged at 500 x g for 5 min to ensure contact of the bacteria with the macrophages. Plates were incubated at 37°C + 5% CO_2 for 1 h. Media was then removed and cells were washed three times with DPBS. Media containing 10 µg/ml penicillin and 100 µg/ml gentamicin was added to kill extracellular bacteria and the plates were incubated for an additional 1 h. Media was then removed, cells were washed three times with DPBS, and 500 µl of 0.025% Triton-X was added to lyse cells. Cells were removed by tituration and dilutions plated to enumerate bacteria;

this represented the 1 h time point. To wells of later time points, fresh media was added, and at the indicated time points, cells were harvested as described. Relative survival was calculated by comparing surviving CFU to the inoculum and normalizing all time points to the M1T1 GAS (AP) 1 h time point.

Murine infection model. Eight-week-old male CD-1 mice were injected intraperitoneally with 2.5 x 10^6 CFU of mid-log phase M1T1 GAS (AP) or M1T1 GAS (AP) Δ*sic* or alternatively, 1.3 x 10^7 CFU of mid-log M49 GAS or M49 GAS expressing SIC, suspended in 200 µl of DPBS + 5% gastric mucin. Survival was monitored for 4 (M1T1) or 7 (M49) days, and time of death was recorded for each mouse.

***In vivo* phenotype switching assay.** To study the influence of SIC on the capacity for *in vivo* phase-switching (*covS* mutation and SpeB inactivation), 8-week-old female C57BL/6J mice or outbred male CD-1 mice (Charles River Laboratories) were subcutaneously inoculated with a nonlethal dose (~10^8 CFU/100 µl) of mid-log phase M1T1 GAS or M1T1 GAS Δ*sic* in DPBS. For the C57BL/6J experiment, separate groups of 10 mice were injected with each strain; for the CD-1 experiment, each of 10 mice was injected on the left flank with the M1 GAS parent strain and on the right flank with the M1T1 GAS Δ*sic* mutant. Three days post-infection, mice were euthanized by CO_2 asphyxiation, skin lesions excised and homogenized in 1 ml DPBS + 1 mm zirconia/silica beads using a Mini-Beadbeater (Biospec Products) and 2 x 1 min homogenization bursts. Homogenates were serially diluted in PBS, plated onto THA, and 50 individual lesion-derived colonies (*n* = 50) were then assayed for SpeB protease by an azocaseinolytic assay (Collin and Olsen, 2000) scaled to a 96-well plate format. Isolates were scored SpeB-negative following negative results in

consecutive assays; for all assays, WT M1T1 GAS and its isogenic Δ*speB* knockout mutant (Aziz et al., 2004) were included as positive and negative controls, respectively.

Statistical analyses. LL-37 kinetics, whole blood, serum, intracellular macrophage survival assays and *in vivo covS* switching were compared using the Student's *t*-test. Mouse intraperitoneal challenge assay was assessed using the log-rank (Mantel Cox) test. All statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). *P* values < 0.05 were considered statistically significant.

Ethics permissions. Permission to collect human blood under informed consent was approved by the UCSD Human Research Protections Program. All animal experiments were conducted according to the guidelines approved by the UCSD Institutional Animal Use and Care Committee.

RESULTS

Creation of bacterial reagents for SIC analysis. To study the role of SIC in the context of the living pathogen, we created a precise in-frame allelic replacement of the *sic* gene in M1T1 GAS with a cassette encoding chloramphenicol acetyltransferase (*cat*). The mutant was then animal-passaged (AP) by murine subcutaneous passage, and identified SpeB-negative colonies were shown to possess an inactivating mutation within *covS*. For gain of function analyses, SIC was heterologously expressed on a plasmid in M49 GAS. To confirm the presence or lack of SIC expression in the generated strains, cell-free culture supernatants were concentrated 50-fold and subjected to western blot analysis (**Figure 2.1A**). As expected, no detectable SIC was identified in supernatants from the two Δ*sic* mutants, and heterologous expression in M49 GAS was successful. Even at a 50-fold concentration, however, the M1T1 GAS strain did not produce sufficient amounts of SIC for detection by western blot, whereas this protein was highly abundant in the M1T1 GAS strain following *covS* mutation and associated transcriptional upregulation. Purified SpeB has been reported to cleave purified SIC (Hoe et al.), so we examined whether this was occurring in the context of the M1T1 GAS strain, resulting in the absence of SIC in the supernatant. We first looked at SpeB expression in the four M1T1 strains and found that it was expressed in the M1T1 GAS and M1T1 GAS Δ*sic* strains but not in the M1T1 GAS (AP) or M1T1 GAS (AP) Δ*sic* strains as expected (**Figure 2.1B**). To demonstrate cleavage of SIC by SpeB in M1T1 GAS, overnight cultures were grown with or without E-64, a cysteine protease inhibitor, concentrated 50-fold and subjected to western blot. When SpeB was inhibited by E-64, full-length SIC was present in the supernatant (**Figure 2.1C**).

Thus, in this representative strain of the invasive M1T1 GAS clone expressing the *sic 1.84* allele, only very small amounts of the putative virulence factor may be present, due to cleavage by SpeB, until the *in vivo* selection event of *covS* mutation that leads to drastically reduced levels of SpeB and increased levels of SIC. Consequently, for many of the ensuing comparative studies of SIC function, the M1T1 GAS (AP) strain and the M1T1 GAS (AP) Δ*sic* mutant were employed. Despite several attempts, we were technically unable to achieve complementation of the M1T1 GAS (AP) Δ*sic* mutant with the expression vector used successfully in M49 GAS. We hypothesize these results derive from either (1) the marked hyperencapsulation of animal-passaged *covS* mutant GAS strains interfering with transformation or (2) potential toxicities of protein overexpression when the *sic* gene is present on a multi-copy number plasmid and simultaneously subject to strong transcriptional upregulation.

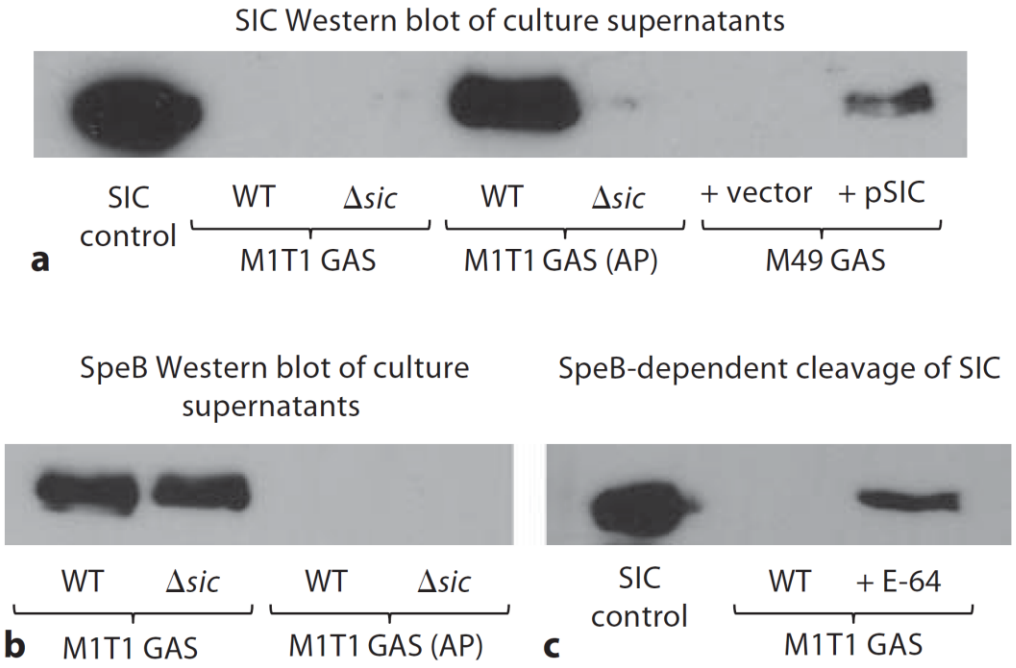

Figure 2.1. Generation of bacterial reagents with altered SIC expression. (A) Western blot analysis of SIC expression following targeted mutagenesis and/or animal-passage in M1T1 GAS strains and heterologous expression in M49 GAS. Concentrated (50-fold) cell-free culture supernatants were probed using polyclonal anti-SIC antisera. (B) Western blot analysis of SpeB expression and (C) SIC cleavage by SpeB in M1T1 GAS strains. SIC control lanes contain 0.5 µg of purified SIC protein.

SIC 1.84 binds to human and murine cathelicidins. While previous studies of SIC protein interaction with human cathelicidin LL-37 were performed with the protein encoded by the *sic 1.01* allele, our M1T1 GAS strain harbors the *sic 1.84* allele. The alleles possess 89.6% sequence identity (**Figure 2.2**). To determine whether a cathelicidin-binding phenotype was retained in the SIC 1.84 protein, we incubated M1T1 GAS (AP) supernatants with either 1 μM LL-37 or the related murine cathelicidin peptide CRAMP, then performed a co-immunoprecipitation by pulling down cathelicidin and immunoblotting for SIC. Increased amounts of SIC were seen in the anti-cathelicidin lanes, compared to the isotype control lanes, demonstrating that our particular *sic* variant binds both LL-37 and CRAMP (**Figure 2.3A**). To our knowledge, this is the first time SIC has been shown to bind murine cathelicidin.

```
89.6% identity in 326 residues overlap; Score: 1584.0; Gap frequency: 4.0%

sic1.01       1 MNIRNKIENSKTLLFTSLVAVALLGATQPVSAETYTSRNFDWSGDDWSGDDWPEDDWSGD
sic1.84       1 MNIRNKIENSKTLLFTSLVAVALLGATQPVSAETYTSRNFDWSGDDWSGDDWPEDDWSGD
                ************************************************************

sic1.01      61 GLSKYDRSGVGLSQYGWSKYGWSSDKEEWPEDWPEDDWSS------DKKDE-------TE
sic1.84      61 GLSKYDRSGVGLSQYGWSKYGWSSDKKDETEDKTRPPYGEALGTGYEKRDDWGGPGTVAT
                ************************    **              * *

sic1.01     108 DKTRPPYGEALGTGYEKRDDWGGPGTVATDPYTPPYGGALGTGYEKRDDWGGPGTVATDP
sic1.84     121 DPYTPPYGGALGTGYEKRDDWGGPGTVATDPYTPPYGGALGTGYEKRDDWGGPGTVATDP
                *   **** **************************************************

sic1.01     168 YTPPYGGALGTGYEKRDDWRGPGHIPKPENEQSPNPSHIPEPPQIEWPQWNGFDGFDGLS
sic1.84     181 YTPPYGGALGTGYEKRDDWRGPGHIPKPENEQSPNPSHIPEPPQIEWPQWNGFDGFDGLS
                ************************************************************

sic1.01     228 SGPSDWGQSEDTPRFPSEPRVTEKPQHTPQKNPQESDFDRGFSAGLKAKNSGRGIDFEGF
sic1.84     241 SGPSDWGQSEDTPRFPSEPRVTEKPQHTPQKNPQESDFDRGFSAGLKAKNSGRGIDFEGF
                ************************************************************

sic1.01     288 QYGGWSDEYKKGYMQAFGTPYTPSAT
sic1.84     301 QYGGWSDEYKKGYMQAFGTPYTPSAT
                ***************************
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Figure 2.2. Sequence comparison of the SIC proteins encoded by the group A streptococcal *sic 1.01* and *sic 1.84* alleles.

SIC and bacterial resistance to cathelicidin killing. Finding that SIC binds both LL-37 and CRAMP, we sought to determine whether SIC expression influences bacterial resistance to killing by these host defense peptides. Strains were incubated with LL-37 or CRAMP, and bacteria were plated and enumerated at 24 h to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). It is important to recognize that other virulence factors implicated in GAS cathelicidin resistance are also altered upon *covS* mutation, including the loss of SpeB protease, capable of degrading LL-37 (Johansson et al., 2008), and preservation of surface-anchored M1 protein, which is capable of binding the peptides via its N-terminus (Lauth et al., 2009). The presence of *sic* was found to promote GAS resistance to LL-37 in both the pre- and post-animal passage background (**Table 2.1**), with the greatest relative difference in MIC observed upon direct comparison of the M1T1 GAS (AP) strain to the M1T1 GAS (AP) Δ*sic* mutant (16 μM vs. 4 μM). Further functional linkage was provided by the observation that expression of SIC in M49 GAS led to increased resistance to LL-37 (**Table 2.1**). In contrast, independent effects of SIC on GAS resistance to the murine cathelicidin CRAMP, if any, were insufficient to discriminate by *in vitro* MIC or MBC assays. Here we acknowledge the limitations of short-term *in vitro* analyses of a secreted protein, which may not be produced *de novo* in sufficient quantities from the freshly prepared bacterial inoculums to replicate levels that could accumulate over longer periods at foci of infection *in vivo*.

To further investigate the role of SIC in resistance to LL-37, we looked at the rates at which the bacteria were killed. To test this, M1T1 GAS (AP) and M1T1 GAS (AP) Δ*sic* were incubated with 32 μM LL-37 and time points were taken over a period

of four hours to allow for enumeration of bacteria. We found that M1T1 GAS (AP) Δ*sic* was killed significantly faster than M1T1 GAS (AP) (**Figure 2.3B**). In addition, we observed that M49 GAS expressing SIC was significantly more resistant to killing by LL-37 over time (**Figure 2.3C**). Thus, we conclude that SIC is necessary and sufficient to promote resistance to the human cathelicidin, LL-37.

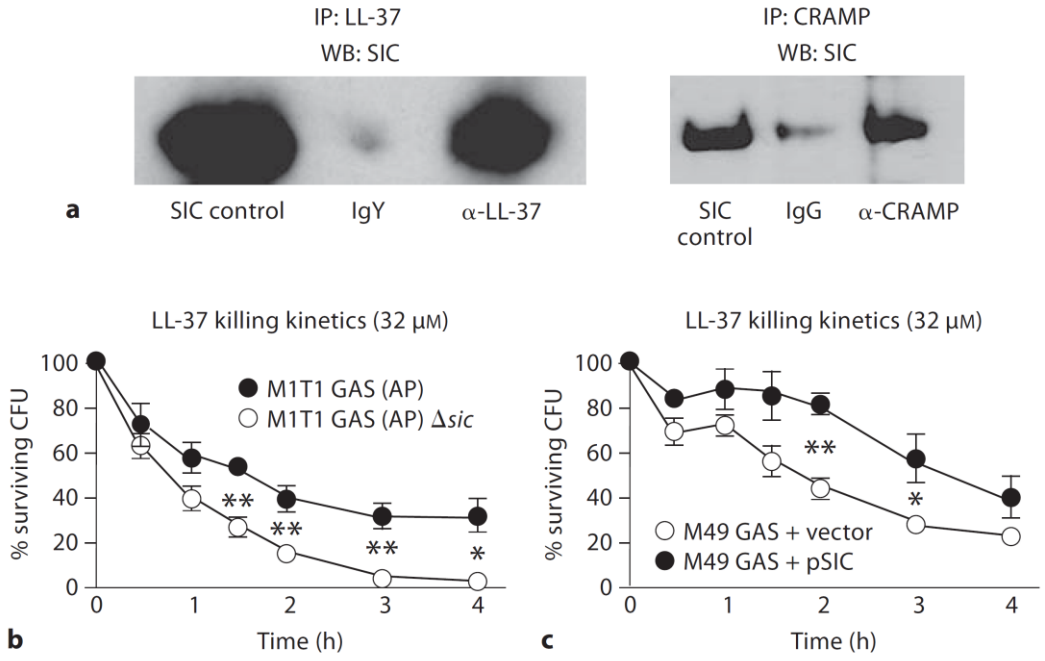

Figure 2.3. SIC interferes with cathelicidin activity by sequestration. (A) SIC 1.84 allelic variant binds cathelicidins LL-37 (human) and CRAMP (murine) as demonstrated by co-immunoprecipitation after incubation of M1T1 GAS (AP) supernatant with 1 μM of the indicated cathelicidin. Samples were immunoprecipitated (IP) for cathelicidin and western blotted (WB) for SIC. (B) M1T1 GAS (AP) and (C) M49 GAS strains lacking SIC were killed significantly better over time by LL-37. Experiments were performed in triplicate and repeated three times with similar results. Percent survival was calculated by dividing the surviving CFU by the initial inoculums and multiplying by 100. A representative experiment is shown with the mean ± SEM. Significance is indicated by the following: * $P < 0.05$, ** $P < 0.01$ according to Student's unpaired *t*-test.

Table 2.1. Activity of human and murine cathelicidin antimicrobial peptides against bacterial strains with altered SIC production.

