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Characterization of the Effect of Serum and Chelating Agents on Staphylococcus aureus Biofilm Formation; Chelating Agents Augment Biofilm Formation through Clumping Factor B

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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List of Abbreviations

°C ..... Degree centigrade
µ ..... micro
µg ..... microgram
µL ..... microliter
A ..... Adenine
Agr ..... accessory gene regulator
AMPs ..... Antimicrobial peptides
ATCC ..... American Type Tissue Collection
Aur ..... Aureolysin
bp ..... base pair
BSA ..... Bovine serum albumin
BSIs ..... Bloodstream infections
C ..... Cytosine
CA-MRSA ..... Community-acquired MRSA
CDC ..... Center's for Disease Control and Prevention
cDNA ..... complementary DNA
CFU ..... colony forming unit
ClfA ..... Clumping factor A
ClfB ..... Clumping factor B
CLS ..... Catheter Lock Solutions
Cm^r ..... Chloramphenicol resistance
C-terminal ..... Carboxy-terminal
CVC ..... Central venous catheter
DEPC ..... Diethylpyrocarbonate
DNA ..... Deoxyribonucleic acid
dNTP ..... deoxynucleotide triphosphate
DTT ..... Dithiothreitol
E. coli ..... *Escherichia coli*
E. faecalis ..... *Enterococcus faecalis*
eDNA ..... extracellular DNA
EDTA ..... ethylenediamine tetraacetic acid
EGTA ..... ethylene glycol tetraacetic acid
Erm^r ..... Erythromycin resistance
EtOH ..... Ethanol
FBS ..... Fetal bovine serum
g ..... gram
G ..... Guanine
gDNA ..... genomic DNA
h ..... hours
HA-MRSA ..... Hospital acquired MRSA

ICU ..... intensive care unit
IPTG ..... Isopropyl β-D-1-thiogalactopyranoside
kb ..... kilobase pairs
kDa ..... kilodaltons
L ..... Liter
LB ..... Luria-Bertani
LMWF ..... low molecular weight fraction
M ..... molar
Mb ..... megabase
MBEC ..... minimum biofilm eradication concentration
min ..... minutes
mL ..... milliliter
mM ..... millimolar
mmol ..... millimole
mol ..... mole
mRNA ..... messenger RNA
MRSA ..... Methicillin resistant *Staphylococcus aureus*
MSCRAMMs ..... microbial surface components recognizing adhesive matrix molecules
msec ..... millisecond
MW ..... molecular weight
MWCO ..... molecular weight cut-off
NaCi ..... Trisodium citrate
nM ..... nano molar
nm ..... nano meter
N-terminal ..... amino-terminal
OD ..... Optical density
P. aeruginosa ..... *Pseudomonas aeruginosa*
PBS ..... phosphate buffered saline
PBS-t ..... PBS supplemented with 0.05% Tween 20
PCR ..... polymerase chain reaction
pM ..... pico molar
PNAG ..... poly-*N*-acetylglucosamine
RT-PCR ..... reverse transcriptase PCR
RNA ..... Ribonucleic acid
rpm ..... revolutions per minute
rRNA ..... ribosomal RNA
RT ..... reverse transcriptase
S. aureus ..... *Staphylococcus aureus*
S. epidermidis ..... *Staphylococcus epidermidis*
S. mutans ..... *Streptococcus mutans*
SarA ..... Staphylococcal accessory regulator A
SDS ..... sodium dodecyl sulphate
SEM ..... Scanning electron microscopy
SERAMs ..... secretable expanded repertoire of adhesive molecules

SNPs.....Single nucleotide polymorphisms
TAE..... Tris-acetate EDTA buffer
TCA..... Tricarboxylic cycle
Tet^r .....Tetracycline resistance
TetM..... Tetracycline resistance M determinant
TSA..... Tryptic soy agar
TSB .....Tryptic soy broth
TSBG ..... TSB supplemented with 1% glucose
U.....Uracil
UV..... Ultraviolet
VCU .....Virginia Commonwealth University
WT .....wildtype

Abstract

CHARACTERIZATION OF THE EFFECT OF SERUM AND CHELATING AGENTS ON *STAPHYLOCOCCUS AUREUS* BIOFILM FORMATION; CHELATING AGENTS AUGMENT BIOFILM FORMATION THROUGH CLUMPING FACTOR B

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Staphylococcus aureus is the causative agent of a diverse array of acute and chronic infections, and some these infections, including infective endocarditis, joint infections, and medical device-associated bloodstream infections, depend upon its capacity to form tenacious biofilms on surfaces. Inserted medical devices such as intravenous catheters, pacemakers, and artificial heart valves save lives, but unfortunately, they can also serve as a substrate on which *S. aureus* can form a biofilm, attributing *S. aureus* as a leading cause of medical device-related infections. The major aim of this work was take compounds to which *S. aureus* would be exposed during

infection and to investigate their effects on its capacity to form a biofilm. More specifically, the project investigated the effects of serum, and thereafter of catheter lock solutions on biofilm formation by *S. aureus*.

Pre-coating polystyrene with serum is frequently used as a method to augment biofilm formation. The effect of pre-coating with serum is due to the deposition of extracellular matrix components onto the polystyrene, which are then recognized by MSCRAMMs. We therefore hypothesized that the major component of blood, serum, would induce biofilm formation. Surprisingly, serum actually inhibited biofilm formation. The inhibitory activity was due to a small molecular weight, heat-stable, non-proteinaceous component/s of serum. Serum-mediated inhibition of biofilm formation may represent a previously uncharacterized aspect of host innate immunity that targets the expression of a key bacterial virulence factor: the ability to establish a resistant biofilm.

Metal ion chelators like sodium citrate are frequently chosen to lock intravenous catheters because they are regarded as potent inhibitors of bacterial biofilm formation and viability. We found that, while chelating compounds abolished biofilm formation in most strains of *S. aureus*, they actually augmented the phenotype in a subset of strains. We investigated the molecular basis of this phenomenon. Deletion and complementation analysis and thereafter antibody based inhibition assays confirmed a functional role for the surface adhesin clumping factor B as the causative determinant associated with the increased biofilm phenotype. Finally, we investigated the regulation of clumping factor B-mediated biofilm formation and the basis for the strain dependence. Regulation was determined to

occur via two novel post-translational networks- one affecting ClfB activity, mediated by Ca^{2+} binding to the EF-Hand domain, and the other affecting protein stability, mediated by the enzymatic activity of the metalloprotease-aureolysin. Polymorphisms within the aureolysin gene sequence, between strains, was identified as the basis for some strains forming robust biofilms within chelated media versus other than do not exhibit this phenotype.

CHAPTER 1

Introduction

Clinical overview of *Staphylococcus aureus*

The staphylococci make up a family of Gram-positive cocci, Staphylococcaceae, which is in the order Bacillales. The staphylococcal bacteria are named for their distinct grape-like clustering morphology with the etiological root of the word "staphylococcus" coming from the Greek word *staphyle*, meaning bunches of grapes. The staphylococci are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or fermentation. Members of this genus are catalase positive and oxidase negative, distinguishing them from the genus *Streptococcus*, which are catalase negative. To date, there are 32 species and eight sub-species in the genus *Staphylococcus* (Kloos and Bannerman, 1994); however, *Staphylococcus aureus* and *S. epidermidis* are the two best-characterized and studied strains. Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot serum (Kloos and Musselwhite, 1975). This distinguishes the coagulase positive strains including *S. aureus* (primarily a human pathogen) and *S. intermedius* and *S. hyicus* (two animal pathogens), from the other staphylococcal species such as *S. epidermidis*, which are coagulase negative (CoNS).

S. aureus is considered a major pathogen that colonizes and infects both hospitalized patients with decreased immunity, and healthy immunocompetent individuals within the community. While *S. aureus* is an important human pathogen, the bacterium is typically found as a commensal. The human anterior nare is the main reservoir with approximately 20% of individuals persistently colonized with *S. aureus* and 30% intermittently colonized (Wertheim, Melles et al., 2005; Kluytmans, van Belkum et al., 1997; Cole, Tahk et al., 2001). However, numerous other sites may be colonized, including the axillae, groin, and gastrointestinal tract. Colonization increases the risk for subsequent infection since it provides a reservoir from which bacteria can be introduced when host defenses are breached, e.g. by shaving, aspiration, insertion of an indwelling catheter or by surgery. von Eiff et al. provided empirical evidence to this in a study of bacteremia, where blood isolates were identical to nasal isolates in 82% of patients (von Eiff, Becker et al., 2001).