	MIC	MBC
LL-37 (human)		
M1T1 GAS	14 μM	24 μM
M1T1 GAS Δ*sic*	10 μM	14 μM
M1T1 GAS (AP)	16 μM	28 μM
M1T1 GAS (AP) Δ*sic*	4 μM	10 μM
M49 GAS + vector	8 μM	16 μM
M49 GAS + pSIC	16 μM	32 μM
CRAMP (mouse)		
M1T1 GAS	4 μM	8 μM
M1T1 GAS Δ*sic*	4 μM	8 μM
M1T1 GAS (AP)	4 μM	16 μM
M1T1 GAS (AP) Δ*sic*	4 μM	16 μM
M49 GAS + vector	4 μM	16 μM
M49 GAS + pSIC	4 μM	16 μM

SIC promotes GAS growth in human whole blood and human serum. The *in vivo* selection of GAS M1T1 *covS* mutants is associated with systemic dissemination and enhanced resistance to phagocyte killing (Cole et al., 2006; Sumby et al., 2006; Walker et al., 2007). Here, we examined whether SIC is an important contributor to GAS proliferation in human blood and serum. Compared to the M1T1 GAS (AP) strain, the isogenic M1T1 GAS (AP) Δ*sic* mutant grew significantly less efficiently in freshly isolated human whole blood (**Figure 2.4A**) and human serum (**Figure 2.4B**). Conversely, expression of SIC in the M49 GAS background significantly enhanced growth in human whole blood (**Figure 2.4C**). We conclude that SIC is both necessary and sufficient to support effective bacterial growth in human blood and necessary for growth in human serum.

SIC promotes GAS survival in murine intraperitoneal macrophages. To begin to investigate whether a murine model of infection might prove useful for assessing the contribution of SIC to GAS systemic virulence, we compared intracellular survival of the M1T1 GAS (AP) and M1T1 GAS (AP) Δ*sic* in thioglycolate-elicited primary murine peritoneal macrophages. GAS were added to the macrophages at MOI = 10:1 and plates were centrifuged to bring bacteria into close contact with the macrophages. After 1 h incubation, aliquots were removed and plated to ensure similar rates of phagocytosis (data not shown), and antibiotic media was added to kill remaining extracellular bacteria. Cells were harvested at the indicated time points and wells were plated for enumeration of bacteria. Absence of SIC in the animal-passaged *covS* mutant GAS strain was associated with a significant intracellular survival defect (**Figure 2.4D**).

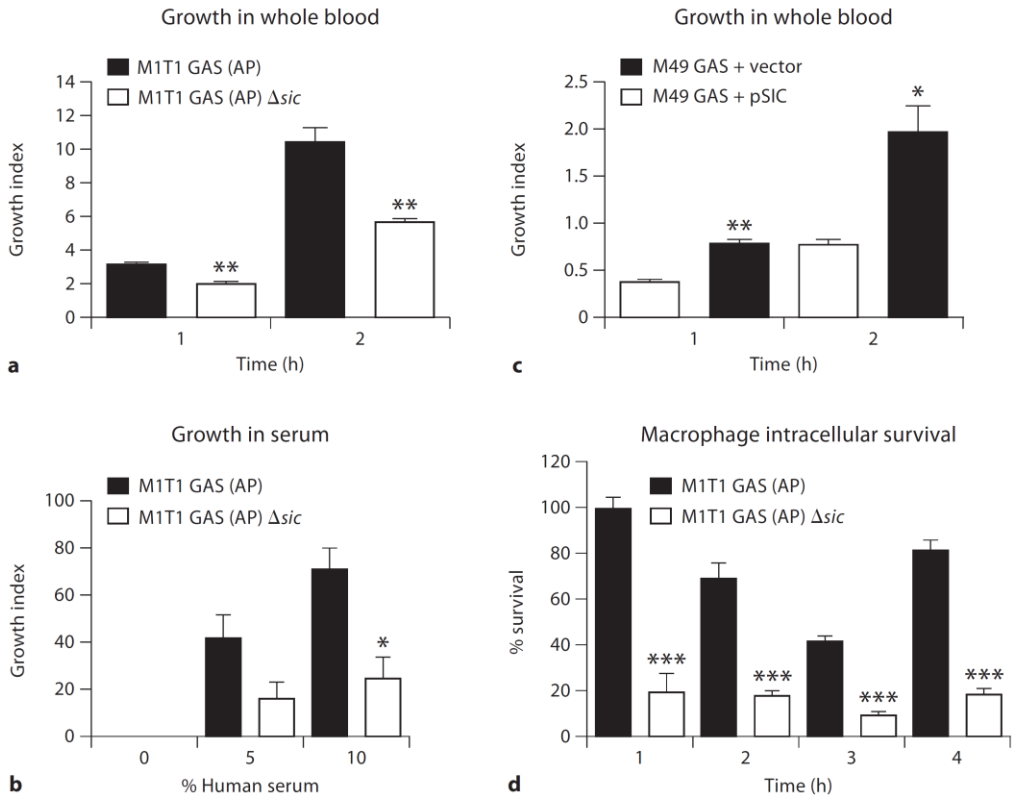

Figure 2.4. SIC is necessary and sufficient to promote bacterial growth in human whole blood and necessary for growth in normal human serum and intracellular survival in primary murine macrophages. Absence of SIC expression in the M1T1 GAS (AP) background is associated with decreased survival in (A) whole blood, (C) normal serum and (D) the macrophage intracellular environment. (B) Heterologous expression of SIC in M49 GAS promotes bacterial growth in whole blood. For whole blood, experiments were performed in triplicate and repeated five times with similar results. Growth index was calculated by dividing the surviving CFU by the initial inoculum. A representative experiment is shown with the mean ± SEM. For serum, experiments were performed in duplicate and repeated four times with similar results. Mean ± SEM of four experiments is shown. For intracellular survival, each experiment was performed with six replicates, then repeated three times with similar results. Relative survival was calculated by comparing surviving CFU to the inoculum and normalizing all time points to the GAS M1T1 (AP) 1 h time point. Significance is indicated by the following: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ according to Student's unpaired *t*-test.

SIC accelerates GAS-induced mortality in a murine systemic model of infection. To determine the effect of SIC expression to the course of systemic GAS infection, 8-week-old CD-1 mice were administered intraperitoneal injections of M1 GAS (AP) or M1 GAS (AP) Δ*sic* or for heterologous expression studies, M49 GAS or M49 GAS expressing SIC, and the kinetics of their survival was monitored over time. Mice injected with the Δ*sic* mutant survived significantly longer than those challenged with the SIC-expressing strain (50% vs. 100% mortality at 24 h), suggesting a significant contribution of SIC to the overall virulence of the hyper-invasive *covS* mutant form of M1T1 GAS (**Figure 2.5A**). In addition, mice injected with M49 GAS survived significantly longer than those infected with M49 GAS expressing SIC, further demonstrating the contribution of SIC in invasive GAS infection (**Figure 2.5B**). We next wondered whether preservation of SIC, which itself is a target of SpeB-mediated proteolytic degradation (Aziz et al., 2004), might be a selective force favoring *in vivo covS* mutations to eliminate *speB* expression. Two strains of mice (CD-1 and C57BL/6J) were injected subcutaneously with either the M1T1 GAS strain or its isogenic Δ*sic* mutant (n = 10 in each of the 4 groups). After 72 h, resulting lesions were excised to identify SpeB-negative colonies indicative of phase-switching to the *covS* mutant phenotype (**Figure 2.5C**). In both genetic backgrounds, SpeB-negative mutants were readily identified among the 50 bacterial colonies isolated from individual mice infected with the M1T1 GAS Δ*sic* mutant, and no statistically significant difference in the frequency of *covS* phase-switching was found comparing the Δ*sic* mutant to the parent M1T1 GAS strain. We conclude that in contrast to findings reported for the bacteriophage-encoded M1T1 GAS DNase Sda1 (Walker et

al., 2007), the presence of SIC is not required to maintain the selection pressure favoring *covR/S* mutation *in vivo*.

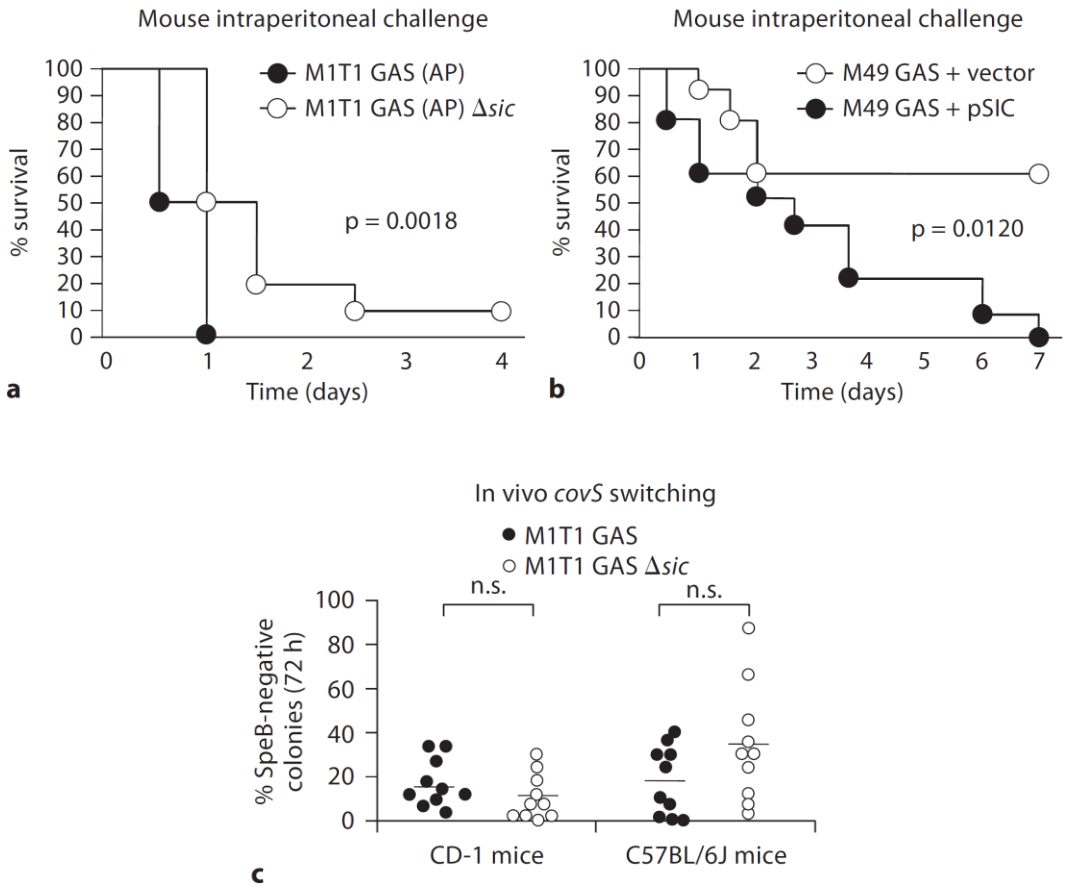

Figure 2.5. SIC promotes GAS virulence in murine models. Eight-week-old male CD-1 mice were injected intraperitoneally with (A) 2.5 x 10^6 CFU of M1T1 GAS (AP) or M1T1 GAS (AP) Δ*sic* + 5% mucin or (B) 1.3 x 10^7 CFU of M49 GAS or M49 GAS expressing SIC + 5% mucin (*n* = 10). Survival was monitored for 4 or 7 days. Statistics were performed using the log-rank (Mantel-Cox) test. (C) *In vivo covS* switching of GAS M1T1 vs. GAS M1T1 Δ*sic* as assessed by loss of SpeB activity 72 h after subcutaneous injection; no significant difference between WT and Δ*sic* mutant in either the CD-1 or C57BL/6J mouse background.

DISCUSSION

We used targeted mutagenesis and heterologous expression to demonstrate that SIC contributes to the human cathelicidin antimicrobial peptide, whole blood, and serum resistance phenotypes of invasive M1T1 GAS. These virulence phenotypes occur upon strong transcriptional upregulation of the *sic* gene following *covS* mutations selected *in vivo* during the transition to invasive infection. Ultimately, *sic* can be functionally validated to be part of critical suite of virulence factor genes that are upregulated during *covS* mutation and invasive M1T1 GAS infection, including those encoding hyaluronic acid capsule (Ravins et al., 2000), DNase Sda1 (Walker et al., 2007), streptolysin O, and interleukin-8 protease SpyCEP, that together promote innate immune resistance and systemic dissemination (Aziz and Kotb, 2008; Sumby et al., 2006).

SIC promoted GAS M1T1 survival in murine macrophages and enhanced the progression of invasive infection during mouse systemic infection. These results suggest at least some limited utility of murine models for further analyzing SIC virulence functions or evaluating therapeutic interventions directed against SIC. We observe that the 1.84 allelic variant of SIC binds both human and murine cathelicidin, although in the intact organism its influence on LL-37 resistance is clearly more pronounced than its influence on CRAMP resistance. Theoretically, a virulence contribution of SIC binding to CRAMP could manifest *in vivo* upon greater accumulation of the secreted virulence factor in established foci of infection or by effects upon other pro-inflammatory or immunostimulatory functions of the cathelicidin peptide (Lai and Gallo, 2009; Radek and Gallo, 2007). The observed SIC contribution to mouse virulence could also derive from enhanced survival in

macrophages, enhanced serum resistance or other yet to be determined pathogenic effects.

Cathelicidin is a critical aspect of host defense against invasive GAS infection (Nizet et al., 2001), and serotype M1 GAS strains show higher intrinsic resistance levels to LL-37 killing than GAS of other serotypes uncommonly associated with invasive infection (Lauth et al., 2009). The expression of two LL-37 binding/sequestering molecules, namely SIC and the N-terminal domain of M1 protein (Lauth et al., 2009), pose a significant challenge for our innate immune system attempting to restrict the spread of M1T1 GAS infection. The unusually high allelic variation of SIC in M1 GAS may parallel that of M protein across all serotype strains -- evidence of an ongoing evolutionary battle of pathogen and host, with M1T1 GAS seeking to preserve pro-survival functions of the SIC peptide while escaping host neutralizing antibody responses.

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CHAPTER 3.

The metalloprotease aureolysin is not essential for cathelicidin resistance or virulence of methicillin-resistant *Staphylococcus aureus*

ABSTRACT

Staphylococcus aureus is a pre-eminent human pathogen with methicillin-resistant (MRSA) strains representing an ever increasing threat to the public health in both hospital and community settings. *S. aureus* produces multiple extracellular proteases including the broad-spectrum metalloprotease aureolysin. Several studies have proposed virulence roles for aureolysin, the majority of them by analysis of the purified protein and its activities. For example, purified aureolysin cleaves human cathelicidin antimicrobial peptide (AMP) LL-37, degrades complement component C3, activates the fibrinolytic system and inhibits IgG production. In this study, we used targeted mutagenesis of the encoding *aur* gene coupled with *in vitro* and *in vivo* model systems to probe potential contributions of this metalloprotease to virulence of contemporary MRSA strains. Although loss of aureolysin diminished the ability of MRSA culture supernatants to degrade human and murine cathelicidin AMPs, no differences were detected in the susceptibility of the Δ*aur* mutants vs. the wild-type (WT) parent strains to cathelicidin killing. Furthermore, WT and isogenic Δ*aur* mutant bacteria demonstrated comparable survival in human serum and whole blood, resistance to neutrophil killing, epithelial cell adherence, and virulence in murine

subcutaneous and systemic challenge models. We conclude that among a large array of virulence and immune evasion factors expressed by contemporary MRSA strains such as the USA300 clone, aureolysin does not likely represent a critical contributor to disease pathogenesis.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a major human bacterial pathogen associated with a wide range of clinical disease, ranging from mild superficial skin conditions to invasive blood-borne infections with high associated morbidity and mortality. Methicillin-resistant strains of *S. aureus* (MRSA) have for decades been a major cause of nosocomial infections in hospitals throughout many parts of the world. In recent years, community-associated MRSA strains, exemplified by the USA300 clone, have attained epidemic status in the United States and are disproportionately associated with severe forms of invasive disease including necrotizing pneumonia, necrotizing fasciitis and sepsis (Francis et al., 2005; Gonzalez et al., 2005; Miller et al., 2005). The high incidence and potential severity of MRSA infections reflect the coordinated activities of several known virulence factors that promote tissue spread, facilitate resistance to innate immune clearance and/or exert toxic effects upon human cells and organ system physiology.

Cathelicidins are cationic antimicrobial peptides (AMPs) produced by epithelial cells and leukocytes of the mammalian host. The human (LL-37) and murine (CRAMP) cathelicidins share similar encoding genes, tissue distribution, amphipathic α-helical structures, spectrum of bactericidal activity, and pro-inflammatory properties (Nijnik and Hancock, 2009; Nizet and Gallo, 2003; Zanetti, 2005). Cathelicidin expression is induced in response to injury or infection, and these peptides provide a critical front line element of innate immunity, evidenced by the increased susceptibility of cathelicidin-deficient mice in a variety of bacterial infection models (Chromek et al., 2006; Huang et al., 2007; Iimura et al., 2005; Nizet et al., 2001). *S. aureus* strongly induces cathelicidin LL-37 expression in cultured human

cells (Braff et al., 2005; Komatsuzawa et al., 2006), and studies suggest that MRSA strains are more resistant to LL-37 killing than are methicillin-sensitive *S. aureus* strains (Midorikawa et al., 2003; Ouhara et al., 2008). Understanding the fundamental mechanisms by which MRSA exhibits reduced susceptibility to cathelicidin AMP killing could inform improved strategies to combat this leading public health challenge.

Aureolysin is the major metalloprotease of *S. aureus.* Initially produced as a prepropeptide, aureolysin is subsequently activated through autocatalysis (Nickerson et al., 2008). The metalloprotease is strongly conserved at the genetic level and occurs in two allelic forms, type I and type II (Sabat et al., 2000). These aureolysin alleles differ by ~10% (Sabat et al., 2008) and display similar or differing activity levels based on the substrate used (Sabat et al., 2008; Takeuchi et al., 2002). Purified aureolysin has been characterized biochemically and found to be capable of degrading host targets such as the fibrinolytic system proteins plasminogen and pro-urokinase (Beaufort et al., 2008) or complement factor C3 (Laarman et al., 2011) and also of blunting lymphocyte production of immunoglobulin (Prokesova et al., 1991).

A key role for aureolysin in *S. aureus* resistance to cathelicidin AMPs was proposed when the purified metalloprotease was shown to cleave human LL-37 in a time- and concentration-dependent manner (Sieprawska-Lupa et al., 2004). Moreover, *in vitro* analyses have demonstrated that aureolysin is produced following phagocytosis of *S. aureus* by human neutrophils (Burlak et al., 2007). Neutrophils represent a critical first line element of host innate immune defense against invasive bacterial pathogens, and possess abundant LL-37 within their specific granules to deploy as part of their antibacterial arsenal. In the present work, we sought to

determine for the first time if aureolysin contributed to *S. aureus* AMP and neutrophil resistance phenotypes in the context of the intact living bacterium, using isogenic strains generated through targeted mutagenesis of the encoding gene (*aur*) in two strains of the hypervirulent USA300 clone of community-associated MRSA (CA-MRSA) as well as a prototypical hospital-associated (HA-MRSA) strain from the epidemic EMRSA-16 clone prevalent in UK health care facilities.

MATERIALS AND METHODS

Cathelicidin cleavage. Supernatants from overnight cultures grown in TSB were filter sterilized through a 0.22 μm syringe-driven filter (Millipore). Eighteen μl of filtered supernatant were incubated with 2 μl of LL-37 or CRAMP (AnaSpec) to give a final concentration of 16 μM (except for the LAC/LL-37 combination, in which 8 μM was used). To determine the contribution of different classes of proteases, the following inhibitors were added: EDTA (5mM) or 1, 10 phenanthroline (10 mM) to inhibit metalloproteases, E-64 (10 μg/ml) to inhibit cysteine proteases and PMSF (1 mM) to inhibit serine proteases. Samples were incubated at 37˚C for 24 h, mixed with 4X sample buffer and 10X reducing agent (Invitrogen), boiled for 10 min, loaded onto a 12% Bis-Tris gel (Invitrogen) and run in MES running buffer at 120 V. Gels were stained with SimplyBlue SafeStain (Invitrogen) and subsequently destained in H_2O overnight.

Bacterial strains and generation of *aur* mutants. The wild-type strains UAMS1182 and LAC are both isolates of the methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 clone. A markerless *aur* deletion was created in each strain by transduction of the pKOR1Δ*aur* plasmid (Kavanaugh et al., 2007) by 80α phage and utilization of the pKOR1 knockout strategy, previously described in (Bae and Schneewind, 2006).

Wild-type MRSA252 (Sanger252) is a hospital-associated MRSA strain. A fragment 5’ to the *aur* gene was amplified using primers upF.EcoRI (5’-gtcagaattccttgtgtacacgtgatac-3’) and upR (5’-tttaaaatttcaggaggaatgaaataaatatataaacaagaagaag-3’), which included a 25 bp overhang for the 3’ region. A fragment 3’ to the *aur* gene was amplified using primers

dnF (5'-attacttcttcttgtttatatatttatttcattcctcctgaaattttaaaaaca-3'), which included a 27 bp overhang for the 5' region, and dnR.EcoRI (5'-gtcagaattcgtcagacattgcatggcatcatgctttc-3'). A "double" fusion PCR product was created by using both fragments as template and primers upF.EcoRI and dnR.EcoRI. The "double" PCR product was TA cloned into pCR2.1-TOPO (Invitrogen) and subsequently subcloned into the pKOR1 knockout vector. A markerless *aur* deletion was constructed using the pKOR1-Δ252*aur* plasmid as described in (Bae and Schneewind, 2006).

For complementation studies, the type II allele of the *aur* gene was amplified from UAMS1182 genomic DNA by polymerase chain reaction (PCR) using primers aurForw.XbaI (5'-gcgtctagagtgaggaaattttcaagatatgcatttacaag-3') and aurRev.BamHI (5'-gcgggatcctttactccacgcctacttcattcca-3'). The PCR product was digested and subcloned into shuttle expression vector pDC123 (Chaffin and Rubens, 1998). The type I allele of the *aur* gene was amplified from the MRSA252 genome using primers aurF (5'-ttactcgacgccaacttcgttcc-3') and aurR (5'-caacgaacaaaaagtataga-3'), the PCR amplicon TA cloned into pCR2.1-TOPO (Invitrogen), then subsequently subcloned into pDC123.

Aureolysin activity. Twenty-five µl of overnight cultures were dropped onto TSA plates containing 15% ultra-high-temperature (UHT) non-fat milk (Gossner Foods, Inc) and incubated at 37˚C overnight to allow for development of zones of clearing.

Cathelicidin killing kinetics. Bacterial cultures were grown to mid-log phase in RPMI + 5% TSB. Bacteria were resuspended to 10^5 CFU/ml in the same buffer, and 225 µl of bacteria were added to 25 µl of 160 µM or 320 µM LL-37 in Eppendorf

tubes, giving a final concentration of 16 μM or 32 μM LL-37. Tubes were placed on a rotisserie at 37°C, and 25 μl was removed at each time point to be diluted and plated for enumeration of CFU.

Adherence to keratinocytes. One day prior to the experiment, HaCaT cells were plated at a density of 2 x 10^5 cells/well in a 24-well plate (500 μl total volume). On the day of the experiment, late-log phase bacteria were resuspended in RPMI + 2% FBS. Two million CFU were added to each well, resulting in a multiplicity of infection (MOI) of 10. Plates were centrifuged at 500 x g for 5 min and subsequently incubated at 37˚C + 5% CO_2 to allow bacteria to adhere to cells. After 30 min, media was removed, and wells were washed 3X with 1 ml of DPBS. One hundred microliters of trypsin-EDTA were added to each well and plates were incubated at 37˚C for 10 min to facilitate detachment of the cells. Four hundred μl of 0.025% Triton X were added to each well, and wells were triturated for 20 sec to lyse cells. Serial dilutions were plated on THA for enumeration of CFU.

Human neutrophil killing. Blood was drawn from healthy donors after informed consent, and neutrophils were isolated using the PolyMorphPrep kit as per the manufacturer's instructions (Axis-Shield, Norway). In 96-well plates, 100 μl containing 2 x 10^5 neutrophils in RPMI + 2% autologous heat-inactivated plasma were added to 100 μl containing 2 x 10^4 CFU of late-log phase bacteria resuspended in the same media. The plates were centrifuged at 500 x *g* for 5 min and incubated at 37°C + 5% CO_2 for 30 min. After incubation, the neutrophils were lysed hypotonically with H_2O, diluted and plated for enumeration of CFU. Percent survival was calculated as [CFU/ml experimental well]/[CFU/ml control well] x 100.

Human whole blood killing. Blood was drawn from healthy donors after informed consent, and 10^5 (MRSA252) or 10^7 (UAMS1182, LAC) CFU of late-log phase bacteria in 100 µl DPBS were added to a) 900 µl heparinized whole blood, b) 800 µl heparinized whole blood + 100 µl supernatant from overnight cultures or c) 900 µl whole blood drawn with lepirudin. Siliconized tubes were placed on a rotisserie at 37°C, and 25 µl was removed at each time point to be diluted and plated for enumeration of CFU.

Human serum assay. Blood was drawn from healthy human volunteers after informed consent. Normal human serum was prepared by pooling sera of four different donors and stored at -80°C. Overnight cultures of bacteria were diluted 1:1,000 in RPMI. Fifty microliters of bacteria were mixed with 50 µl of serum in RPMI in 96-wells plates. Plates were incubated, shaking, for 6 h at 37°C, and surviving bacteria were enumerated by plating serial dilutions on THA. Percent survival was calculated by normalizing to a no serum control.

Murine skin abscess model. Eight-week-old female CD-1 mice (Charles River Laboratories, *n* = 10) were shaved and treated with Nair for hair removal on the day prior to injection. Mice were injected subcutaneously with 3.6 x 10^6 CFU of mid-log phase UAMS1182 or UAMS1182 Δ*aur* bacteria suspended in 100 µl of DPBS. Lesion sizes were monitored for 4 d, at which time mice were sacrificed. Lesions were excised, weighed and homogenized in a Mini-BeadBeater-8 (Biospec Products). Homogenates were serially diluted in PBS and plated on THA for enumeration of bacteria in the lesion.

Murine systemic infection model. Eight-week-old female CD-1 mice (*n* = 8) were injected intraperitoneally with 9 x 10^6 CFU of mid-log phase UAMS1182 or

UAMS1182 Δ*aur* bacteria suspended in 200 µl of DPBS + 5% mucin. Survival was monitored for 4 d, and time of death was recorded for each mouse.

Plasminogen and C3 cleavage. Human and mouse plasminogen were purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany) and Haematologic Technologies Inc (Essex Junction, Vermont, USA) respectively. Human C3 was purified from human plasma as described previously (Rooijakkers et al., 2009). Murine C3 was purified from mouse serum as described (Van den Berg et al., 1989). Plasminogen (1 µM) was incubated with aureolysin (0.1 or 1 µM, Biocentrum) for 5 or 24 h at 37°C in Veronal Buffered Saline containing 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$ (VBS^{++}). C3 (0.1 µM) was incubated with aureolysin (0.1 or 1 µM) for 1 hour at 37°C in VBS^{++}. Reactions were stopped by adding SDS sample buffer and proteins were analyzed by SDS-PAGE and Instant Blue (Gentaur) staining.

Statistical analyses. LL-37 kinetics, whole blood, serum, neutrophil and adherence assays were compared using the Student's *t*-test (LAC and MRSA252 strains) or one-way ANOVA with Bonferroni post-test (UAMS1182 strains). Lesion size and CFU counts for the murine abscess model were compared using the Student's *t*-test. The murine intraperitoneal challenge assay was assessed using the log-rank (Mantel Cox) test. All statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). *P* values < 0.05 were considered statistically significant.

Ethics permissions. Permission to collect human blood under informed consent was approved by the UCSD Human Research Protections Program. All

animal experiments were conducted according to the guidelines approved by the UCSD Institutional Animal Use and Care Committee.

RESULTS

MRSA produces a metalloprotease capable of cleaving cathelicidin. To verify that a representative CA-MRSA USA300 strain harbors proteolysin activity capable of targeting cathelicidin peptides, we incubated overnight cell-free supernatant isolated from cultured MRSA with human cathelicidin LL-37 or the related murine cathelicidin peptide CRAMP. A proteolytic activity targeting both cathelicidin compounds was observed, and this activity could be blocked by an inhibitor of metalloproteases but not by inhibitors of cysteine or serine proteases (**Figure 3.1**). This result corroborated earlier findings with purified recombinant aureolysin, and encouraged us to pursue targeted gene deletion and construct aureolysin-deficient mutants (Δ*aur*) in three different MRSA backgrounds.

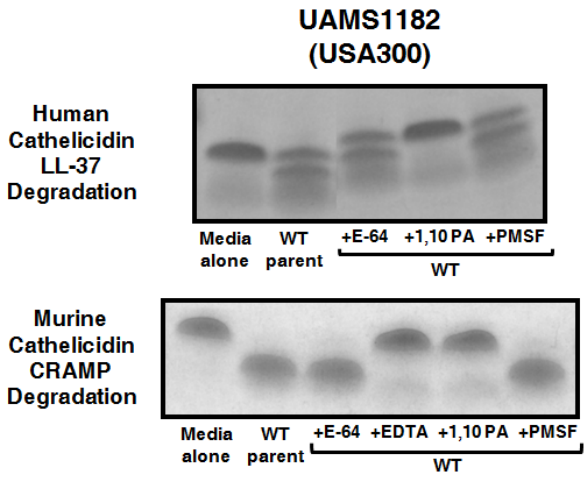

Figure 3.1. MRSA produces a metalloprotease with cathelicidin degrading properties. Overnight cell-free supernatants from WT USA300 MRSA strain UAMS1182 were incubated with cathelicidins LL-37 or CRAMP in the presence of E-64 to inhibit cysteine proteases, ETDA and 1, 10PA to inhibit metalloproteases or PMSF to inhibit serine proteases. Degradation of LL-37 or CRAMP was analyzed by SDS-PAGE. Abbreviation: 1,10 PA - phenanthroline

Generation of isogenic aureolysin deficient strains. To further investigate the role of aureolysin in the context of the living pathogen, we created a precise in-frame deletion of *aur* in two separate CA-MRSA USA300 backgrounds, UAMS1182 and LAC, as well as in a HA-MRSA background, MRSA252. In addition, UAMS1182 Δ*aur* was complemented with its own type II allele (p1182*aur*), and MRSA252 Δ*aur* was complemented with its own type I allele (p252*aur*). To confirm the presence or absence of aureolysin activity, overnight cultures were dropped onto TSA milk plates to evaluate zones of clearing resulting from casein degradation. Wild-type (WT) strains harboring the *aur* gene created zones of clearing, while the mutants lacking *aur* did not (**Figure 3.2A**). Additionally, complementation of the *⊿aur* mutant strains with the *aur* gene expressed on a plasmid vector restored substantial caseinolytic activity, though not fully equivalent to that produced by the WT strain.

Aureolysin cleaves cathelicidins but does not determine MRSA resistance to AMP activity. To demonstrate that aureolysin cleaves cathelicidin, we incubated cell-free supernatants from overnight cultures with either 8 or 16 μM LL-37 or 16 μM CRAMP. Samples were subsequently analyzed by SDS-PAGE. Strains containing the *aur* gene were able to cleave both LL-37 and CRAMP, while the isogenic mutant strains lacking the *aur* gene displayed absent or markedly reduced activities against each peptide (**Figure 3.2B**).

Upon demonstrating that aureolysin is capable of cleaving cathelicidin, we sought to determine if it contributes to MRSA resistance against these peptides. Strains were incubated with decreasing concentrations of LL-37 or CRAMP, and the absorbance at 600 nm (A_{600}) read at 24 h to determine the minimum inhibitory concentrations (MICs). A small aliquot from each well was subsequently plated and

incubated overnight to determine the minimum bactericidal concentrations (MBCs). Though we demonstrated that aureolysin cleaves cathelicidin, loss of aureolysin did not contribute to an increased sensitivity to cathelicidin the context of the living bacterium (**Table 3.1**). For further verification, we repeated the MIC and MBC testing for the LAC WT and Δ*aur* mutant strains with the addition of overnight supernatants, in case accumulated secreted aureolysin absent in the first round would produce a different result. However, even with the addition of supernatants, the MICs remained the same, and there was a slight increase in the MBC of LAC Δ*aur* against CRAMP. In the MRSA252 background, no differences were seen in resistance to LL-37, and the mutant was two-fold more resistant, rather than sensitive, to CRAMP (MIC 32 μM vs. 16 μM, **Table 3.1**).

In addition to MIC/MBC assays, we further investigated whether aureolysin expression might delay the kinetics of LL-37 killing of MRSA by monitoring bacterial survival over time. WT and Δ*aur* mutant MRSA strains were incubated with 16 μM LL-37, and CFU plated at specific time points over 3 h to determine the rate of killing. We found no difference in the LL-37 killing kinetics in any of the three backgrounds (**Figure 3.3A-C**).