Once *S. aureus* crosses the epithelial boundary, the opportunistic nature of this pathogen, compounded with the armamentarium of virulence factors, facilitates it to infect a wide variety of body tissue. These infections vary in severity from minor, generally self-limiting, purulent soft-tissue skin infections including folliculitis, impetigo and carbuncles (boils) to a variety of life-threatening, potentially fatal, infections including necrotizing pneumonia, osteomyelitis, infective endocarditis and bacteremia. *S. aureus* is the most common cause of bloodstream, skin and soft tissue, and nosocomial lower respiratory tract infections in the United States and Canada (Diekema, Pfaller et al.

2001). The economic impact on the U.S. healthcare system from *S. aureus* infections is $14.5 billion annually (Noskin, Rubin et al., 2007).

S. aureus is also the leading cause of hospital-associated infections in the United States (Styers, Sheehan et al., 2006). This is a direct corollary with the organisms' ability to become multi-drug resistant. With the emergence of antibiotic resistant strains of *S. aureus*, including, Methicillin-resistant *Staphylococcus aureus* or MRSA, control of such infections has become a paramount clinical and pharmacologic battle (Bamberger and Boyd 2005; Grundmann, Aires-de-Sousa et al., 2006). There are nearly 300,000 cases of *S. aureus* infections in hospitalized patents each year in the U.S. Of these, roughly 43% are related to MRSA (Kuehnert, Hill et al., 2005). The prevalence of MRSA infections in hospitals has been increasing from 2% of all staphylococcal intensive care unit infections in 1974, to 22% in 1995 and 64% in 2004 (Kuehnert, Kruszon-Moran et al., 2006). Until recently, MRSA was thought to be a significant threat only in the hospital setting; however, it has been shown that normal healthy individuals can become infected with community acquired MRSA (CA-MRSA); thought to have evolved separately from the hospital acquired clones (Voyich, Otto et al., 2006; Moroney, Heller et al., 2007; Tenover, McDougal et al., 2006). The prevalence of MRSA has also been documented within livestock. A report examining the prevalence of MRSA carriage in swine and swine farmers in the Midwestern U.S. showed that the carriage rate for the animals on average was roughly 50%. It was also found that 45% of the human workers tested positive for MRSA colonization (Smith, Male et al., 2008). These numbers provide evidence for the large threat that *S. aureus* poses as a significant human pathogen,

especially its antibiotic resistant derivative, MRSA.

Two antibiotics currently in circulation to treat MRSA are vancomycin and daptomycin (Chambers and Deleo, 2009; Appleman and Citron, 2010). Vancomycin is a glycopeptide antibiotic that acts to inhibit cell wall synthesis in Gram-positive bacteria The large hydrophilic molecule forms hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the *N*-acetylglucosamine (NAG)/ *N*-acetylmuramic acid (NAM)-peptides. This binding of vancomycin to the D-Ala-D-Ala prevents cell wall synthesis prevents the synthesis of the long polymers of NAM and NAG that form the backbone strands of the bacterial cell wall, and it prevents the backbone polymers that do manage to form from cross-linking with each other. Daptomycin, on the other hand differs from vancomycin and has a distinct mechanism of action by binding to the membrane causing rapid depolarization, resulting in a loss of membrane potential leading to inhibition of protein, DNA and RNA synthesis, which results in bacterial cell death Although these second line antibiotics are used sparingly so as not to promote the spread of resistant strains, in 2002, the first vancomycin resistant *S. aureus* (VRSA) infection was documented in a patient in the U.S. (Sievert, Boulton et al., 2002). This strain was shown to carry the *van* gene cassette, suggesting that the resistance determinant might have been acquired through the genetic exchange of material between vancomycin resistant enterococci and *S. aureus*. The spread of vancomycin resistance worldwide is inevitable and raises fears of a return to pre-antibiotic era. Hence, the need to identify novel virulence-based bacterial targets is a critical pre-requisite in the search for new antibiotics and prophylaxis strategies.

Catheter-related infections and Catheter Lock Solutions (CLS)

Primary bloodstream infections (BSIs) are the most common cause of morbidity and mortality in hospitalized patients and are attributed to the increased use of intravascular devices. It is estimated that there are over 400,000 catheter-related nosocomial BSIs that occur annually in the United States (Maki, Kluger et al., 2006; Raad and Hanna, 2002; Ramos, Reitzel et al., 2010). These infections account for 10-20% of all nosocomial infections; they increase health care costs, prolong patient stay and increase mortality rates (Hugonnet, Sax et al., 2004). These infections add $33,000-$65,000 per case to healthcare costs with a yearly impact in the U.S. estimated to be $1.8 billion (Orsi, Di Stefano et al., 2002; Mayor, 2000). Most central venous catheter (CVC)-related BSIs occur in patients in intensive care units (ICUs), followed by patients with long-term catheters placed for cancer and hemodialysis. A recent study by Warren and colleagues identified vascular device associated infections increased ICU length of stay by 2.41 days and hospital length of stay by 7.54 days (Warren, Quadir et al., 2006). The crude mortality rate of patients with CVC-related BSIs ranges from 12-35% (Pittet, Tarara et al., 1994; Dimick, Pelz et al., 2001; Rello Ochagavia et al., 2000). Staphylococci are the most common cause of CVC-related BSIs. While CoNS *S. epidermidis* is a more frequent isolate, *S. aureus*, which accounts for 20% of BSIs, is associated with higher morbidity and mortality due to severe hematogenous complications, systemic sepsis and invasive infections such as endocarditis (Walz, Memtsoudis et al., 2010). *S. aureus* catheter-related BSIs are associated with greater health-related costs than any other isolated species (Walz, Memtsoudis et al., 2010) and

such infections result in a 6-fold increase in mortality rate in hospitalized patients (Berger, Diab-Elschahawi et al., 2010).

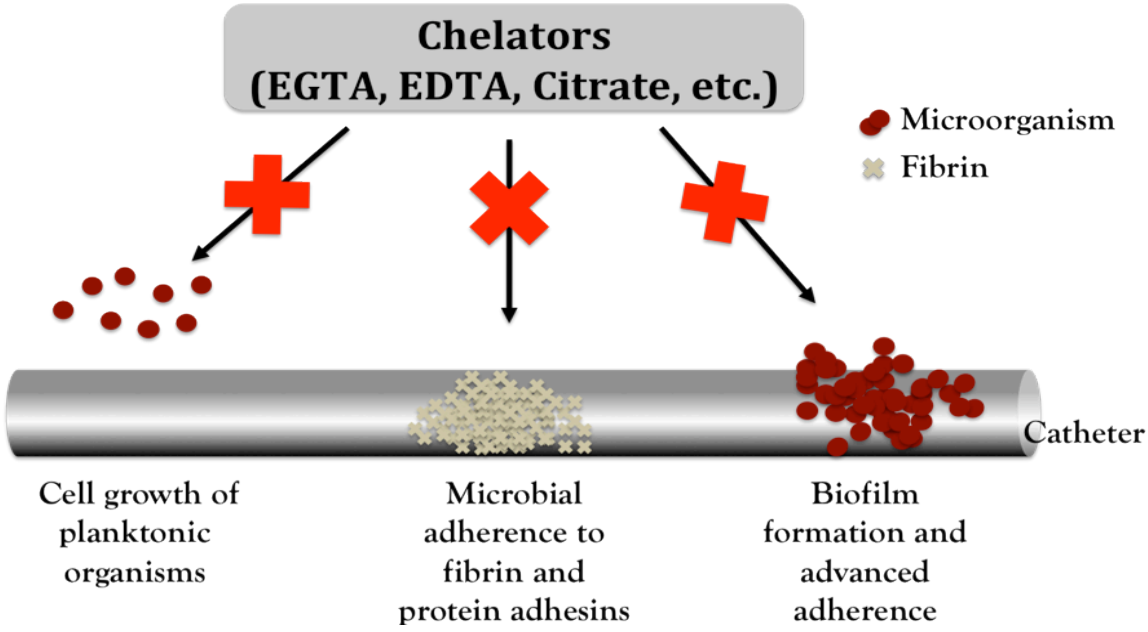
With short-term intravascular catheters, such as peripheral IVs, bacterial colonization and, in turn, contamination is most likely to result from skin microorganisms (75-90%) (Sherertz, 2005; Sherertz, 2000). Such contamination often occurs on the extraluminal surface of the catheter. These catheters can often be removed and replaced, which, along with antibiotic therapy, results in resolution of the infection. However, long-term catheters like CVC often become contaminated intraluminally through contaminated catheter hubs (66%) or by the skin commensals (26%). Several studies have demonstrated that clinical findings correlate well with quantitative catheter cultures and that, the higher number of microorganisms isolated from the surface of the catheter, the greater likelihood of insertion site erythema and/or catheter-related BSIs (Sherertz, Raad et al., 1990; Cleri, Corrado et al., 1980; Brun-Buisson, Abrouk et al., 1987; Siegman-Igra, Anglim et al., 1997). Thus, removal and replacement of such devices is often complicated (Crnich and Maki, 2002).