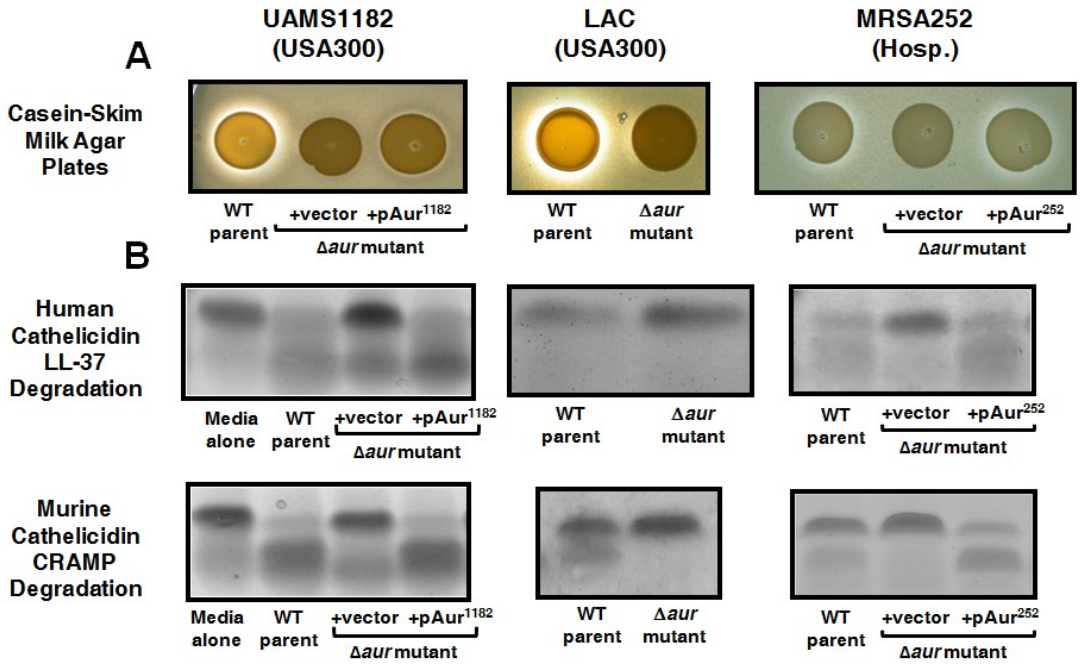

Figure 3.2. Effect of aureolysin on casein and cathelicidin cleavage in different MRSA backgrounds. (A) Protease activity as assessed by zones of clearance on TSA milk plates. (B) Degradation of LL-37 or CRAMP by overnight cell-free supernatants analyzed by SDS-PAGE.

Table 3.1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of human (LL-37) and murine (CRAMP) cathelicidin antimicrobial peptides against wild-type (WT) methicillin-resistant *Staphylococcus aureus* (MRSA) strains, isogenic aureolysin-deficient (Δ*aur*) mutants, and plasmid complemented strains.

	Human Cathelicidin LL-37		Murine Cathelicidin CRAMP	
MRSA Strain	**MIC (μM)**	**MBC (μM)**	**MIC (μM)**	**MBC (μM)**
UAMS1182 (USA300)	8	32	4	16
Δ*aur* mutant	8	32	4	16
Δ*aur* + pAur^{1182}	8	32	4	16
LAC (USA300)	16	32	8	32
Δ*aur* mutant	16	32	8	32
WT + supernatant	16	32	16	32
Δ*aur* + supernatant	16	32	16	64
MRSA252 (Hosp.)	8	64	16	> 64
Δ*aur* mutant	8	64	32	> 64
Δ*aur* + pAur^{252}	8	64	16	> 64

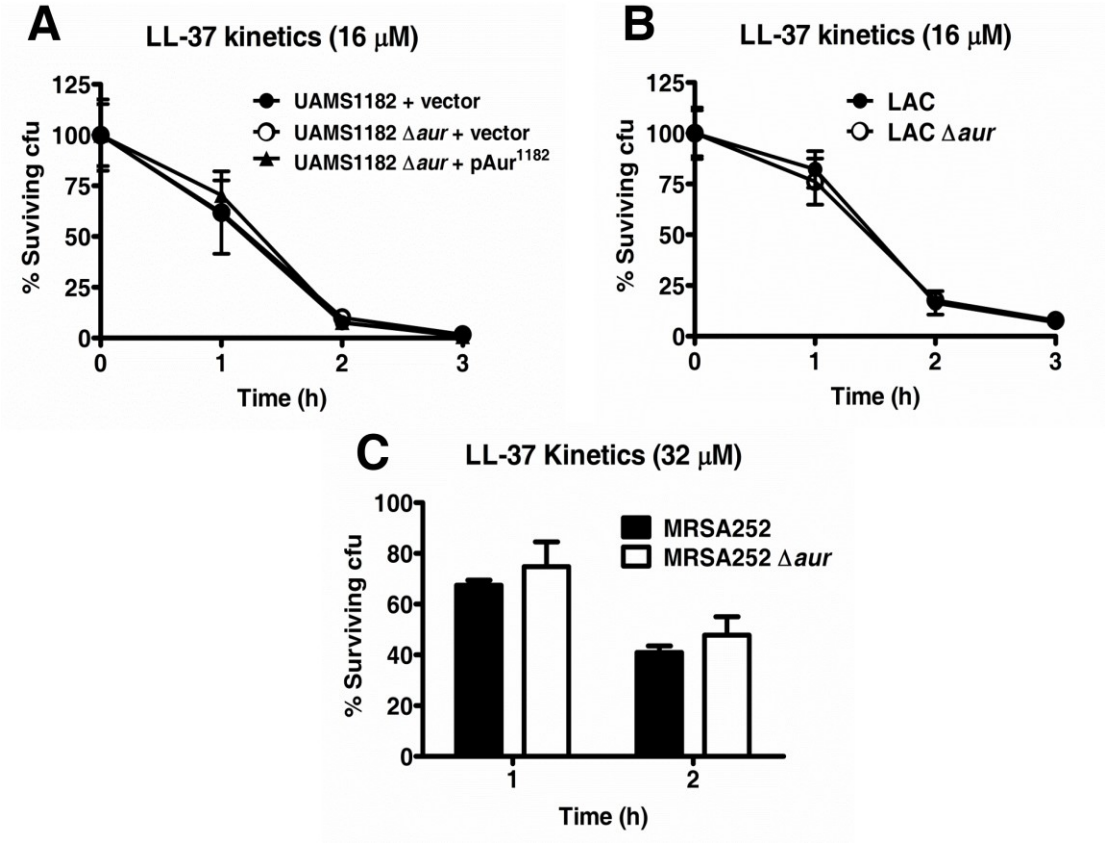

Figure 3.3. Aureolysin does not contribute to MRSA LL-37 resistance. MRSA strains (A) UAMS1182, (B) LAC and (C) MRSA252 were incubated with the specified concentrations of LL-37. Experiments were repeated three times with similar results. A representative experiment is shown, including the mean ± SD.

Aureolysin does not contribute to MRSA survival in human whole blood. To extend our analysis of a potential role of aureolysin in MRSA innate immune resistance beyond individual cathelicidin peptides, we examined the contribution of aureolysin to bacterial survival in human whole blood. We found no difference in the kinetics of killing in heparinized whole blood between the WT and Δ*aur* strains in any of the MRSA backgrounds (**Figure 3.4A-C**). As performed before with the MICs, the assay was repeated for the USA300 LAC strain pair with the addition of overnight culture supernatants, but still no survival difference was observed (**Figure 3.4D**). As an alternative to heparinized blood, we also tested blood drawn with lepirudin, which does not interfere with complement activation. However, WT and Δ*aur* mutant bacterial survival rates remained the same (**Figure 3.4E**). Thus, we conclude that aureolysin expression does not contribute significantly to MRSA survival in human whole blood.

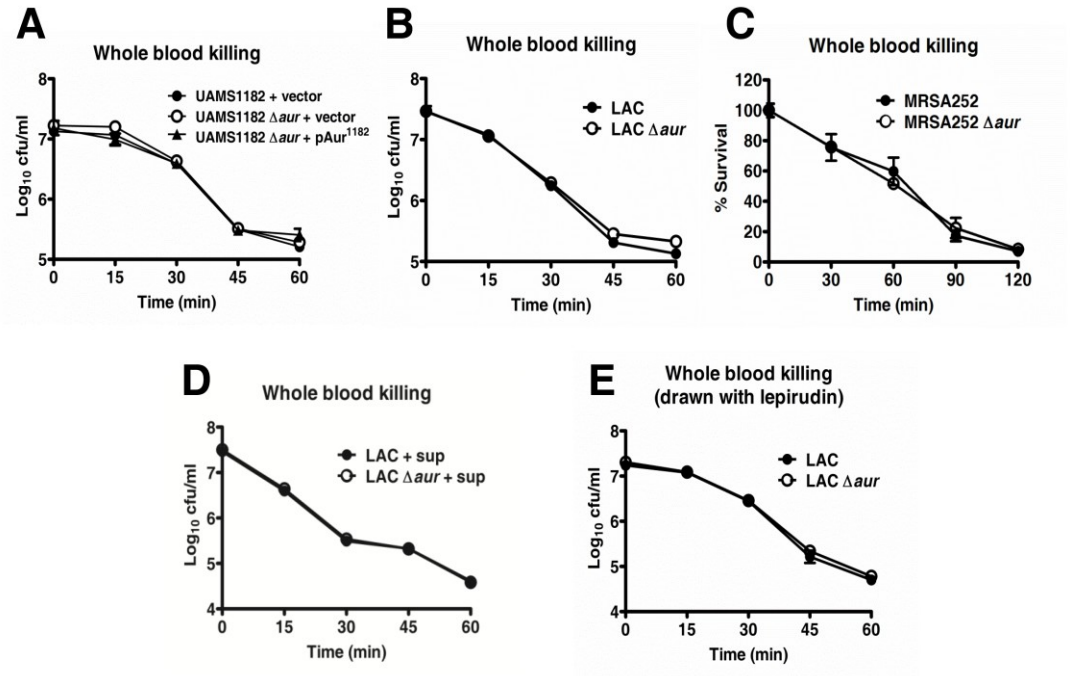

Figure 3.4. Aureolysin does not contribute to MRSA survival in human whole blood. (A) UAMS1182, (B) LAC and (C) MRSA252 strains were incubated with heparinized blood. (D) The experiment was repeated for the LAC strains with the addition of bacterial supernatants. (E) LAC strains were incubated with blood drawn with lepirudin to preserve complement factors. Experiments a, b, d and e were performed in triplicate and repeated three times with similar results. A representative experiment is shown with the mean ± SD. The MRSA252 experiment was performed in triplicate and repeated two times with similar results. Pooled data from both experiments is shown with the mean ± SEM.

Aureolysin does not contribute to MRSA resistance to human neutrophils or serum. As human neutrophils are a significant source of antimicrobial molecules, we tested whether WT and Δ*aur* mutant bacteria were differentially sensitive to neutrophil killing. No significant differences were seen between WT and Δ*aur* mutant in any of the MRSA backgrounds (**Figure 3.5A**). Additionally, to test complement-mediated killing, bacteria were incubated with normal human serum pooled from four donors and incubated for 6 h. However, once again, no difference in bacterial survival was observed (**Figure 3.5B**). Thus, we conclude that aureolysin is not required for survival with human neutrophils or serum.

Aureolysin does not contribute to MRSA adherence to human keratinocytes. A proximal step in MRSA pathogenesis is adherence to the human upper respiratory tract or skin epithelium. MRSA were brought into contact with cultured HaCaT keratinocytes and incubated for 30 min. Cells were subsequently lysed and CFU enumerated. We observed no differences in adherence rates between parental strains and their corresponding *aur* mutants (**Figure 3.5C**).

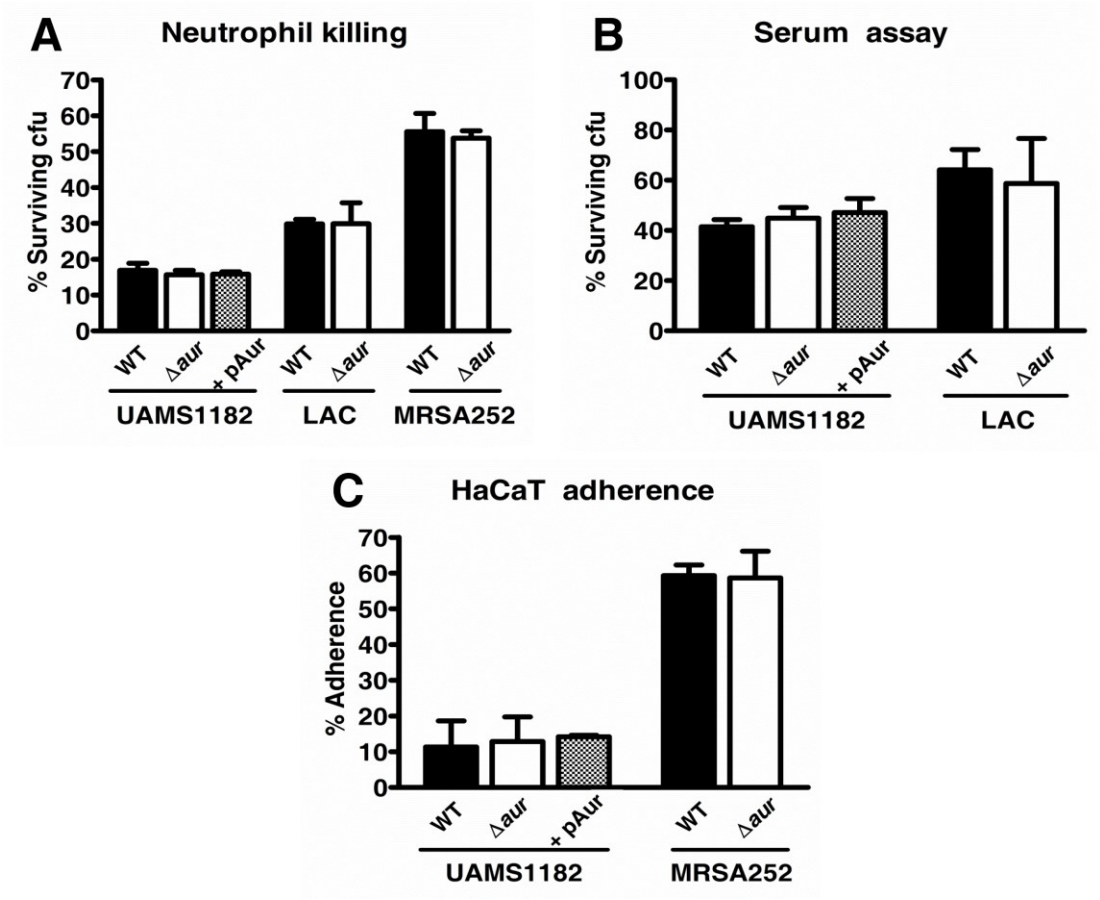

Figure 3.5. *In vitro* analyses of aureolysin. The aureolysin mutant does not differ significantly from the parental strain in susceptibility to (A) human neutrophil killing or (B) killing by human serum. (C) Wild-type and Δ*aur* strains adhere to HaCaT keratinocytes at similar levels. Experiments were performed in triplicate and repeated three times with similar results. A representative experiment is shown with the mean ± SD.

Aureolysin does not contribute to MRSA virulence *in vivo*. To determine whether aureolysin contributes to virulence in an animal model, we first employed a murine skin abscess model, as USA300 is a major cause of skin and soft tissue infections (King et al., 2006). CD-1 mice were injected subcutaneously on the left flank with WT CA-MRSA strain UAMS1182 and on the right flank with the corresponding isogenic Δ*aur* mutant. Lesion size was monitored for 4 d, and on the fourth day, mice were sacrificed and bacterial numbers were enumerated. No differences were observed between the WT and Δ*aur* MRSA strains in lesion sizes or CFU recovered (**Figure 3.6A, B**).

As a model of invasive systemic MRSA disease, we infected mice intraperitoneally with UAMS1182, its corresponding *aur* mutant, or the complemented strain. Survival was monitored every 12 h for 4 d. We observed no difference in survival rates between the isogenic MRSA strains (**Figure 3.6C**), indicating that aureolysin does not play a measureable role in the pathogenesis of invasive disease in this murine model.

Aureolysin cleaves mouse plasminogen but not mouse C3. To investigate whether species specificity could explain the lack of virulence in mice, we incubated purified aureolysin with human and mouse plasminogen as well as human and mouse complement C3, two substrates that aureolysin has previously been shown to cleave. We demonstrated cleavage of human plasminogen as reported in (Beaufort et al.) and showed that aureolysin was able to cleave mouse plasminogen as well (**Figure 3.7A**).

As for complement degradation, we observed cleavage of human C3 as previously published in (Laarman et al., 2011). However, mouse C3 was not cleaved

by aureolysin (**Figure 3.7B)**. This may be due to sequence variation at the proposed cleavage site. In human C3, the cleavage site is RSN#LDE where in mouse the same site contains RSE#LEE. The presence of a glutamic acid residue in place of the asparagine residue may make the site no longer recognizable by aureolysin.

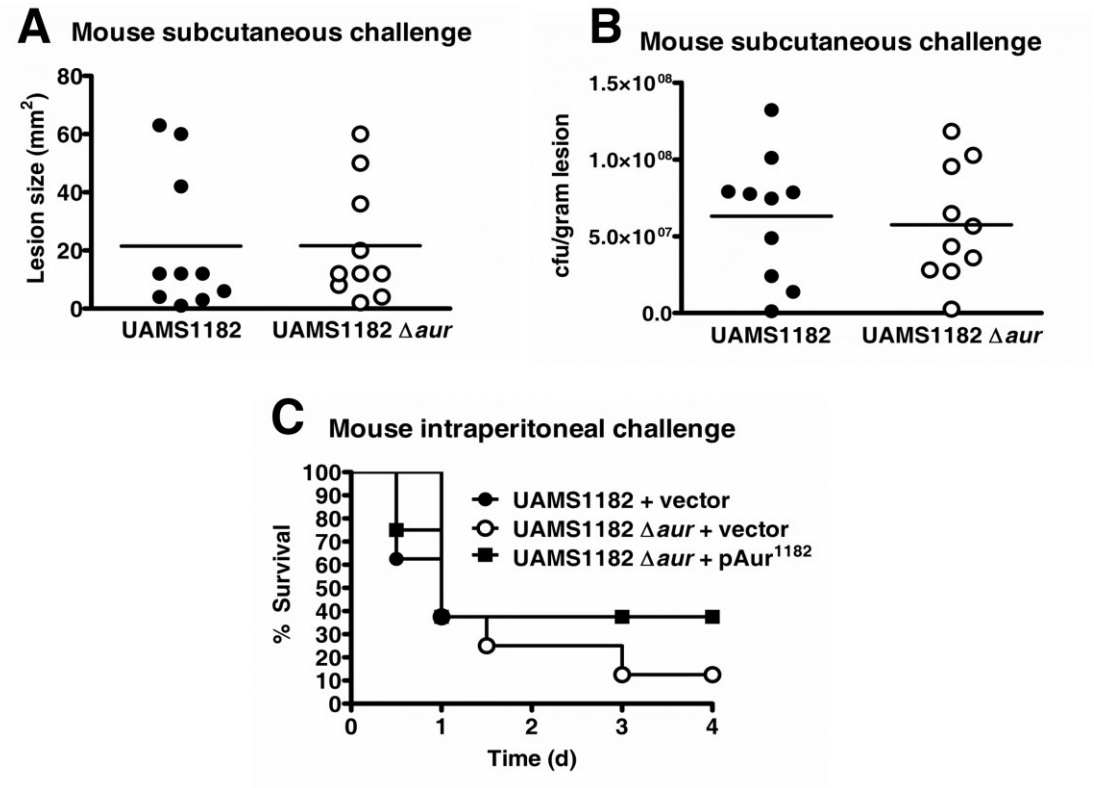

Figure 3.6. Aureolysin does not contribute to MRSA virulence *in vivo*. Eight-week-old female CD-1 mice were injected subcutaneously on one flank with WT MRSA and on the alternating flank with Δ*aur* (*n* = 10). (A) Lesion size was monitored each day for 4 days. (B) On day 4, bacteria were enumerated. (C) As a systemic model, mice were injected intraperitoneally with WT MRSA, Δ*aur* or the complemented strain (*n* = 8). Survival was monitored for 4 days.

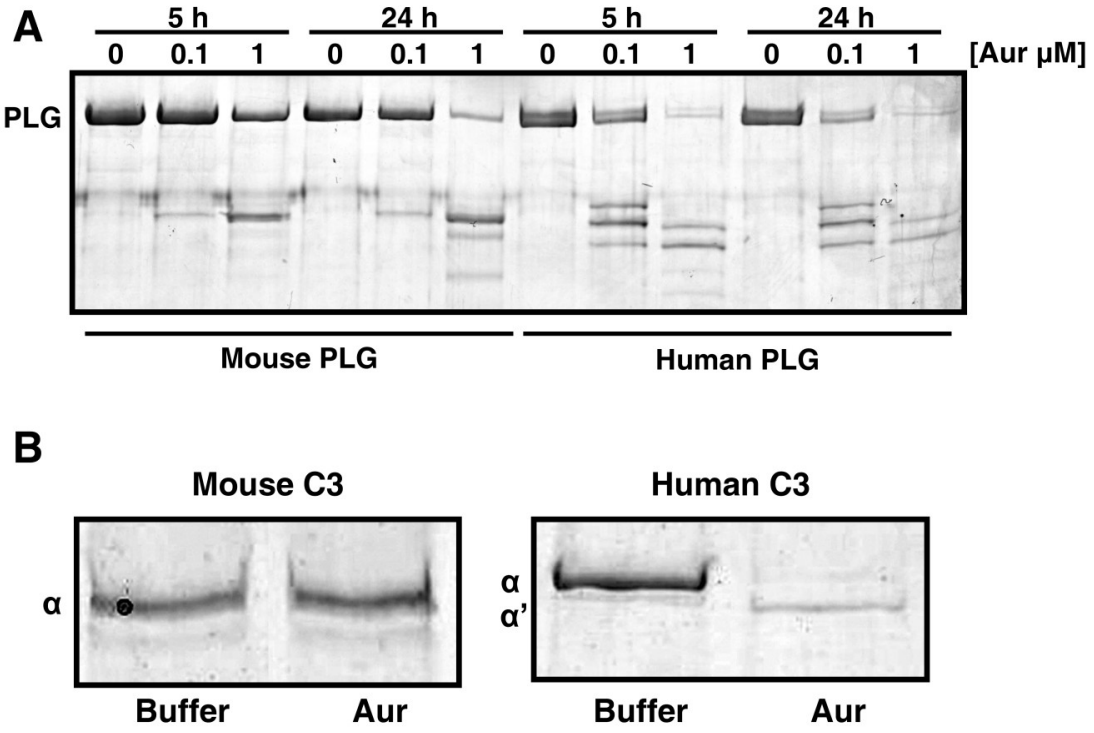

Figure 3.7. Aureolysin cleaves mouse plasminogen but not mouse C3. Aureolysin was incubated with (A) mouse or human plasminogen or (B) mouse or human C3 and results were analyzed by SDS-PAGE and Instant Blue staining.

DISCUSSION

Biochemical and genetic studies can play complementary roles in analysis of bacterial virulence factors. Previous biochemical analyses had indicated that purified *S. aureus* metalloprotease aureolysin can degrade host cell targets such as cathelicidin LL-37, plasminogen and complement factor C3 (Beaufort et al., 2008; Laarman et al., 2011; Sieprawska-Lupa et al., 2004), prompting speculation of a prominent role in immune evasion or virulence of the pathogen. Here we used allelic replacement mutagenesis, *in vitro* and tissue culture assays, and murine infection studies to assess whether aureolysin contributed to immune resistance phenotypes of two strains of the CA-MRSA USA300 clone and a prototypical HA-MRSA strain. While we found that deletion of the *aur* gene produced clear reductions in caseinolytic activity around bacterial colonies and that supernatants from the mutant bacteria had markedly diminished ability to degrade human and murine cathelicidins, loss of the metalloprotease did not produce a significant change in the overall cathelicidin resistance, whole blood survival, neutrophil resistance, serum resistance, skin epithelial cell adherence, nor animal virulence phenotypes of the MRSA strains. If the lack of a phenotype in mouse models is due to species specificity, aureolysin-associated virulence must be due mainly to C3 cleavage since we have demonstrated that aureolysin is unable to cleave mouse C3 but is able to cleave mouse plasminogen and the mouse cathelicidin, CRAMP. Alternatively, aureolysin may not inhibit IgG production in mice as it does in human cells (Prokesova et al., 1991), but this was not explored in this study.

S. aureus, and the USA300 CA-MRSA clone in particular, is a pre-eminent human pathogen capable of causing serious infections even in previously healthy

individuals. This fact reflects the coordinated action of numerous immune evasion factors including but not limited to cytolytic toxins (e.g. α-hemolysin, phenol soluble modulins, Panton-Valentine leukocidin), immunoglobulin Fc-binding proteins (e.g. protein A), oxidant resistance factors (e.g. catalase, staphyloxanthin pigment), fibrinogen binding proteins (e.g. clumping factor), complement and chemokine inhibitors (e.g. CHIPS, Efb). Furthermore, aureolysin is one of twelve proteases produced by *S. aureus*, which may provide significant functional redundancy. One principle conclusion of the present study is that while aureolysin can indeed degrade key host factors such as cathelicidin, its net contribution to the overall fitness of the organism is relatively minor within this large suite of virulence phenotypes. In earlier work in the methicillin-sensitive *S. aureus* strain 8325-4, aureolysin was not essential for virulence in a murine model of septic arthritis (Calander et al., 2004). Furthermore, while loss of aureolysin did lead to a detectable decrease in intracellular survival of methicillin-sensitive *S. aureus* strain Newman within macrophages *in vitro*, the contribution was much less prominent that that observed upon loss of the α-hemolysin toxin (Kubica et al., 2008).

Aureolysin has been shown to be responsible for activation of the *S. aureus* serine protease SspA (V8 protease), which in turn is responsible for maturation of another protease, staphopain B (Nickerson et al., 2007; Rice et al., 2001). Thus the effects of deletion of the *aur* gene will have downstream effects on the overall balance of protease activity of the pathogen, and it is likely that these activities influence not only host cell targets but the bacterium's own surface and secreted proteins. Thus it is theoretically possible that aureolysin degradation of host immune effector proteins (e.g. cathelicidin) is offset by proteolytic degradation of specific *S.*

aureus immune resistance or virulence proteins. Such a phenomenon is present for the human pathogens group A *Streptococcus* (GAS), which expresses a cysteine protease (SpeB) that targets host cathelicidin antimicrobial peptides, but also cleaves GAS virulence proteins such as M protein, streptolysin O, streptokinase, and the DNAse Sda1 (Aziz et al., 2004; Cole et al., 2011; Cole et al., 2006). In earlier work, aureolysin was indeed shown to cleave clumping factor B (ClfB), a cell wall-associated protein virulence factor involved in binding to fibrinogen and cytokeratin 10, the latter of which is involved in colonization of the nares (McAleese et al., 2001; O'Brien et al., 2002).

Finally, an important consideration is that the amount of aureolysin produced during infection is unknown, and it is possible that the results obtained in previous biochemical studies, and with culture supernatants in the current study, have evaluated supraphysiological amount of accumulated protease. In this context, it is noteworthy that development of antibodies against aureolysin was not detected following a CA-MRSA murine abscess model infection, suggesting expression of the toxin *in vivo* may be relatively low (Burlak et al., 2007). Our studies in one animal species (mouse) of course do not exclude that there are certain as yet untested infectious scenarios or tissue compartments in which aureolysin expression can exceed a threshold level to contribute to pathogen survival and virulence.

MRSA infections present a critical challenge to the public health, and new strategies to treat or prevent serious infections with this pathogen are urgently needed. Increased understanding of specific pathogenic mechanisms can provide new opportunities for virulence factor inhibitor based pharmaceuticals or for subunit antigens to be included in a *S. aureus* vaccine formulation. Our present work

suggests that the metalloprotease and candidate virulence factor aureolysin does not represent an attractive target for novel MRSA therapeutics.

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CHAPTER 4.

The novel role of the β-lactamase repressor BlaI as a virulence factor that renders *Staphylococcus aureus* more resistant to the innate immune defense

ABSTRACT

BlaI is the repressor of BlaZ, the β-lactamase responsible for penicillin resistance in *Staphylococcus aureus*. Through use of a transposon library in *S. aureus* Newman, we discovered BlaI as a novel antimicrobial peptide resistance factor. Additionally, through integrational mutagenesis in *S. aureus* Newman and MRSA252 strains, we confirmed the role of BlaI in resistance to antimicrobial peptides and demonstrated that it contributes to virulence in whole blood and murine mouse models. We further demonstrate that BlaI may be a target for antimicrobial therapies; by removing BlaI through subinhibitory concentrations of 6-aminopenicillanic acid, we were able to sensitize *S. aureus* to killing by human cathelicidin, LL-37.

INTRODUCTION

Staphylococcus aureus is a principal source of community- and hospital-associated infections, which range from superficial syndromes to a diverse array of life-threatening conditions such as sepsis and endocarditis (Lowy, 1998). Penicillin has been the drug of choice for treatment of *S. aureus* infections since the 1940s; however, penicillin-resistant *S. aureus* strains were reported as early as 1942. Today over 95% of human *S. aureus* isolates are resistant to penicillin (Fuda et al., 2005). The β-lactamase-resistant penicillin derivate methicillin was introduced in 1961, and the first methicillin-resistant *S. aureus* (MRSA) strains occurred shortly thereafter. Recent reports of *S. aureus* isolates with intermediate or complete vancomycin resistance may have heralded the imminent arrival of an era in which effective treatment of *S. aureus* infections may in the near future no longer be available. Therefore, new treatment measures and the identification and characterization of additional targets for anti-staphylococcal therapy are urgently needed.

The β-lactamase-mediated penicillin resistance in *S. aureus* has been thoroughly investigated (Fuda et al., 2005). The inducible PC1 β-lactamase of *S. aureus* is encoded by *blaZ*, and the transcription of *blaZ* is controlled by the BlaZ-BlaR1-BlaI system. The genes for BlaZ, its repressor BlaI and the signal transducer-sensor protein BlaR1 are clustered together. They are located either on a plasmid or in the bacterial chromosome, e.g. in MRSA252 (Holden et al., 2004). In the absence of β-lactams, the DNA repressor BlaI represses *blaZ* by binding to the conserved DNA motif TACA/TGTA, which is located in the promoter region of *blaZ* (Safo et al., 2005). The detection of β-lactam molecules by BlaR1 initiates a signaling cascade, ultimately resulting in de-repression of *blaZ* (illustrated in **Figure 4.1**).

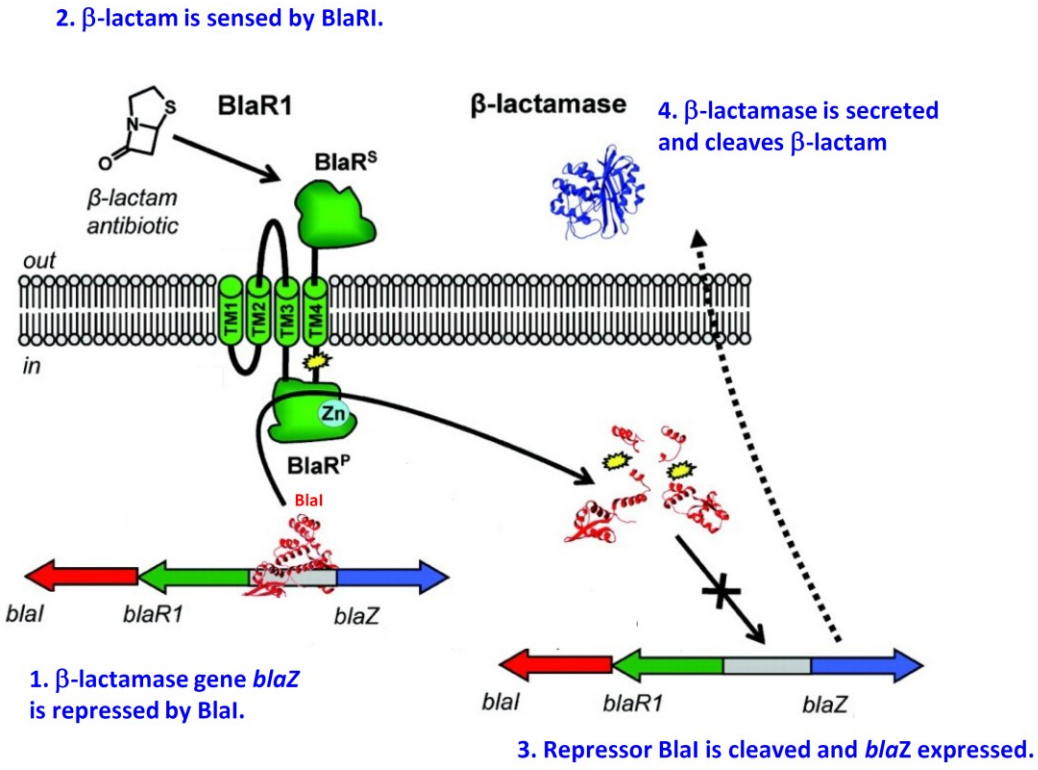

Figure 4.1. Model for β-lactamase regulation in *S. aureus.* (1) In the absence of β-lactams, the *blaZ-blaR1-blaI* genes are repressed by BlaI. (2) When β-lactam molecules are sensed by BlaR1, the cytoplasmatic domain of the transmembrane protein is autoproteolytically cleaved. (3) Following this event, the repressor protein BlaI is proteolytically cleaved and dissociates from its binding site, enabling transcription of the β-lactamase-encoding gene *blaZ*. (4) Finally, the active β-lactamase BlaZ is secreted, leading to hydrolysis of the β-lactam molecules. (Modified from Wilke *et al,* J Biol Chem, 2004)

Antimicrobial peptides (AMPs) are fundamental components of the mammalian innate immunity that control microbial infections and coordinate host responses to infection. The AMPs identified in humans are members of the cathelicidin, defensin and histatin families. The sole cathelicidin found in humans is LL-37, while a functional homolog, CRAMP, is found in mice. Bacterial pathogens have evolved countermeasures to decrease their susceptibility to host AMPs including (i) decreased affinity to AMPs through cell envelope modifications, (ii) active efflux pumps, (iii) external trapping of AMPs, (iv) production of AMP degrading proteases and (v) interference with host AMP production (Nizet, 2006; Peschel and Sahl, 2006). In the last several years, it has been established that the degree of resistance to host AMPs correlates with the potential of pathogenic bacteria to cause disease. Consequently, others and we suggest that AMP resistance factors could be targets for novel anti-bacterial drugs (Nizet, 2007).