Factors affecting catheter colonization and infection can be divided into three major categories: those that relate to the host (i.e. immunosuppression, staphylococcal colonization of the nose, severity of underlying disease), the catheter (including duration of catheterization, site of insertion catheter composition and antimicrobial coating), and the causative microorganism. Microbial adherence to a biomaterial is an early, essential event in the pathogenesis of a prosthetic device infection. Microbial risk factors that mediate catheter colonization, as it pertains to *S. aureus*, include surface-associated

MSCRAMMs (Microbial surface components recognizing adhesive matrix molecules), biofilm formation, and a variant subpopulation of *S. aureus* that grow slowly called small-colony variants. These microbial factors aid in the initial stages of adherence and biofilm formation and persistence towards infection. Adhesion and aggregation are mediated by specific adhesin-receptor interactions. *S. aureus* adheres to host proteins including fibrinogen, fibronectin, fibrin, collagen, bone sialoprotein, laminin, elastin, and extracellular matrix proteins commonly found on intravascular devices (Herrmann, Vaudaux et al., 1988). Exopolysaccharides like PNAG (poly-*N*-acetylglucosamine) promote biofilm maturation and some studies suggest that it contributes to virulence (Arciola, Baldassarri et al., 2001; Peacock, Moore et al., 2002; Peacock, de Silva et al., 2002).

Recent guidelines for the prevention and treatment of catheter-related BSIs have proposed the use of intraluminal antimicrobial lock solutions (Mermel, Farr et al., 2001; O'Grady, Alexander et al., 2002; Raad, Costerton et al., 1993). During interdialytic periods, the catheter lumen is locked with an anti-coagulant to prevent intraluminal thrombosis. Some of these Catheter Lock Solutions (CLS), contain cationic chelators like tri-sodium citrate (NaCi), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), which serve as effective anti-coagulants and have recently been shown to possess added antimicrobial and antibiofilm properties (Banin, Brady et al., 2006; Percival, Kite et al., 2005; Shanks, Sargent et al., 2006). As shown in **Figure 1**, the antimicrobial activity of these cationic chelators stems from several mechanistic activities. First, metallic cations are necessary for prokaryotic

Figure 1. The role of chelators in inhibiting bacterial growth, disrupting surface adherence, and preventing biofilm formation. Chelators play an important role in inhibiting biofilm formation through their capabilities of chelating metallic cations that are vital in bacterial cell growth and microbial adherence to fibrin and biofilm formation.



division and the integrity of the bacterial cell wall. EDTA, EGTA and NaCi chelate metallic cations like calcium (Ca^{2+}) and magnesium (Mg^{2+}) leading to inhibition of bacterial growth. Secondly, Ca^{2+} chelators inhibit the formation of fibrin, which plays an important role in adherence of microbial organisms to the surface of an indwelling catheter. Additionally, these divalent cations can stimulate cell-cell adhesion and thus promote aggregation through the shared binding of divalent cations by cell wall teichoic acids; this increases the number of organisms adhering to a finite surface area (Dunne and Burd, 1992; Hall-Stoodley, Costerton et al., 2004). The third mechanism relates to the negative effect chelators have on biofilm formation; this property has been utilized in treating biofilm-related infections by several research groups (Banin, Brady et al., 2006; Raad, Chatzinikolaou et al., 2003; Percival, Kite et al., 2005). In *S. aureus,* sub-inhibitory doses of chelators prevent biofilm formation, at least in part by targeting PNAG (Juda, Paprota et al., 2008; Ozerdem Akpolat, Elci et al., 2003; Shanks, Sargent et al., 2006; Weijmer, Debets-Ossenkopp et al., 2002). While a very low level of NaCi has been shown to augment *S. aureus* biofilm formation, seemingly by acting as a TCA cycle intermediate within the bacterial cell, concentrations of NaCi $\geq$ 0.5% effectively inhibit the biofilm phenotype (Shanks, Meehl et al., 2008; Shanks, Sargent et al., 2006). Therefore many CLS, such as Citra-Lock ™, Dura-Lock-C ™, and Loxxit ™, are now composed solely of the chelating agent tri-sodium citrate.

***Staphylococcus aureus* virulence and regulation**

Genome sequencing of the staphylococci has provided unprecedented insight into

their success as significant bacterial pathogens. Within the past decade, genomes of 13 *S. aureus*, two *S. epidermidis*, one *S. haemolyticus*, and one *S. saprophyticus* have been sequenced (Baba, Bae et al., 2008; Baba, Takeuchi et al., 2002; Diep, Gill et al., 2006; Gill, Fouts et al., 2005; Herron-Olson, Fitzgerald et al., 2007; Holden, Feil et al., 2004; Kuroda, Ohta et al., 2001; Kuroda, Yamashita et al., 2005; Taeuchi, Watanabe et al., 2005). The first sequenced *S. aureus* genomes were published in 2001 by the Hiramatsu group comparing two methicillin-resistant strains, N315 and Mu50 (Kuroda, Ohta et al., 2001). These genome sequences were followed in quick succession over the next 7 years by publication of the genome sequences of MW2 (Baba, Takeuchi et al., 2002), MRSA252 and MSSA476 (Holden, Feil et al., 2004), COL (Gill, Fouts et al., 2005), USA300-FPR3737 (Diep, Gill et al., 2006), USA300-HOU-MR (Highlander, Hulten et al., 2007), NCTC8325 (Gillaspy, Worrell et al., 2006), ET3-1 (Herron-Olson, Fitzgerald et al., 2007), JH1 and JH9 (Mwangi, Wu et al., 2007) and, most recently, Newman (Baba, Bae et al., 2008). Comparative genome analyses between the sequenced strains have identified close evolutionary relationships; analyses have revealed the presence of not only core metabolic functions shared by all *S. aureus* strains, but also previously unidentified virulence factors with key roles in pathogenesis. The arsenal of virulence factors of *S. aureus* is extensive with both structural and secreted products playing a role in the pathogenesis of infections.

Biofilms

Many pathogenic and commensal bacteria are capable of transitioning between life in the environment and in the human host, and all must be able to adapt to sudden

shifts in nutrient availability as well as primary and secondary host immune defenses. One particularly important and clinically relevant example of bacterial adaptation through systematized gene expression is the ability to grow as part of a sessile, exoploymer-enshrouded community referred to as a biofilm. A biofilm is an adherent community of bacteria with an organized structure and chemistry, surrounded by an extracellular biochemical polymer. Scientific interest in the process of biofilm formation has erupted in recent years since this mode of growth has been recognized as distinct from its planktonic or free-floating counterpart and vital for bacterial growth in nature or in infectious diseases (O'Toole, Kaplan et al., 2000; Lindsay and van Holy, 2006).

Biofilm formation is an important aspect of many, if not most bacterial diseases including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients (Donlan and Costerton, 2002). Biofilms impart numerous advantages to their resident bacteria that would encourage this mode of growth. Biofilms impart, on its constituent bacteria, resistance to antimicrobial agents (Passerini de Rossi, Garcia et al. 2009), disinfectants (Kim, Ryu et al. 2007), drying (Vanderlinde, Harrison et al. 2009), ultraviolet light (Bak, Ladefoged et al. 2009), acid exposure (Kubota, Senda et al. 2009), phagocytosis (Cerca, Jefferson et al. 2006) and sheer stress (Giao, Azevedo et al. 2008). Increased resistance to antimicrobials and immune defenses frequently makes biofilm-related infections chronic and/or recurrent. In addition, the physiological heterogeneity throughout the biofilm results in a form of "division of labor", which endows the community with a higher metabolic potential than an equivalent planktonic

population increasing their overall fitness. While both *S. aureus* and CoNS *S. epidermidis* can form biofilms, *S. aureus* harbors more virulence factors and is therefore associated with more severe disease manifestations (George and Muir, 2007). The serious and pervasive clinical impact of *S. aureus* biofilms has inspired focused research and study of this developmental process with the ultimate goal of pinpointing targets for chemotherapeutic agents.

As with any developmental process, biofilm formation is associated with a series of complex but distinct and well-regulated steps. While the exact molecular mechanisms differ from organism to organism, transitioning from a planktonic to a biofilm state, the stages are similar across a wide range of microorganisms. Described schematically in **Figure 2**, the steps associated with this process can be arbitrarily divided into five stages: (i) initial attachment, (ii) irreversible attachment mediated by bacterial surface adhesins, (iii) aggregation through the expression of intracellular adhesins, (iv) maturation and development of biofilm architecture, and (v) dispersion of cells from the biofilm (Stoodley, Sauer et al., 2002). Certain stressors including temperature, osmolarity shock, low oxygen tension, changes in the availability of glucose, glucosamine and *N*-acetylglucosamine, changes in cyclic-di-GMP levels, and exposure to toxic chemicals such as ethanol and antibiotics can induce global changes in gene expression initiating the switch to a sessile biofilm growth modality (Shanks, Donegan et al., 2005; Cramton, Ulrich et al., 2001; Lim, Jana et al., 2004; Resch, Rosenstein et al., 2005; Beenken, Dunman et al., 2004).

Figure 2. Schematic representation of the stages of biofilm formation in *Staphylococcus aureus*. Biofilm formation by *S. aureus* can be divided into five stages. (i) Initial attachment: Cells make their first points of contact with their substratum of choice. These interactions are mediated primarily via non-specific charge interactions and cell surface hydrophobicity. (ii) Irreversible attachment: A stronger, more intimate contact is made between the cells and their surface. Adhesion to host extracellular matrix components is propagated by various surface-associated and secreted adhesins. (iii) Aggregation: After adhesion to the surface, microcolony formation occurs promoted by surface proteins including biofilm-associated protein (Bap), *S. aureus* surface protein G (SasG), and accumulation/adhesion-associated protein (Aap). Exopolysaccharide production (PNAG) along with accessory macromolecules, such as teichoic acids, aid intracellular aggregation. (iv) Maturation: Cell communication (potentially through the *agr* quorum sensing system) leads to cell clusters reaching maximum thickness via production of copious amounts of exopolysaccharide (PNAG). Cells lysis occurs releasing eDNA. (v) Dispersion: Release of bacteria from the biofilm architecture through shearing or production of exoenzymes (alginate lyase in *P. aeruginosa*) to return to a planktonic lifestyle or to seed another location for biofilm formation.

(i) Initial attachment

Adherence via cell surface hydrophobicity and non-specific charge interactions

(ii) Irreversible attachment

Attachment to host matrix proteins

Expression of MSCRAMMs

(iii) Aggregation

Expression of intercellular adhesins- Bap, SasG, Aap

Polysaccharide production (PNAG)

(iv) Maturation

Copious amounts of polysaccharide (PNAG)

Lysing bacteria (eDNA)

Cell-cell adhesion and proliferation

(v) Dispersal

Detachment through shearing or exoenzymes (alginate lyase)

In the initial stage of biofilm formation, bacteria reversibly adhere to a solid surface via nonspecific charge-related interactions and hydrophobic interactions between the cells and their substratum of choice. This is quickly followed by the second stage wherein the bacteria irreversibly attach to the surface. This step is critical in the pathogenesis of all bacterial infections since the bacteria must adhere to the host tissue to avoid clearance by host defenses that physically remove non-self entities, such as the mucociliary escalator. *S. aureus* produces numerous proteinaceous adhesins called MSCRAMMs, which by definition, are surface associated proteins that specifically bind to some component of the host extracellular matrix (Foster and Hook, 1998). The formation of a biofilm on the surface of a subvenous catheter has been extensively researched. When inserting a catheter into the bloodstream, bacteria can become attached to the tip and length of the catheter surface. Rapidly, blood plasma proteins, platelets, fibrin and leukocytes bind to the surface of the catheter; these host products serve as scaffolding for the developing biofilm and receptors for newly arriving bacteria (Cappelli, Tetta et al. 2005). *S. aureus* tightly adheres to proteins like fibrinogen and fibronectin that coat the surface of the catheter using their MSCRAMM proteins thereby promoting irreversible attachment (Arciola, Campoccia et al. 2005; O'Neill, Humphreys et al. 2009).

The third stage of biofilm formation distinguishes adherent bacteria from bacteria that establish themselves within a biofilm. At this stage, the staphylococci initiate expression of intercellular adhesins that allow the bacterial cells to adhere to one another and to aggregate, thereby forming microcolonies. Staphylococcal intercellular adhesins

that promote biofilm development include surface proteins such as biofilm-associated protein (Bap), *S. aureus* surface protein G (SasG), accumulation/adhesion-associated protein (Aap) and a carbohydrate based adhesin known as polysaccharide intercellular adhesin (PIA) in *S. epidermidis* or PNAG in *S. aureus* (Mack, Fischer et al., 1996; Maira-Litràn, Kropec et al.,2002; Corrigan, Rigby et al., 2007).

The fourth stage of development involves the biofilm architecture becoming larger and more porous. The exopolymeric matrix may be composed predominantly of PIA/PNAG, however, depending on strain background and environmental conditions, staphylococcal biofilms that lack such polysaccharides and are primarily proteinaceous, can form (Frank and Patel, 2007; Rohde, Burandt et al., 2007). A recent study by Hennig et al. described a spontaneous switch from a polysaccharide based biofilm to biofilm formation mediated by proteins in an *icaC* insertion mutant within *S. epidermidis* (Hennig, Nyunt Wai et al. 2007). More recently, extracellular bacterial DNA (eDNA), presumably released by lysed bacteria, has also been observed as a component of the staphylococcal biofilm matrix (Qin, Ou et al., 2007; Rice, Mann et al., 2007).

Finally, in order to colonize new surfaces the bacteria within the biofilm detach and disseminate so as to either resume the planktonic lifestyle or to establish a new biofilm at an alternate location (Stoodley, Wilson et al., 2001). Dispersal is accomplished by shearing, where pieces of the biofilm can break off in the flow of blood or urine or by enzymatic dispersal. An example of enzymatic dispersal is seen in *Pseudomonas aeruginosa*, which produces an enzyme called alginate lyase that digests the polysaccharide component of its biofilm, releasing bacteria into the environment (Boyd

and Chakrabarty 1994). However, such active dispersal processes have not been shown to occur in the staphylococci.

The matrix of staphylococcal biofilms can be composed of various components including polysaccharides, proteinaceous adhesins and/or eDNA. These matrix components are unique in that the bacteria can utilize each of these components individually or synergistically in order to establish a successful biofilm. The growing condition and the environmental milieu play critical roles in determining, and potentially altering, the matrix architecture. Therefore, the intrinsic variability of staphylococcal biofilms directly relates to, biofilm-related infections that appear to respond to a therapeutic course of antibiotics, may relapse weeks or even months later, making surgical removal and replacement of the infected tissues or medical device a frequent and unfortunate necessity.

Surface-associated virulence factors

Surface-associated virulence factors of *S. aureus* include structural components of the cell wall, extracellular polysaccharides, and surface-exposed proteins. Collectively, these virulence factors play important roles in aiding *S. aureus* to avoid host defenses and promote colonization. Colonization is an important aspect of staphylococcal pathogenesis and, as infection is frequently by autoinoculation, colonization usually precedes infection. During colonization, the host is not adversely affected although colonization is oftentimes associated with acute local immune response. Therefore, while colonization is not likely to harm a healthy individual, it is a risk factor for disease and infection once normal protective barriers or defenses are weakened. The surface of *S. aureus* is coated with a

variety of adhesins capable of binding to different host proteins in plasma and/or the extracellular matrix: proteins that mediate colonization. These proteins are of paramount importance in the initial attachment steps associated with biofilm formation.