The work described in this paper provides evidence that BlaI goes beyond the scope of controlling β-lactamase expression in *S. aureus* by acting as a virulence factor that is effectively contributing to diminished susceptibility to cathelicidin AMPs and virulence in *S. aureus*. Opening an option for the treatment of infections caused by β-lactamase producing *S. aureus* strains, pharmacological removal of BlaI by low concentrations of β-lactams rendered the penicillin-resistant bacteria more susceptible to innate immune defenses.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The staphylococcal strains used for this study were *S. aureus* Newman and MRSA252 (Sanger252) the chromosomes of which have been recently sequenced (Baba et al., 2008; Holden et al., 2004). As a control for the screening of cathelicidin susceptible *S. aureus* Newman transposon mutants, an AMP susceptible *S. aureus* Newman *dltA* mutant (Peschel et al., 1999) was used. *Tn*917 mutants and *blaI* mutants were generated as described below, and 5-10 µg/mL erythromycin (Em) was employed for antibiotic selection. For complementation experiments, 10 µg/mL chloramphenicol (Cm) was used for culture of strains carrying the empty complementation vector pD123 or pDC123 with a copy of *blaI* (pBlaI). For production of knockout and complementation vectors, *Escherichia coli* strains were used as host and grown in Luria-Bertani broth (LB); the antibiotic selection employed 100 µg/mL ampicillin, 500 µg/mL Em or 10 µg/mL Cm. The restriction deficient intermediate host strain *S. aureus* strain RN4220 (Fairweather et al., 1983) was used to replicate plasmids produced in *E. coli* before transfer into the staphylococcal target strains.

Preparation of *S. aureus* Newman transposon mutant library. Random transposon mutagenesis of *S. aureus* Newman was performed using *Tn*917 essentially as described for Group B *Streptococcus* (Doran et al., 2005). Briefly, the temperature sensitive suicide plasmid pTV_1OK, which has a kanamycin (Km) resistance marker in the vector backbone and harbors *Tn*917 carrying an Em resistance gene, was introduced via electroporation into the *S. aureus* strain RN4220. Plasmid DNA was isolated and transferred into the target strain *S. aureus* Newman. Several transformants were grown overnight in Todd Hewitt broth (THB)

with 1 mg/mL Km at 30°C, a temperature permissive for pTV_1OK replication. Overnight cultures were diluted 1/1,000 into THB without antibiotics, the temperature shifted to a non-permissive temperature (37°C) and incubated overnight yielding several potential random *Tn*917 mutant libraries which were stored at -80°C in THB, 35% glycerol. The randomness of *Tn*917 transposition in selected libraries was subsequently determined. To that aim, serial dilutions of the libraries were plated on Todd Hewitt agar (THA) plates with 5 µg/ml Em and incubated overnight at 37°C. The chromosomal *Tn*917 transposition events were identified in single colonies exhibiting Em resistance. The DNA of 10 randomly picked colonies per library was digested with *Hin*dIII and integration of *Tn*917 was probed for by Southern blot analysis using a digoxigenin-labeled transposon specific probe. The analysis of transposon mutant library #2 demonstrated a random chromosomal distribution of *Tn*917 insertion and a single integrated copy of *Tn*917 in at least 90% of the mutant strains (data not shown).

For subsequent phenotypic screens, 4,800 transposon mutants of library #2 were picked, grown up overnight in 100 µl THB with 5 µg/ml Em and stored in a total of fifty 96-well plates at -80°C in THB + 40% glycerol.

Screen for cathelicidin susceptible transposon mutants. A 96-well microtiter plate assay was established to screen transposon library #2 for mutants susceptible to the murine cathelicidin CRAMP. Notably, *S. aureus* is known to be highly resistant to cathelicidin AMPs in commonly used complex growth media such as THB or tryptic soy broth (TSB), but more susceptible in carbonate-containing solutions such as RPMI-1640 (Dorschner et al., 2006). First, we showed that *S. aureus* Newman WT and the cathelicidin susceptible *S. aureus* Newman *dltA* mutant

strain grew well in RPMI-1640, 10% LB medium. The minimal inhibitory concentration (MIC) of commercially synthesized CRAMP in this medium was determined to be 3 μM for the *dltA* mutant, a concentration at which the WT strain was not inhibited. Subsequently, stationary cultures of the 4,800 *S. aureus* Newman *Tn*917 mutants were tested for their growth in the presence or absence of 3 μM CRAMP in order to identify genes that contribute to the cathelicidin resistance of the WT strain. Putative CRAMP susceptible candidate mutants identified in primary screens were subjected to additional testing to verify cathelicidin susceptibility. Their growth was compared to that of the WT strain in THB, and exponential phase mutant bacteria with no growth defect were re-tested for their cathelicidin susceptibility in CRAMP killing assays as described below.

Identification of *Tn*917 insertion sites. The site of *Tn*917 insertion in CRAMP susceptible mutants was determined by using the following protocol. In a first PCR, DNA obtained from the mutant strains was subjected to PCR with a random primer (5'-GGCCACGCGTCGACTAGTACA TTACTAGCTACGCC-3') and the *Tn*917 specific primer 1st_left_*Tn*917 (5'-CCATGTTAAACCCATAGATAA-3'). The resulting amplification products were purified using standard procedures and subjected to a second and third PCR using the random primer mentioned above and the *Tn*917 specific primer 2nd_left_*Tn*917 (5'-ACACCTGCAATAACCGTTACC-3'). The binding site of 2nd_left_*Tn*917 is located downstream of 1st_left_*Tn*917 of the *Tn*917 sequence and thus was expected to specifically react with primary PCR products containing parts of the *Tn*917 and the disrupted Newman gene sequence. The final PCR products were purified and sequenced at Eton Bioscience Inc. (San Diego, CA) using the sequencing primer 3rd_left_*Tn*917 (5'-

AGAGAGATGTCACCGTCAAG-3'). The obtained sequences were compared with published gene sequences in the GenBank database using the BLAST algorithm. In the transposon mutant G2E3, the insertion of *Tn*917 in *blaI* was verified by two PCRs using the primer *blaI*-integ (5'-AGATCTTGTGTTGGGTTATTGAACA-3') in combination with either 1st_left_*Tn*917 or 2nd_left_*Tn*917.

Targeted mutagenesis. Plasmid insertional mutagenesis was performed to inactivate *blaI* in *S. aureus* Newman and MRSA252 as described (Datta et al., 2005). For generating the *blaI* mutants, genomic DNA preparations from Newman and MRSA252 were used as templates. The primers *blaI*_ko_f_*Xba*I (5'-GCTCTAGATGGCCAATAAGCAAGTTGAA-3') and *blaI*_ko_r_*Bam*HI (5'-CGGGATCCACTAATATCATTTAAAATGTC-3') with overhangs constructing *Xba*I and *Bam*HI restriction sites into the PCR product were employed to amplify the first 351 bps of *blaI* by PCR. Please note that the *blaRI* gene, upstream of *blaI*, would be left intact using the described knockout strategy. The PCR products were cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions and propagated in *E. coli*. The plasmids were isolated, and the knockout constructs were digested with *Bam*HI/*Xba*I. For knocking out *blaI* in *S. aureus* Newman, the constructs were then ligated with *Bam*HI/*Xba*I digested temperature sensitive suicide vector pHY304, which confers Em resistance. The knockout vectors where then propagated in *E. coli*, subsequently transferred into *S. aureus* RN4220 and finally into the target strain Newman by electroporation using standard methods. The target strains carrying the knockout plasmids were grown under Em selection at 30°C, a temperature permissive for pHY304 replication. The cultures were shifted to a non-permissive temperature to allow only for survival of mutants with integrated plasmids

in the presence of Em. PCR analysis for single Em resistant colonies was used to confirm the targeted disruption of *blaI* in *S. aureus* Newman. The same cloning strategy was used for generating MRSA252 Δ*blaI*. However, since MRSA252 is instrinsically Em resistant, the *Bam*HI/*Xba*I digested PCR products were cloned into the suicide vector pVE6007 (Maguin et al., 1992), which carries a Cm resistance gene, and subsequently utilized to knock out *blaI* as described above for the pHY304 construct.

Complementation analysis. For complementation of *blaI in trans* in *S. aureus* Newman, *blaI* plus flanking DNA was PCR amplified from a Newman genomic DNA preparation using primers *blaI*_complementation_f_*Sph*I (5'-CGGCATGCGAAAAGTATGAACTGTATGG-3') and *blaI*_complementation_r_*Bam*HI (5'-CGGGATCCGAGTCAAGCATAGTTTACA-3'); the PCR product was cloned directionally into the expression vector pDC123 (Chaffin and Rubens, 1998) which confers Cm resistance, yielding plasmid pBlaI. pBlaI was propagated in *E. coli*, transferred into *S. aureus* RN4220 and finally the *S. aureus* Newman *blaI* mutant as described above. As control strains for comparative functional analyses, the empty expression vector pDC123 was also put into the *S. aureus* Newman WT and *blaI* mutant strains.

Nitrocefin test. Nitrocefin is a chromogenic cephalosporin used to determine β-lactamase activity (O'Callaghan et al., 1972). When cleaved, it changes from yellow to red in color. Bacteria were incubated with 50 μg/ml nitrocefin (Calbiochem) and incubated at 37˚C for 30 min in the dark. The A_{490} was read to determine the level of nitrocefin cleavage. Increased absorbance corresponds to increased β–lactamase activity.

Minimum inhibitory concentration. For penicillin susceptibility, MICs were performed by broth microdilution. Mid-log phase bacteria were resuspended to 5 x 10^6 CFU/ml in Mueller Hinton Broth (MHB) supplemented with Ca^{2+} and Mg^{2+}. Ten microliters were added to 80 µl MHB and 10 µl penicillin G in triplicate in round bottom 96-well plates. Plates were shaken at 37˚C for 24 h, at which time the A_{600} was read. The MIC was determined to be the lowest concentration of antibiotic that inhibited bacterial growth detected at A_{600}.

For daptomycin susceptibility, the MICs were determined by standard Etest. Cultures were resuspended to A_{600} = 0.2 and spread on MHB plates. An Etest strip for daptomycin was placed on the center of the plate, and plates were incubated at 37˚C overnight.

Cathelicidin killing assays. Mid-log phase bacteria were resuspended to 10^7 CFU/ml in Dulbecco's phosphate-buffered saline (DPBS) + 10% TSB + 50 mM $NaHCO_3$, and 225 µl were added to 25 µl of CRAMP or LL-37 and incubated at 37˚C. At the indicated timepoint, 25 µl were removed, diluted in PBS and plated on THA for enumeration of CFU.

Human whole blood assay. Blood was drawn from healthy donors after informed consent, and 10^6 CFU of mid-log phase bacteria in 10 µl of DPBS + 10% TSB were added to 990 µl heparinized whole blood in siliconized tubes. Tubes were placed on a rotisserie at 37°C for 1 h, at which point 25 µl was removed, diluted in ddH_2O to lyse blood cells and plated for enumeration of CFU.

Murine skin abscess model. Eight-week-old female CD-1 mice (Charles River Laboratories) were shaved and treated with Nair for hair removal on the day prior to injection. Mice were injected subcutaneously with 5 x 10^7 CFU of mid-log

phase Newman or Newman Δ*blaI* bacteria suspended in 100 µl of DPBS + 0.5 µg/ml cytodex beads. Lesion sizes were monitored for 7 days.

Murine systemic infection model. Eight-week-old female CD-1 mice were injected intraperitoneally with 1.3 x 10^7 CFU (Newman) or 6 x 10^8 CFU (MRSA252) of mid-log phase WT or Δ*blaI* bacteria suspended in 200 µl of DPBS + 5% gastric mucin (MP Biomedicals). Survival was monitored every 12 h for 3 d, and time of death was recorded for each mouse.

FITC-labeled poly-L-lysine binding. Poly-L-lysine (PLL) is a positively charged molecule used to measure surface charge. The assay was modified from a previously described method (Mukhopadhyay et al., 2007). Briefly, overnight cultures were washed twice with HEPES (20 mM, pH 7.25) and suspended to an A_{578} of 0.3. The bacterial suspension was incubated with 1 ug/ml FITC-labeled PLL (Sigma) for 15 min at room temperature and subsequently washed. The amount of bound PLL was determined using flow cytometry, and this inversely reflected the relative positive surface charge. A total of 10,000 events were counted and analyzed using a BD FACSCalibur system (Becton Dickinson).

Hydrophobicity. The hydrophobicity of bacterial cells was tested using a modified version of the MATH (microbial adhesion to hydrocarbons) assay (Rosenberg et al., 1980). Stationary phase bacteria were resuspended to A_{600} = 1.0 in DPBS. Nine hundred microliters were added to 300 µl hexadecane in triplicate. Tubes were vortexed for 2 min and subsequently left to stand on the benchtop for 35 min to allow separation of hydrophobic and aqueous layers. Twenty five microliters were subsequently removed from the aqueous layer, diluted in PBS and plated to allow for enumeration of CFU.

Cathelicidin cleavage. Supernatants from overnight cultures grown in TSB were filter sterilized through a 0.22 μm syringe-driven filter (Millipore). Eighteen μl of filtered supernatant were incubated with 2 μl of CRAMP or LL-37 (AnaSpec) to give a final concentration of 16 μM CRAMP or 8 μM LL-37. Samples were incubated at 37˚C for 24 h, mixed with 4x sample buffer and 10x reducing agent (Invitrogen), boiled for 10 min, loaded onto a 12% Bis-Tris gel (Invitrogen) and run at 120 V in MES running buffer. Gels were stained with SimplyBlue SafeStain (Invitrogen) and subsequently destained in H_2O.

Treatment with 6-aminopenicillinic acid (6-APA). Bacteria were grown to mid-log phase in DPBS + 10% TSB and resuspended to 10^7 CFU/ml in the same buffer. Bacteria were incubated alone or in the presence of a subinhibitory dose of 6-APA in siliconized tubes on a rotisserie for 1 h at 37˚C. Subsequently, bacteria were incubated with 24 μM (Newman) or 2 μM (MRSA252) LL-37 or water in a 96-well round bottom plate shaking for 1 h at 37˚C.

Statistical analyses. Nitrocefin tests were analyzed using one-way ANOVA with Bonferroni post-test. Cathelicidin kinetics were compared using repeated measures two-way ANOVA with Bonferroni post-test except for MRSA252 + CRAMP, which was analysed using Student's unpaired *t*-test. The whole blood assay was analysed using one-way ANOVA with Bonferroni post-test. Lesion sizes for the murine abscess model were compared using the Student's paired *t*-test. The murine intraperitoneal challenge assays were assessed using the log-rank (Mantel Cox) test. Poly-L-lysine-FITC binding was compared one-way ANOVA with Bonferroni post-test. Hydrophobicity was analzyed using one-way ANOVA (Newman) or Student's unpaired *t*-test (MRSA252). All statistical tests were performed using GraphPad

Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). *P* values < 0.05 were considered statistically significant.