There are at least 28 different *S. aureus* surface-associated proteins that promote the binding of at least 18 different host proteins. These are broadly classified into MSCRAMMs and SERAMs (secretable expanded repertoire adhesive molecules) (Clarke and Foster, 2006). These adhesins are critical in establishing infection through the intimate binding of host-derived proteins. MSCRAMMs bind host extracellular matrix components including collagen, fibronectin, and fibrinogen. Different *S. aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections (Patti, Allen et al., 1994; Foster and Hook, 1998). Specific properties of *S. aureus* surface proteins that define them as MSCRAMMs include the presence of a C-terminal sorting signal containing an LPXTG motif followed by a hydrophobic membrane-spanning region and a positively charged cytoplasmic tail. The LPXTG motif serves to covalently anchor the MSCRAMM to the cell wall peptidoglycan via a reaction catalyzed by sortase A (Schneewind, Mihaylova-Petkov et al., 1993). This group of adhesins include- Protein A (Spa), collagen adhesin (Cna), the fibronectin-binding proteins (FnbA and FnbB) and the fibrinogen-binding clumping factor proteins (ClfA and ClfB). While MSCRAMMs promote the early stages of biofilm formation, they also initiate endovascular infections, bone and joint infections, and prosthetic-device infections (Otto, 2008).

S. aureus Protein A (Spa) classically functions to prevent antibody-mediated opsonization by binding to the Fc region of IgG. However, Spa also binds von Willebrand factor, a large serum glycoprotein that mediates adherence of platelets upon endothelial damage (Hartleib, Kohler et al., 2000). FnbA and FnbB are MSCRAMMs that promote binding to fibronectin, a high molecular weight glycoprotein of the host extracellular matrix. Strains encoding both genes are found more commonly associated with invasive disease (Peacock, Fay et al., 2000). *S. aureus* produces two primary fibrinogen-binding clumping factor proteins designated ClfA and ClfB. Unlike the fibronectin-binding proteins, the fibrinogen-binding proteins are not contiguous within the *S. aureus* chromosome and appear to be fairly well conserved among different strains (Tristan, Ying et al., 2003). Both ClfA and ClfB promote platelet aggregation, and this has been shown to contribute to the development of infective endocarditis (Moreillon and Que, 2004). ClfA, and to a lesser extent ClfB, was shown to promote virulence in an experimental endocarditis model (Entenza, Foster et al., 2000; Vernachio, Bayer et al., 2003).

The ability of *S. aureus* to adhere to plasma and extracellular matrix proteins deposited on biomaterials is a significant factor in the pathogenesis of device-associated infections. Once *S. aureus* adheres to host tissue or prosthetic material, encased within host extracellular matrix components using the above mentioned surface-associated proteins, the bacteria can develop into tenacious biofilms which are refractory to the immune system and antimicrobials leaving device removal or tissue replacement as the lone therapeutic alternative.

Regulation of virulence factors

In order to control its normal cellular functions involved in metabolism and growth, *S. aureus* must have efficient ways to respond quickly to an infinite number of environmental stimuli including antibiotics, phagocytes, cell density, complement, etc. These external signals and stimuli exert their appropriate phenotypic effects through the coordinated action of internal regulators. Thus, *S. aureus* regulates its plethora of virulence factors in a manner that is specific and appropriate for the current stage of colonization or infection. To accomplish this large feat, *S. aureus* has multiple regulatory mechanisms involving three sigma factors, 16 two-component systems, a quorum sensing system through the Agr/RNAIII pathway and a large repository of transcriptional regulators (George and Muir, 2007; Somerville and Proctor, 2009; Pragman and Schlievert, 2004). While these regulatory elements control a wide variety of genes involved in the pathogenic potential of *S. aureus*, this review will be limited to the coordinated activity of two major global regulatory elements: *sarA* (staphylococcal accessory regulator A) and *agr* (accessory gene regulator) as they relate to biofilm formation (**Figure 3**).

The staphylococcal accessory regulator A, SarA is the prototype for the 11-member Sar family of DNA binding regulators that *S. aureus* uses to respond to changing local environments (Cheung, 2008). SarA is a DNA binding protein that is known to regulate the expression of many virulence factors, especially extracellular proteases, cell wall proteins and biofilm formation (Bronner, Monteil et al., 2004; Beenken, Blevins et al., 2003; Karlsson and Arvidson, 2002; Chien, Manna et al., 1999). Expression of SarA

Figure 3. Balance between SarA and Agr during *S. aureus* biofilm formation. Two major regulators associated with biofilm formation- Agr and SarA work in concert with one another. Staphylococcal accessory regulator A (SarA) is a global regulator that is primarily expressed during early-mid exponential phase of growth. SarA positively regulates virulence factor expression through increased expression of surface-associated proteins involved in initial colonization and biofilm formation while simultaneously downregulating expression of extracellular proteases and exoenzymes. Crowding during post-exponential phase activates the accessory gene regulator (agr) system that stimulates toxic exoprotein production enabling the bacteria to release themselves from the biofilm and spread to new sites of colonization.

is dependent upon growth phase, with its greatest expression observed during the early to mid-exponential growth phase. Thus, it can regulate virulence determinants in a temporal manner, upregulating surface-associated proteins involved in initial colonization and biofilm formation like the fibronectin-binding proteins (FnbA and FnbB) (Cheung, Koomey et al., 1992; Xiong, Bayer et al., 2004). SarA acts by either directly binding to the promoter region, stabilizing mRNA, or indirectly through other regulators such as the Agr system (Bronner, Monteil et al., 2004). In addition, SarA is a positive regulator of *ica* (intercellular adhesin) operon transcription, which is associated with PNAG production and thus enhances polysaccharide production and biofilm formation (Tsang, Cassat et al., 2008). Mutation of *sarA* leads to decreased biofilm formation and increased antimicrobial susceptibility in *S. aureus* (Weiss, Spencer et al., 2009). One can theorize that SarA is turned on when cells are at a high enough density where adherence to tissues is desired.

In considering biofilm formation and adaptive physiology of *Staphylococcus*, quorum sensing is an important phenomenon. Many bacterial behaviors including growth, biofilm formation, and antibiotic resistance are regulated by quorum sensing (Keller and Surette, 2006). Quorum sensing is the perception of a critical density of a secreted molecule (autoinducer) by a bacterial cell that results in a change in gene expression. This will occur when the bacterial population reaches a certain density or "quorum" within a confined space. Staphylococci possess two quorum-sensing systems, the *agr* system and the *luxS* system that negatively influence biofilm formation. The *agr* locus contains two divergent promoters, P2 and P3, which encode for the RNAII and RNAIII transcripts respectively. RNAIII is the effector molecule of the *agr* response and

is responsible for upregulating extracellular proteases and exoenzymes including *hla* (α-toxin) (Morfeldt, Taylor et al., 1995; Oscarsson, Kanth et al., 2006) and *sspA* (serine proteinase) (Oscarsson, Tegmark-Wisell et al. 2006), and downregulating of cell wall associated proteins, such as the fibronectin-binding proteins (FnbA and FnbB) and *spa* (protein A) (Novick, Ross et al., 1993) during the post-exponential phase.

As illuminated in **Figure 3**, the two global regulators- SarA and Agr play opposing roles within *S. aureus* biofilm formation. During early stages of the biofilm development through exponential phase, SarA accounts for increased expression of surface-associated adhesins and repression of extracellular enzymes including proteases and nucleases. As the biofilm matures, the increasing cell density turns 'on' the Agr quorum sensing system, which serves to prevent the biofilm from becoming too large and acts as a mechanism for dispersal through decreased expression of surface proteins and increased expression of secreted protease.

Research Objectives

Due to the clinical gravity and high morbidity and mortality associated with *S. aureus* infections, identification of compounds that the bacterium would encounter *in vivo* and their effects on biofilm formation is paramount. That said, this project first elucidates the role of host serum components, and thereafter, the effect of chelators and catheter lock solutions on *S. aureus* biofilm formation. These objectives have resulted in identification of novel host-derived serum components that successfully inhibit strains of *S. aureus* from establishing biofilms. Additionally, this project has revealed a novel

outlook on the role of chelators and their proposed antibiofilm properties; being limited only to a subset of strains. This study revealed a newly defined subgroup of strains that counter the inhibitory effects of these chelators and catheter lock solutions. Knowledge and understanding the role these compounds have within *S. aureus* biofilm formation can provide new insight into alternative therapeutics against this, clinically relevant, developmental process.

CHAPTER 2

Materials and Methods

Strains and culture conditions. Bacterial strains used in these studies are listed in **Table 1**. *S. aureus* strains were grown on Tryptic Soy Agar plates (TSA) (BD, Sparks, MD), or TSA plates, supplemented with the appropriate antibiotic as required for selection, at 37 °C overnight, or as indicated. Liquid cultures of *S. aureus* were grown in either Tryptic Soy Broth (TSB) (EMD Chemicals Inc, Gibbstown, NJ) with or without addition of 1% D-Glucose (TSBG) (Fisher Scientific, Fair Lawns, NJ) at 37 °C overnight with shaking at 200 rpm. *E. coli* strain CH3 Blue was grown in LB (EMD Chemicals Inc, Gibbstown, NJ), either as broth or on plates at 37 °C overnight. The following antibiotics were incorporated into the media when appropriate: 10 μg/mL erythromycin (Erm), 100 μg/mL ampicillin (Amp), 35 μg/mL chloramphenicol (Cm), 5 μg/mL tetracycline (Tet). Primers used throughout these studies are listed in **Table 2**.

Static biofilm assay. Biofilm assays were performed essentially as described previously by Christensen et al. (Christensen, Simpson et al., 1985). Briefly, overnight planktonic cultures of *S. aureus* were diluted to a final OD_{600nm} of 0.015 in fresh media and 200 μL of the culture was aliquoted into individual wells of a 96 well Cell Culture plates (Cellstar, Greiner Bio-one, Monroe, NC). The cells were grown in Tryptic Soy Broth

Table 1. Bacterial strains used in this study

***S. aureus* Strains**	**Description**	**Source**
SA113	**ATCC 35556; PNAG-dependent biofilm producer**	**Iordanescu and Surdeanu, 1976**
SA113 Δ*ica::tet*	***ica* mutant of *S. aureus* SA113 (Tet^r)**	**Cramton, Gerke et al., 1999**
MN8	**Clinical isolate from nonmenstrual toxic shock syndrome case**	**Schlievert and Blomster, 1983**
MN8 Δ*ica::tet*	***ica* mutant of *S. aureus* MN8 (Tet^r)**	**Jefferson, Cramton et al., 2003**
RN450	**NCTC8325 cured of Φ11, Φ12, and Φ13**	**Novick, 1967**
RN450 Δ*agr::tetM*	***agr* mutant of *S. aureus* RN450 (Tet^r)**	**Novick, Ross et al., 1993**
RN4220	**Restriction defective derivative of RN450**	**Kreiswirth, Lofdahl et al., 1983**
RN4220 Δ*clfB::erm*	***clfB* mutant of *S. aureus* RN4220 (Erm^r)**	**This study**
10833	**ATCC 25904; Clinical isolate from a throat swab, clumping factor-positive variant of Newman**	**Cramton, Gerke et al., 1999**
10833 Δ*clfB::erm*	***clfB* mutant transduced from RN4220 Δ*clfB::erm* (Erm^r)**	**This study**
10833 Δ*clfB* / pCL15-*clfB*	**Complementation of *clfB* mutant *in trans* using pCL15 (Cm^r)**	**This study**
10833 Δ*aur::Tn*	***aur* Tn mutant transduced from NE163 (Erm^r)**	**This study**
10833 Δ*aur* / pCL15- 10833-*aur*	**Complementation of *aur* mutant *in trans* using pCL15 and 10833 *aur* sequence (Cm^r),**	**This study**
10833 Δ*aur* / pCL15- Newman-*aur*	**Complementation of *aur* mutant *in trans* using pCL15 and Newman *aur* sequence (Cm^r),**	**This study**
Newman	**ATCC 31153; Clinical isolate from osteomyelitis, produces clumping factor**	**Duthie and Lorenz, 1952**
Newman Δ*agr::tetM* (Colony A)	**Single colony from *agr* mutant transduced from RN450 Δ*agr::tetM* (Tet^r)**	**This study**
Newman Δ*clfB::erm*	***clfB* mutant transduced from RN4220 Δ*clfB::erm* (Erm^r)**	**This study**
Newman Δ*clfB* / pCL15-*clfB*	***clfB* mutant of *S. aureus* Newman (Erm^r) complemented *in trans* using pCL15 (Cm^r)**	**This study**
Newman Δ*aur::Tn*	***aur* Tn mutant transduced from NE163 (Erm^r)**	**This study**
Newman Δ*aur* / pCL15- 10833-*aur*	**Complementation of *aur* mutant *in trans* using pCL15 and 10833 *aur* sequence (Cm^r),**	**This study**

Newman Δ*aur* / pCL15- Newman-*aur*	**Complementation of *aur* mutant *in trans* using pCL15 and Newman *aur* sequence (Cm^r),**	**This study**
USA300 JE2	**USA300 LAC (MRSA) cured of 3 plasmids pUSA03, pUSA02, pUSA01**	**Kennedy, Otto et al., 2008**
NE391 (Δ*clfB*)	**Transposon (*bursa aurealis*, a mariner-based transposon) insertion of USA300 JE2 within *clfB***	**NARSA Repository**
NE543 (Δ*clfA*)	**Transposon (*bursa aurealis*, a mariner-based transposon) insertion of USA300 JE2 within *clfA***	**NARSA Repository**
NE186 (Δ*fnbA*)	**Transposon (*bursa aurealis*, a mariner-based transposon) insertion of USA300 JE2 within *fnbA***	**NARSA Repository**
NE551	**Transposon (*bursa aurealis*, a mariner-based transposon) insertion of USA300 JE2 within a putative fibrinogen/fibronectin binding protein**	**NARSA Repository**
NE163 (Δ*aur*)	**Transposon (*bursa aurealis*, a mariner-based transposon) insertion of USA300 JE2 within *aur***	**NARSA Repository**
VF Strain #19 Δ*clfB::erm*	**Clinical cardiac device-associated isolate-VF Strain #19, *clfB* mutant transduced from RN4220 Δ*clfB::erm* (Erm^r)**	**This study**
VF Strain #24 Δ*clfB::erm*	**Clinical cardiac device-associated isolate-VF Strain #24, *clfB* mutant transduced from RN4220 Δ*clfB::erm* (Erm^r)**	**This study**

***E. coli* Strains**	**Description**	**Source**
CH3 Blue	**Chemically competent cells, derivative of *E. coli* K12, Cloning strain (Amp^r)**	**Bioline (Tauton, MA)**

Table 2. Primers used in this study.

Name	Sequence
16S RT Fwd	5'-TCCGGAATTATTGGGCGTAA-3'
16S RT Rev	5'-CCACTTTCCTCTTCTGCACTCA-3'
icaA RT Fwd	5'-AAACTTGGTGCGGTTACAGG-3'
icaA RT Rev	5'-GTAGCCAACGTCGACAACTG-3'
sarA RT Fwd	5'-TTGCTTTGAGTTGTTATCAATGG-3'
sarA RT Rev	5'-TTTCTCTTTGTTTTCGCTGAT-3'
sasG RT Fwd	5'-ACCACAGGGTGTAGAAGCTAAATC-3'
sasG RT Rev	5'-CGAGCTTTTCTAACCTTAGGTGTC-3'
sigB RT Fwd	5'-TTATGGGGCAACAAGATGAC-3'
sigB RT Rev	5'-TAAACCGATACGCTCACCTG-3'
ccpA RT Fwd	5'-AGTGTCGCGTGTTGTTAATG-3'
ccpA RT Rev	5'-AAGTCCACGAGCAAGTTGTG-3'
fnbA RT Fwd	5'-ATCAGCAGATGTAGCGGAAG-3'
fnbA RT Rev	5'-TTTAGTACCGCTCGTTGTCC-3'
fnbB RT Fwd	5'-AAGAAGCACCGAAAACTGTG-3'
fnbB RT Rev	5'-TCTCTGCAACTGCTGTAACG-3'
clfA RT Fwd	5'-CTGGGCTTCAGTGCTTGTAG-3'
clfA RT Rev	5'-TCCTGTTGTGCTGGATTTTG-3'
clfB RT Fwd	5'-TCGAACGATACAACGCAATC-3'
clfB RT Rev	5'-TGTTGAAGCTGGCTCTGTTG-3'
clfB Soe 1	5'-AGATCTGCACAAGGTAAGTTTGTGGA-3'