Ethics permissions. Permission to collect human blood under informed consent was approved by the UCSD Human Research Protection Program. All animal experiments were conducted according to the guidelines approved by the UCSD Institutional Animal Care and Use Committee.

RESULTS

Inactivation of *blaI* leads to increased β-lactamase activity and cathelicidin susceptibility in *S. aureus.* To identify staphylococcal genes involved in cathelicidin resistance, a random mutant library of *S. aureus* Newman was generated by *Tn*917 transposition. Individual mutants were screened for increased susceptibility to the murine cathelicidin AMP CRAMP. Of 4,800 *Tn*917 mutants screened, 19 showed increased sensitivity to CRAMP.

Putative CRAMP susceptible mutants identified in the primary screen were subjected to additional testing to confirm their phenotype by characterizing (i) their susceptibility to CRAMP in exponential and stationary phase and (ii) by demonstrating that the mutants exhibited no replication defects in growth curves compared to the wild-type (WT) strain in THB (data not shown).

In one of these mutants, named G2E3, the transposon insertion was mapped between base pairs (bps) 12 and 13 of the β-lactamase repressor gene *blaI*, which is part of the *blaZ-blaR1-blaI* locus. The insertion of the transposon in the *blaI* gene was further confirmed by PCRs with *blaI* and *Tn*917 specific primer pairs (data not shown).

The *blaZ-blaR1-blaI* locus is not found in the published chromosome sequence of *S. aureus* Newman (Baba et al., 2008) and thus must be located in an extrachromosomal locus such as a plasmid. The organization of the *blaZ-blaR1-blaI* locus for several *S. aureus* strains is well characterized (Fuda et al., 2005) and was found to be the same in *S. aureus* Newman as determined by PCRs. **Figure 4.2A,B** illustrate the organization of the *blaZ-blaR1-blaI* locus in *S. aureus* Newman and the location of the *Tn*917 insertion in the cathelicidin susceptible mutant G2E3. Of note,

in sequenced *S. aureus blaZ-blaR1-blaI* loci such as that of MRSA252 (Holden et al., 2004), *blaRI* overlaps with the *blaI* gene by 11 bps. Thus, we assumed that the transposon insertion in mutant G2E3 occurred 1 bp downstream of *blaR1*.

Next, BlaI was confirmed as a cathelicidin resistance factor by targeted integrational mutagenesis of *blaI* and *in trans* complementation of the resulting *blaI* mutant strain in *S. aureus* Newman. In addition, *blaI* was inactivated in MRSA252, in which the *bla* operon is located on the bacterial chromosome (Holden et al., 2004).

The β-lactamase activities of the genetically engineered *blaI* mutant strains were compared to those of the respective WT strains using nitrocefin as the test reagent. The disruption of the β-lactamase repressor *blaI* led to elevated β-lactamase activity in both *S. aureus* Newman and MRSA252 (**Figure 4.2C,D**). Additionally, we tested the minimum inhibitory concentration (MIC) of penicillin G against the strains as a second readout of β-lactamase activity. Compared to the WT strains, we observed an increase in the MICs of the *blaI* mutants in both the Newman (64 μg/ml vs. >128 μg/ml) and MRSA252 (64 μg/ml vs. 128 μg/ml) backgrounds. The Newman complemented strain had an MIC similar to the mutant (>128 μg/ml) inferring that BlaI activity may not be completely restored in that strain. These results indicated that the expression of the β-lactamase BlaZ was de-repressed through inactivation of the *blaZ* repressor BlaI. Futhermore, to determine if BlaI played a role in resistance to daptomycin, we tested the susceptibility of the Newman strains using a standard Etest. The Δ*blaI* strain had a slightly decreased MIC (0.125 μg/ml) compared to the WT (0.190 μg/ml) and complemented strain (0.250 μg/ml).

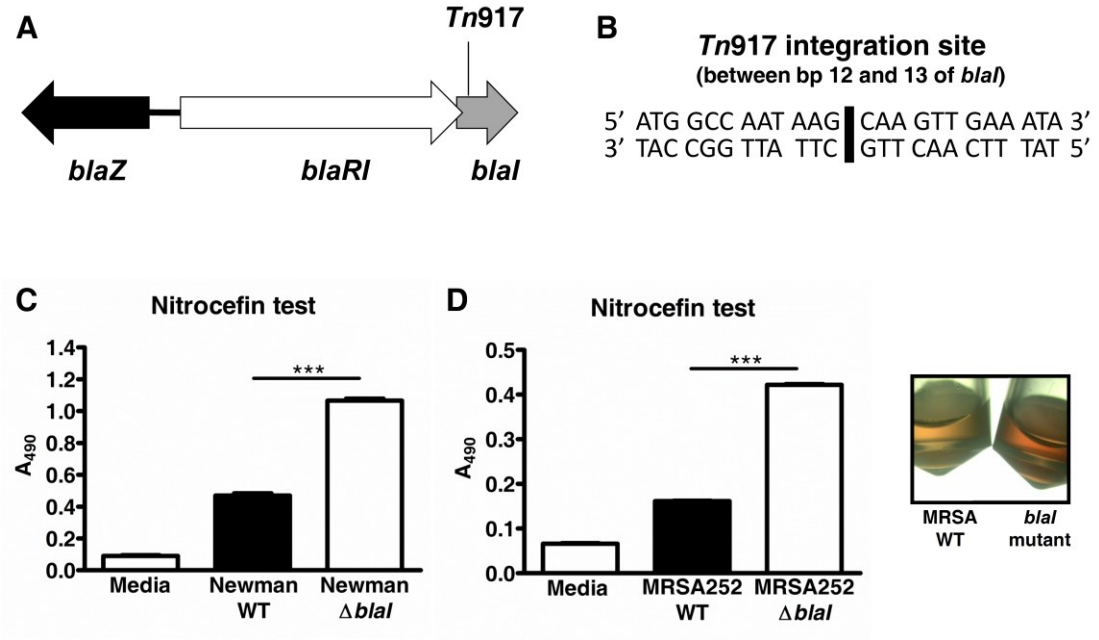

Figure 4.2. Mapping of the *Tn*917 mutant and subsequent inactivation of *blaI* leading to elevated β-lactamase production. (A) Organization of the *blaZ-blaR1-blaI* locus in *S. aureus* Newman. *blaR1* and *blaI* are located in a two-gene operon. *blaZ* is divergently transcribed. (B) In the cathelicidin susceptible *S. aureus* Newman mutant G2E3, *Tn*917 integration occurred 12 bp downstream of the *blaI* start codon. (C) *S. aureus* Newman WT and *blaI* mutant or (D) MRSA252 WT and *blaI* mutant were incubated with 50 μg/ml nitrocefin for 30 min at 37°C, and the A_{490} was read. A higher A_{490} value reflects higher β-lactamase activity. Mean A_{490} values $\pm$ SD of duplicates of one representative experiment of at least three performed for each strain is shown. ***, $p<0.001$

Next, we performed killing kinetics with cathelicidin AMPs. Compared to their respective *S. aureus* Newman and MRSA252 WT strains, the *blaI* mutants were found to be more susceptible to the murine cathelicidin CRAMP and human LL-37 (**Figure 4.3**). Complementation of the *S. aureus* Newman *blaI* mutant with the plasmid pBlaI led to restoration of WT levels for both CRAMP and LL-37 resistance (**Figure 4.3A,B**).

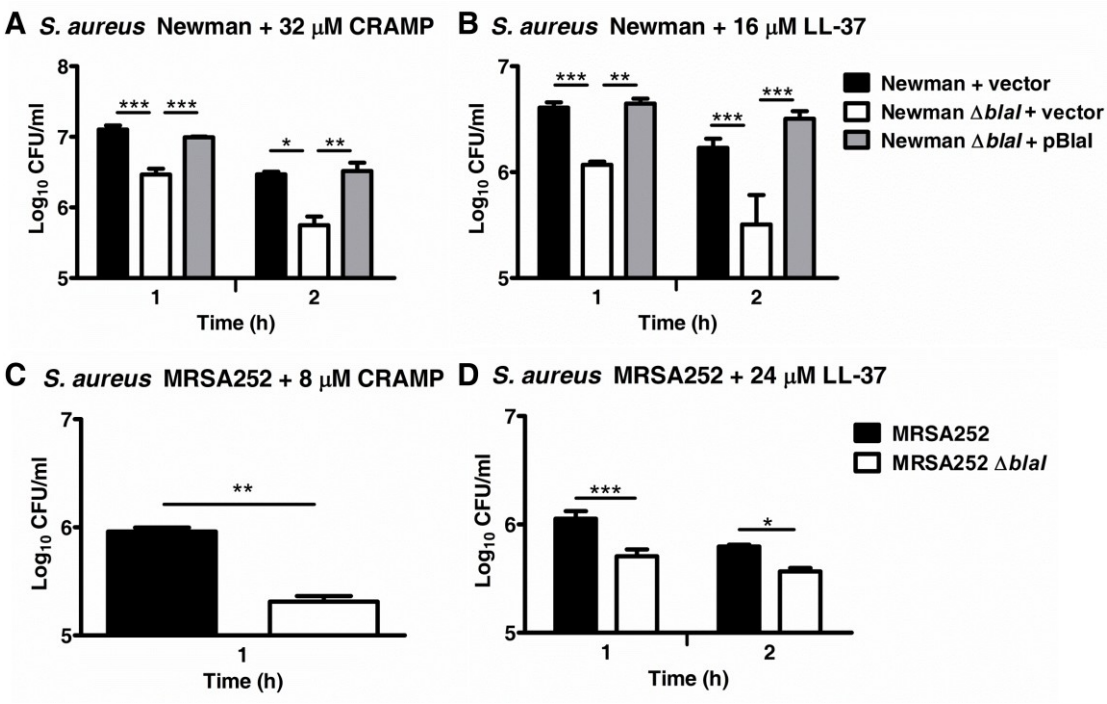

Figure 4.3. Effect of *bla1* on the cathelicidin susceptibility of *S. aureus*. (A,B) *S. aureus* Newman and (C,D) MRSA252 strains were incubated with CRAMP or LL-37 and the numbers of surviving CFUs were determined at the indicated timepoints. Samples were run in triplicate, and one representative experiments of at least two performed is shown. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Inactivation of *blaI* promotes survival of *S. aureus* in human whole blood and virulence in murine infection models. To establish whether the *blaI*-mediated cathelicidin resistance could lead to increased fitness of *S. aureus* in the host, we performed killing assays with human whole blood and compared the virulence of WT and *blaI* mutant bacteria in murine abscess and septicemia models. As shown in **Figure 4.4**, the *S. aureus* Newman *blaI* mutant was killed significantly better by human whole blood than the WT and complemented strains. In a murine skin abscess model, lesions formed by *S. aureus* Newman were significantly larger than those created by the *blaI* mutant (**Figure 4.5A**), and in murine sepsis models, the *blaI* mutant was found strongly attenuated in *S. aureus* Newman (**Figure 4.5B**) and moderately impaired in MRSA252 (**Figure 4.5C**). Taken together, the human whole blood *ex vivo* and the mouse *in vivo* data suggested that BlaI contributes to the pathogenic potential of *S. aureus.*

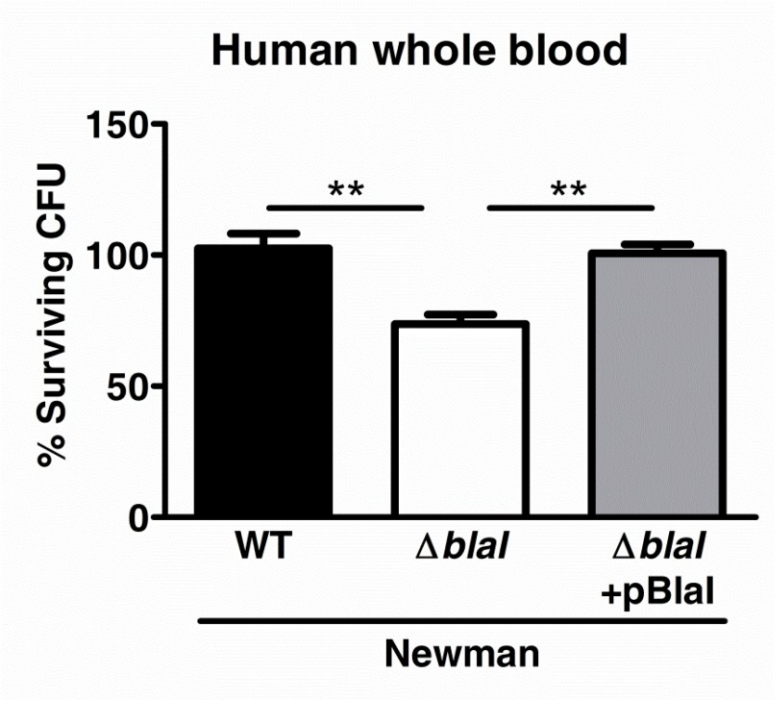

Figure 4.4. Survival of *S. aureus* Newman in human whole blood. *S. aureus* Newman strains were incubated for 1 h in human whole blood. Samples were run in triplicate, and the average ± SD is depicted. A representative experiment of three performed is shown. **, $p<0.01$

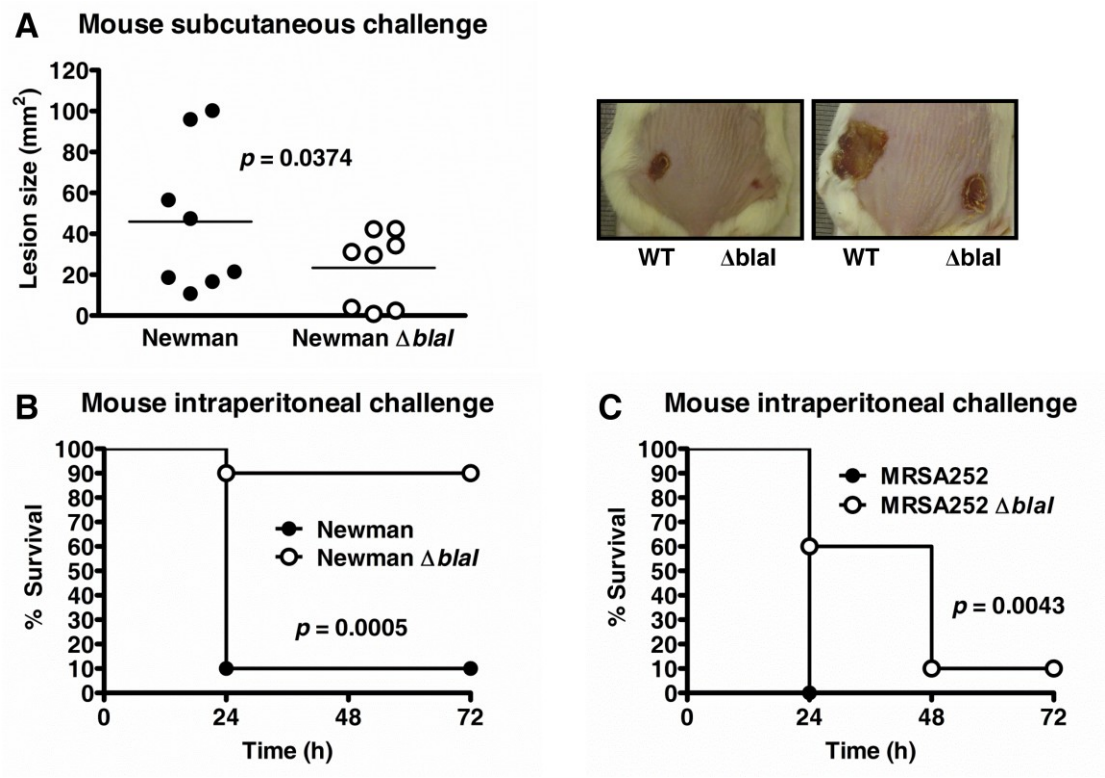

Figure 4.5. BlaI contributes to virulence *in vivo.* (A) CD-1 mice (*n* = 10) were injected subcutaneously on one flank with *S. aureus* Newman WT and on the opposite flank with *ΔblaI*, and lesion sizes were monitored for 7 days. Survival of CD-1 mice (*n* = 10) after intraperitoneal infection with (B) 1 x 10^6 CFU of *S. aureus* Newman WT or Δ*blaI* or (C) 6 x 10^8 CFU of *S. aureus* MRSA252 or Δ*blaI*. Survival was monitored for 3 days.

The surface charge of *S. aureus* is somewhat altered by *blaI* inactivation in MRSA252. After establishing that BlaI contributes to cathelicidin resistance, whole blood survival and the virulence potential of *S. aureus*, we aimed to determine the mechanism through which BlaI exerts these actions.

A common AMP resistance mechanism among Gram-positive and Gram-negative bacteria is to decrease the net negative surface charge through cell envelope modifications in order to repel positively charged AMPs (Peschel and Sahl, 2006). However, the Newman strains did not differ significantly in surface charge as assessed by poly-L-lysine-FITC binding (**Figure 4.6A**) or cytochrome *c* binding assays (data not shown). Additionally, MRSA252 Δ*blaI* showed a decreased binding of poly-L-lysine (indicative of a more positively charged surface) compared to its respective WT, which is opposite of what would be expected if surface charge were playing a role in resistance to AMPs.

Hydrophobicity is slightly affected by loss of *blaI.* Another mechanism by which bacteria resist AMPs is through increased hydrophobicity of their cell membrane. To test if there was a difference in hydrophobicity between WT and Δ*blaI* strains, we utilized a version of the microbial adhesion to hydrocarbons assay. We observed small, though significant, differences in hydrophobicity between the Newman strains in stationary phase and between both the Newman and MRSA252 strains in mid-log phase (**Figure 4.6B,C**). However, the differences were minor and differing in direction based on growth phase; it was unlikely that hydrophobicity was playing a major role in BlaI-mediated AMP resistance.

Proteolytic activity is decreased in MRSA252 *ΔblaI.* Proteolytic cleavage is a third mechanism bacteria utilize to resist AMPs. To test if there were differing

levels of cathelicidin cleavage between strains, we incubated overnight cell-free supernatants with CRAMP or LL-37 for 24 h and subsequently ran the samples on an SDS-PAGE gel. We did not discern any differences between Newman strains but did observe a decrease in cleavage by the MRSA252 *blaI* mutant compared to its parental strain (**Figure 4.6D**).

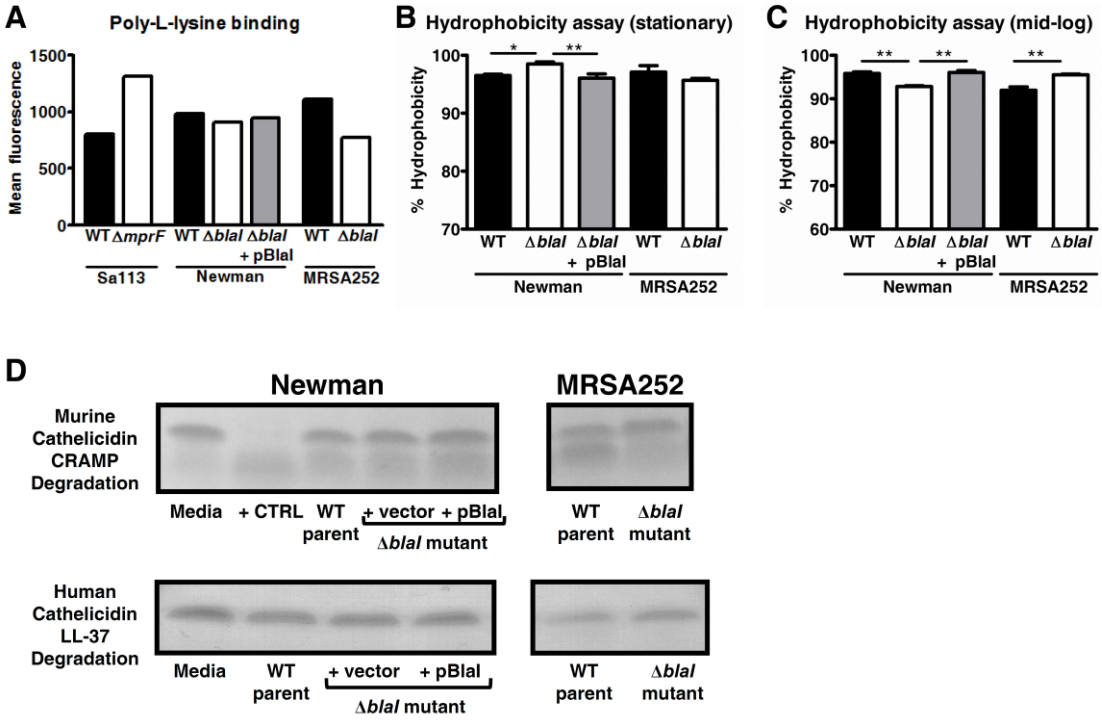

Figure 4.6. Mechanisms of cathelicidin resistance. (A) Surface charge of *S. aureus* Newman and MRSA252 strains was compared by poly-L-lysine binding. (B,C) Hydrophobicity was measured using a modified version of the MATH (microbial adhesion to hydrocarbons) assay. (D) Degradation of CRAMP or LL-37 by overnight cell-free supernatants analyzed by SDS-PAGE. *, $p<0.05$; **, $p<0.01$

Subinhibitory concentrations of 6-aminopenicillinic (6-APA) renders penicillin-resistant *S. aureus* more susceptible to cathelicidin-mediated killing. We hypothesized that the exposure of *S. aureus* Newman and MRSA252 to 6-APA would force the bacteria to remove their β-lactamase repressor BlaI and in consequence would lead to de-repression of their β-lactamase expression and to increased susceptibility to cathelicidin AMPs.

To address this possibility, *S. aureus* Newman and MRSA252 were incubated with subinhibitory concentrations of 6-APA, which is the active core structure of all penicillins. Subsequently, the killing by LL-37 was tested as compared to untreated cells. As can be seen in **Figure 4.7B,C**, subinhibitory concentrations of 6-APA rendered both staphylococcal strains more susceptible to LL-37. The increased susceptibility correlated with increased β-lactamase activity as assessed by the nitrocefin assay (**Figure 4.7A**).

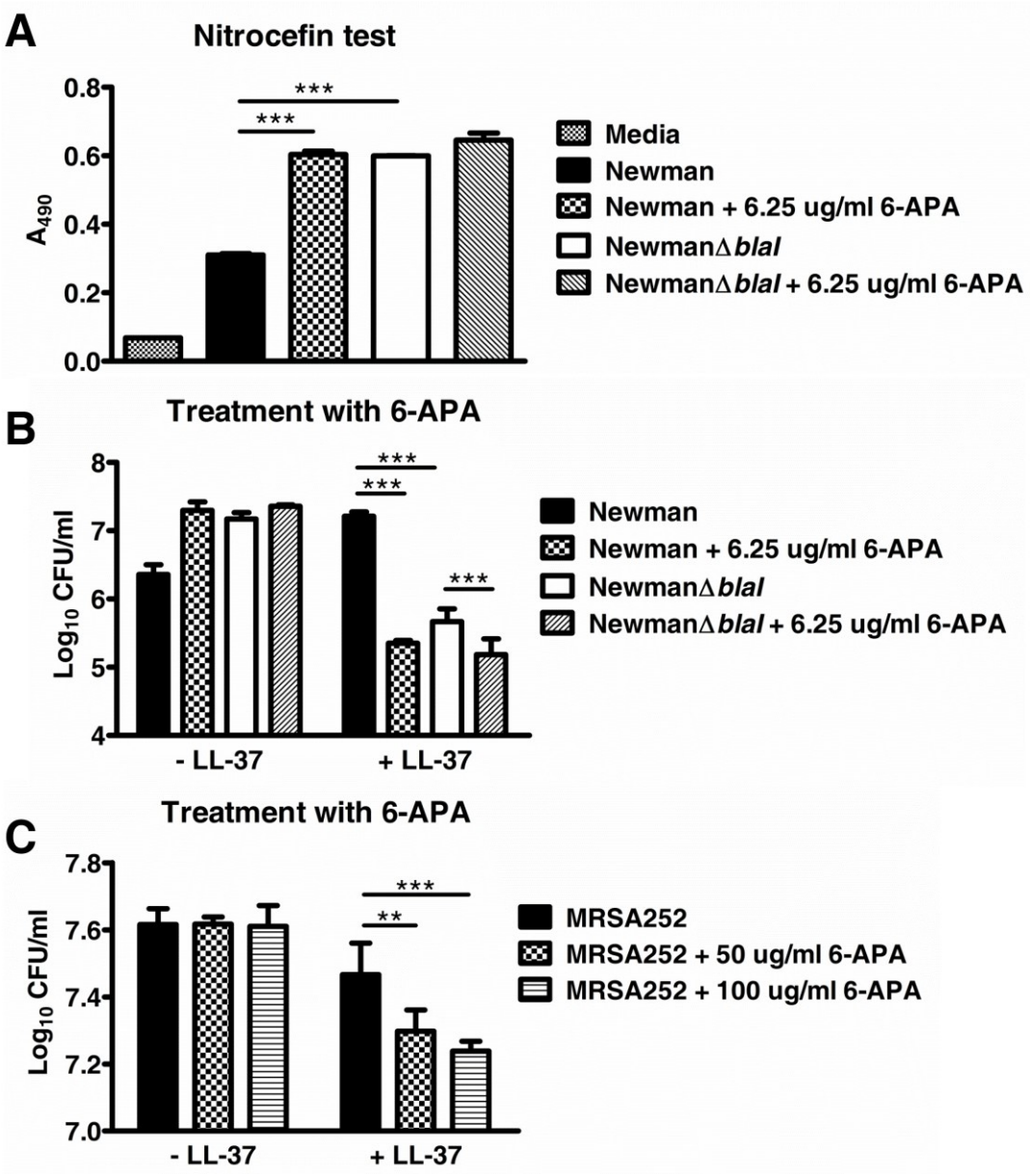

Figure 4.7. Pre-incubation with subinhibitory concentrations of 6-APA increases the β-lactamase activity and LL-37 susceptibility of *S. aureus* Newman and MRSA252. Bacteria were incubated for 60 min at 37˚C in the absence or presence of 6-APA. For an aliquot of each sample the β-lactamase activity was subsequently determined with nitrocefin as test reagent (A). The residual bacteria were incubated with or without LL-37 for another 60 min and the surviving CFUs were quantified (B,C). Samples were run in triplicate and one representative experiment of three performed is shown. **, $p<0.01$ ***, $p<0.001$

DISCUSSION

It has been established that the degree of resistance to host antimicrobial peptides (AMPs) correlates with the potential of pathogenic bacteria to cause disease (Nizet, 2007). Thus, the identification and characterization of AMP resistance mechanisms in bacteria with pathogenic potential is of interest because the responsible genes and gene products could be promising targets for novel antibacterial agents.

Our work described here demonstrates that BlaI renders *S. aureus* more resistant to the cathelicidin AMPs CRAMP and LL-37 as well as to whole blood killing. Additionally, it promotes virulence in mice, thus providing evidence that BlaI contributes to the virulence potential of β-lactam resistant *S. aureus* strains.

It has been shown that increased surface charge contributes to CRAMP resistance in *S. aureus* (Kristian et al., 2003). However, the Newman strains did not differ significantly in surface charge and the MRSA252 *blaI* mutant had an increased positive charge, which is opposite of what would be expected if it were a factor in AMP resistance. Additionally, there were small, significant differences in hydrophobicity in both backgrounds, but the differences do not appear large enough to play a significant role in susceptibility to AMPs. We did observe a decreased cleavage of CRAMP and LL-37 in the MRSA252 Δ*blaI* strain, which may be contributing to increased susceptibility to AMPs, but we did not see this difference between the Newman strains. As of now, it is unclear through what mechanism BlaI acts to increase virulence, but as BlaI has multiple potential binding sites throughout the *S. aureus* genome (data not shown), it may the combination of several factors.

It has been shown that MecI and BlaI bind to the same DNA binding motif and have co-regulatory effects on the expression of β-lactamase and PBP2a: MecI can repress the BlaI target gene *blaZ* and BlaI can repress the MecI target gene *mecA* (Lewis and Dyke, 2000). With respect to the similarities between MecI and BlaI, we speculate, that MecI could contribute to the cathelicidin resistance and virulence potential of MRSA strains.

Today, more than 95% of *S. aureus* isolates are resistant to penicillin (Fuda et al., 2005). It is commonly accepted that the bacteria acquired the BlaZ-BlaR1-BlaI-encoding genes through mobile genetic elements as a result of survival pressure following the introduction of penicillin as an anti-staphylococcal medication in the early 1940's. The experimental observation that *blaI* contributes to staphylococcal resistance to host innate immune molecules and virulence extends the number of possible explanations for why so many staphylococcal isolates harbor and keep the *blaZ-blaR1-blaI* genes: In addition to the continuing selection by β-lactam antibiotics, the β-lactamase regulatory system also might contribute to the fitness of *S. aureus* within the host by modulating the expression of virulence genes by BlaI. Heterologous expression of the BlaZ-BlaR1-BlaI system in β-lactamase-negative *S. aureus* strains would allow to follow-up on this hypothesis.

Finally, we showed that subinhibitory concentrations of 6-aminopenicillinic acid render β-lactam resistant bacteria more susceptible to innate host defense molecules, which could open an avenue of treatment regimes for difficult to treat *S. aureus* infections: attempt to treat infections with minute amounts of β-lactams to render the bacteria more susceptible to host defense.

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CHAPTER 5.

Conclusions and Perspectives

GAS and *S. aureus* are two of the leading pathogens that can cause disease in otherwise healthy adults. In the preceding works, we used molecular genetics to evaluate the roles of three genes: *sic*, *aur* and b*laI* in AMP resistance and virulence of GAS and *S. aureus*.

STREPTOCOCCAL INHIBITOR OF COMPLEMENT (SIC)

We demonstrated that SIC is necessary and sufficient for resistance to cathelicidin and confirmed that this was through direct binding using co-immunoprecipitation. In addition, we were able to show that SIC is necessary and sufficient for survival in human whole blood and necessary for survival in human serum and in primary murine macrophages. In a mouse model of systemic GAS infection, survival was decreased when injected with strains containing SIC. Thus, SIC has been verified as a virulence factor and has earned its place among a suite of others in invasive GAS.

SIC could represent a potential target for therapy in cases of invasive GAS. Though SIC is only found in M1 and M57 serotypes, M1 strains are the leading isolates in cases of invasive disease (Aziz and Kotb, 2008). However, one potential drawback of anti-SIC therapy is the level of polymorphism of the gene. To date, there have been nearly 300 allelles discovered, making it the most polymorphic gene in the

GAS genome (Hoe et al., 2001). A drug or antibody targeting SIC would have to be screened against the nearly 300 alleles to determine if it is active against all of them.

AUREOLYSIN

We studied the role of aureolysin in resistance to AMPs and virulence in three different MRSA strains but were unable to demonstrate a contribution to either phenotype in any of the strains. Perhaps in abscesses or other closed off infections, the amount of secreted aureolysin may be concentrated to levels high enough to contribute to virulence. However, it is also a possibility that aureolysin is not a virulence factor at all. This study demonstrates the importance of analyzing genes in the context of the living pathogen and not simply through use of purified protein. While discoveries made using biochemical studies are often recapitulated in genetic models, this is not always the case as seen here with aureolysin.

β-LACTAMASE INHIBITOR BLAI

We discovered a novel AMP resistance factor, the β-lactamase inhibitor BlaI, through screening of a transposon library in *S. aureus*. However, it is still undetermined through which mechanism BlaI is acting to increase resistance, and as BlaI is a DNA binding protein, resistance may be due to multiple mechanisms. In addition to AMP resistance, we also demonstrated a virulence role for BlaI in both human whole blood and in two different mouse models of staphylococcal disease. As a potential therapy we were able to sensitize bacteria to killing by LL-37 when treated with subinhibitory doses of 6-APA. The presence of 6-APA causes removal of BlaI, thus mimicking the *blaI* mutant and resulting in bacteria that are more susceptible to

host innate defenses. Such a potential therapy is exciting as antibiotic resistance continues to increase in *S. aureus* as we have seen with the development of vancomycin-insensitive *S. aureus* (VISA) as well as vancomycin-resistant *S. aureus* (VRSA) in the last decade (Howden et al., 2010).

It is imperative to study pathogenic organisms in order to better understand their propensity for virulence as well as to determine which genes are the most promising for targeted therapies. We have explored three genes in GAS and *S. aureus*: *sic*, which could be a potential target but also has a significant drawback due to its level of polymorphism, *aur*, which would not be a potential target due to its lack of involvement in virulence and *blaI*, which is the most promising of the three. As organisms continue to become resistant to more antibiotics and few new antibiotics are developed, targeted therapies could provide a promising treatment route.

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