clfB Soe2	5’-CTCGAGCGTCTAATCGAATACTTATT-3’
clfB Soe3	5’-CAGAATAAGTATTCGATTAGACGCTC-3’
clfB Soe4	5’-AGATCTCCTTGTTCAATTCAGCAATGA-3’
clfB-pCl15 Fwd	5’-GGGAAAGGGCTCCAGTTGAAAAAAAG-3’
clfB-pCl15 Rev	5’-GGGAAAGGGGAATTCTTACGCTTTTT-3’
RNAIII RT Fwd	5'-AATTAGCAAGTGAGTAACATTTGCTAGT-3'
RNAIII RT Rev	5'-GATGTTGTTTACGATAGCTTACATGC-3'
*clfB*PROFWD	5’-TGTTGTTAAAGATCATAAAAATTGGTT-3’
*clfB*PROREV	5’-TTACGCTTTTTCTTTATGATCTTGCTTG-3’
ClfBRACE	5’-GTACCTACTGTAAAACGTCTAATCG-3’
ClfBRACEnest	5’-TCGAATACTTATTCTGCTTATTCGAC-3’
aur-pCl15 Fwd	5'-GTGAGGAAATTTTCAAGATATG-3'
aur-pCl15 Rev	5'-TTACTCCACGCCTACTTCATTCC-3'
aur-Seq-ExtFwd	5'-CGATTATTGCGTCTTACATAGTTG-3'
aur-Seq-ExtRev	5'-AGACAACCCTCACACTCCTCTC-3'
aur-Seq-IntFwd	5'-GTATTGACGGTGGATTTAGCC-3'
aur-Seq-IntRev	5'-GCACGTTGCTCATCTTTTACG-3'

(TSB) supplemented with 1% glucose (TSBG). Additional reagents or compounds were supplemented as per the requirements for each individual experiment. The plates were incubated at 37 °C for 18 h, spent medium was removed, and the wells were washed once with 200 μL of sterile water, dried and stained with safranin. The biofilms were visually analyzed using a flatbed HP color LaserJet 2820 scanner (Hewlett-Packard Company) and quantified by dissolving the stain with 200 μL 33% (v/v) acetic acid and determining the OD_{415nm} using a 96-well plate spectrophotometer (BioTek 800 Plate reader, Winooski, VT). Replica plates were used to determine growth at OD_{595nm}. Results for these experiments were compiled from three biologic replicates each of which contained five technical replicates.

Dispersal/Protection Assay. *S. aureus* biofilms were pre-established on individual wells of a 96-well Cell Culture plates using the appropriate strain inoculated into the respective media condition as described above. After 18 h incubation, the wells were removed off their media and replaced with an equivalent volume of varying concentrations of metallic cations, diluted in sterile deionized water, or recombinant Aureolysin metalloprotease synthesized from *S. aureus* strain V8-bc10 (Axxora LLC., San Diego, CA), diluted in 1X PBS, and incubated for another 18 h at 37 °C without any shaking. The biofilms retained on the plate were then treated as before i.e. washed for any non-adherent or dispersed cells with 200 μL of sterile water, dried and stained with safranin. Quantification of any detectable biofilm was thereafter performed as described above.

Quantification of free calcium (Ca^{2+}). The concentration of free calcium ions (Ca^{2+}) in TSB, TSBG, and TSB supplemented with different concentrations of EGTA was determined using the QuantiChrom™ Calcium Assay kit (BioAssay Systems, Hayward, CA) according to manufacturer's instructions. TSB with varying concentrations of added Ca^{2+} was used to ensure accuracy and efficiency of the kit.

Confocal Microscopy of S. aureus biofilms. Diluted overnight cultures of *S. aureus* strain SA113 were grown for biofilms within wells of a 6-well plate (Cellstar, Greiner Bio-one, Monroe, NC) under calcium replete (TSBG + 12.5 mM Ca^{2+}), calcium deplete (TSBG + 12.5 mM EGTA) or calcium reconstituted (TSBG + 12.5 mM Ca^{2+}+ 12.5 mM EGTA) conditions. Biofilms were grown overnight at 37 °C for 18 h without any shaking. The wells were then washed for their media and non-adherent cells with an equivalent volumes of sterile water. Biofilms were stained with 3 μL SYTO® 9 (Green) and 3 μL propidium iodide (Red) of the Live/Dead BacLight™ stain kit (Invitrogen, Carlsbad, CA) diluted in 3 mL of PBS for 15 min in the dark. Green-fluorescent SYTO® 9 stain when used alone labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. Biofilms were then washed twice with PBS solution, and confocal imaging was performed with a multiphoton confocal scanning laser microscope (Zeiss LSM 510; Microscopy Core Facility, Department of Anatomy and Neurobiology, VCU, Richmond, VA).

Scanning Electron Microscopy of S. aureus biofilms. Overnight cultures of strain 10833 were grown for biofilms within TSBG or TSBG supplemented with 12.5 mM EGTA on glass microscope coverslips that were placed within individual wells of a 6-well plate (Cellstar, Greiner Bio-one, Monroe, NC). The plate was allowed to incubate at 37 °C for 18 h. The plate and coverslips were washed gently for non-adherent bacteria with sterilized water. The coverslips were sterilely removed from the wells and placed in a new 6-well plate. Processing of the cover slips was performed by Ms. Judy Williamson (Department of Anatomy and Neurobiology, VCU, Richmond, VA). The processed cover slips were visually analyzed and imaged using a Zeiss EVO 50 XVP scanning electron microscope (Microscopy Core Facility, Neuroscience Department, VCU, Richmond, VA).

Characterization of serum components. Small molecular weight components were separated from FBS by adding 15 mL FBS to Amicon Ultra filter units (Millipore, MA) with a 3000 MWCO. The samples were centrifuged at 4000 rpm for approximately 60 min at 4 ºC. For some experiments, heat labile components in the <3,000 kDa fraction were destroyed by heating at 100 ºC for 5 min. In others, proteinaceous serum components were destroyed by incubating with 0.4 mg/mL proteinase K at 65 ºC for 1 h followed by incubation at 100 ºC for 5 min to inactivate the protease. All fractions were filter sterilized prior to use.

Adherence to catheter tubing. Overnight cultures of 10833 and Newman were diluted to an OD_{600nm} of 0.015, inoculated into test tubes with TSBG or TSBG containing 12.5 mM EGTA and a ~2 cm piece of intravenous catheter tubing (BD, Franklin Lakes, NJ) and incubated at 37 °C for 18 h without shaking. After overnight incubation, the catheter tubes were sterilely removed, washed once with deionized water, sonicated in 1 mL of 1X PBS and plated on TSA for cfu counts. The assay was performed in triplicate and the results are represented as the mean cfu count.

Clumping assay. Overnight cultures of bacteria were diluted to a final OD_{600nm} of 0.015 in 2 mL TSBG or TSBG containing 12.5 mM EGTA. The cultures were incubated, without shaking, at 37 °C for 18 h. Tubes were lightly vortexed and visually inspected for clumping and imaged using a Nikon CoolPix S80 digital camera (Nikon).

Assessment of δ-hemolysis by S. aureus strains. Strains of *S. aureus* were grown overnight on TSA plates supplemented with the appropriate antibiotic, if needed. A single colony was isolated to be restreaked onto Brucella Blood agar plates (Oxyrase, Mansfield, OH) and grown overnight at 37 °C for 18 h. A zone of clearing around the streaked cultures was deemed a positive identification of hemolytic activity. The plates were set atop a light box and imaged.

RNA Isolation. Samples were separated by centrifugation at 4,000 rpm at 4 °C for 15 min in an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany), the spent media was

discarded and pellets were frozen immediately at -80 °C until use. RNA was extracted using the Qiagen RNeasy® mini kit (Qiagen, Valencia, CA) with some modifications. Samples were resusupended in 500 µL of Qiagen Buffer RLT and added to 2 mL tubes containing 1 mm glass beads (Research Products International Corp., Mount Prospect, IL) or Lysing Matrix B® tubes (MP Biomedicals, Solon, OH). 500 µL cold acid phenol was added and the cells were lysed twice using FastPrep FP120 (Thermo, Pittsburgh, PA) at a speed setting of 6.0 for 45 s. The tubes were cooled for 5 min on ice between the runs. Samples were centrifuged at 14,000 rpm for 10 min at 4 °C. The aqueous phase was collected and 500 µL 100% ETOH (Invitrogen, Carlsbad, CA) was added and mixed. Samples were then loaded into the RNAeasy column (Qiagen). Following the manufacturers' instructions, columns were washed with 700 µL RW1 (Qiagen) followed by 2X washes of RPE (Qiagen). The RNA was eluted twice with 50µl nuclease-free H_20. Contaminating DNA was digested with Turbo DNase (Ambion, Austin, TX) for 1 h at 37 °C. Turbo DNAse was inactivated and removed using DNAse Inactivation Reagent (Ambion) according to manufacturers instructions. The absorbance of each RNA sample, post DNase treatment, was measured at 260 and 280 nanometers using either a NanoDrop 1000 (Thermo Scientific, Milford, MA) and the ratio of these two values was used to determine the relative purity and concentration of the RNA samples.

5' Race Analysis. The *clfB* transcriptional start site was determined using the FirstChoice RLM-RACE kit (Ambion). 10 µg RNA was incubated in 1X reaction buffer in a 20 µL total volume with 1 U of Terminator enzyme (Epicentre Biotechnologies, Madison, WI),

a 5' mono-phosphate dependent exonuclease, to degrade rRNA, tRNA, and partially degraded mRNA for 1 h at 37 °C. The reaction was then terminated by phenol extraction and ethanol precipitation and the RNA was subjected to 5'-RACE analysis according to manufacturer's protocol. Primer ClfBRACE was used for the initial PCR reaction and ClfBRACEnest was used for the nested PCR. The nested PCR product was cloned in pCR4TOPO (Invitrogen) and sequenced (Eurofins MWG Operon).

cDNA synthesis. cDNA was synthesized from 4 μg of isolated mRNA by a two-step reverse transcriptase reaction using the Bioline Tetro cDNA synthesis kit (Bioline, Tauton, MA) according to manufacturer's instructions. The RNA and 10 pmol of gene specific reverse primer (**Table 2**) in a 10 μL volume were heated at 70 °C for 10 min and then incubated on ice for 2 min. The reverse transcriptase master mix containing 5X RT Buffer, 10 mM dNTPs, RNase Inhibitor, and RT enzyme is aliqouted into each sample for an additional volume of 10 μL. The reactions were incubated at 42 °C for 2 h, followed by 70 °C for 15 min. Control reactions lacked reverse transcriptase enzyme.

Quantitative RT-PCR. Realtime RT-PCR reactions contained 2 μL cDNA or no-RT control (diluted 1:500 for 16S reactions and 1:5 for other genes), 1 pmol forward and reverse primers, 8.5 μL nuclease free deionized water and 12.5 μL SYBR Green Sensimix™ (Bioline, Tauton, MA). Realtime RT-PCR was performed using an iQ5 Multicolor Realtime PCR (BioRad, Hercules, CA) under the following conditions: 94 °C for 2 min, 40 cycles of 94 °C for 10 s, 54 °C for 15 s, 72 °C for 15 s. The normalized

amount of transcript for each gene was expressed as the n-fold difference relative to the control gene ($2^{\Delta CT}$, where ΔCT represents the difference in threshold cycle between the target gene and the 16S rRNA gene). Averages were obtained from technical replicates and standard deviation was determined. Biological triplicates were performed for each sample.

gDNA Extraction. Genomic DNA was extracted from *S. aureus* cells streaked onto TSA plates supplemented with the appropriate antibiotic, if necessary. Post overnight incubation, a small loop of culture was removed from the respective plate and resuspended in 200 µL Lysostaphin (5.0 mg/mL) (Sigma, St. Louis, MO) diluted in 1X TE buffer (10mM Tris-HCl pH 7.5, 1 mM EDTA) and incubated for 30 minutes at 37ºC. Samples were then incubated with 25 µl of Qiagen Proteinase K solution (20 µg/mL stock) (Qiagen, Valencia, CA) and 200 µL Qiagen Buffer AL at 56 ºC for 30 min. The samples were treated with 200 µl of EtOH and transferred into Qiagen DNeasy Mini Spin columns. DNA was thereafter extracted according to the Qiagen Blood and Tissue Kit protocol. DNA was eluted off the columns twice with 50 µL of Qiagen Buffer AE.

DNA and Plasmid manipulations. DNA was gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) as described by the manufacturer. All restriction enzymes, specific buffers and BSA used for restriction enzyme digests of miniprep DNA or PCR fragments were purchased from New England Biolabs (Ipswich, MA) and used as recommended by the supplier. Removal of phosphoryl groups from the 5' ends was

accomplished with Antarctic Phosphatase (New England Biolabs, Ipswich, MA) using the supplied buffer. Ligations used Ready-To-Go™ T4 DNA Ligase and Ligase Buffer (Amersham Biosciences, Piscataway, NJ). PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) as recommended by the manufacturer. Plasmid mini-preps were prepared from individual *E. coli* transformants that were grown overnight in 3 ml of LB broth with ampicillin, using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA,) as described by the manufacturer.

Agarose gels. Agarose gels were prepared by dissolving agarose in 1X Tris-Acetate-EDTA (TAE) buffer (Fisher Scientific, Pittsburgh, PA). Ethidium bromide was added to 0.15 µg/mL prior to pouring the gel. PCR products, diluted in 10X BlueJuice gel loading buffer (Invitrogen) for a final concentration of 1X, were run on a 1.0% agarose gels. Gels were run at 125 volts until the dye front had migrated roughly ¾ of the length of the gel and then visualized under UV light. DNA size was measured against the Hyperladder (BioLine, Taunton MA) series of DNA MW markers.

Competent cell preparation. Electrocompetent *S. aureus* RN4220 cells were prepared from overnight cultures grown in TSB at 37 °C with shaking. Cultures were subcultured in fresh media (1:100 dilution) and were grown to exponential phase (OD_{600nm} ~ 0.5). The cultures were collected by centrifugation for 15 min at 4,000 rpm at 4 °C. Care was taken to ensure all subsequent steps were performed on ice. The pellet was then washed three times with 25 mL of ice cold sterile water. The pellet was then washed one time in 30 mL

of ice cold sterile 10% glycerol. The pellet was then resuspended in 15 mL of ice-cold sterile 10% glycerol and incubated at room temperature for 15 min. The final bacterial pellet was resuspended in 500 µL sterile 10% glycerol. Aliquots (50µl) were then flash frozen in a dry ice/EtOH bath and were stored at -80 °C until use.

Transformation. One vial of chemically competent CH3-Blue *E. coli* cells (50 µL) was thawed out on ice. Ligation reactions (3 µL) were added competent *E. coli* cells mixed gently and incubated on ice for 10 min. The tubes were then transferred to a water bath set to 42 °C for 30 s and then put back on ice for 5 min. The cells were then added to 0.25 ml of SOC media (2% wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 85.5 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) and incubated for one hour at 37°C with shaking. Following this recovery period the cells were plated on LB plates with appropriate antibiotic selection.

Plasmid DNA isolated as described above was used to transform electrocompetent *S. aureus* RN4220. Plasmid DNA (7.5 µL) was added to a 50 µL aliquot of electrocompetent *S. aureus* and incubated on ice for 10 min. The mixture was then transferred to an electroporation cuvette with a gap length of 0.1 cm and pulsed one time using the MicroPulser (Bio-Rad, Hercules, CA) pre-set *S. aureus* setting Sta (1.8 kV, 2.5 msec, 25 µF). Following electroporation, 0.5 mL of TSB supplemented with the appropriate antibiotic was added immediately to the cuvette and this mixture was then transferred to a glass culture tube. The antibiotic to be used for selection was added at a

subinhibitory concentration (erythromycin = 0.05 μg/mL) and the cells were incubated for 1.5 h with shaking at 37 °C. Aliquots (100 μL and 200 μL) were then spread on TSA supplemented with appropriate antibiotic selection and incubated at 30 °C for 48 h.

Phage Transduction. The generalized transducing phage 80α was used to create allelic exchange mutations in *S. aureus* RN4220. A freezer stock of the respective donor strain carrying the mutation to be mobilized was inoculated onto TSA supplemented with the appropriate antibiotic, and grown overnight at 37 ºC. A single colony was subcultured onto a new plate and allowed to grow overnight at 37 ºC. All of the culture was removed from the plate and resuspended in 0.5 mL of TSB in a small tube. 200 μL of this culture was added to 0.4% soft agar in a new tube, along with 5 μL of 2M $CaCl_2$ and either 75 μL of phage 80α or no phage. This mixture was plated onto TSA plates and monitored for lysis. Following lysis, the soft agar was collected, centrifuged, and the supernatant was filter sterilized through a 0.45 μm filter. Aliquots of this lysate, either 75 or 150 μl, were then mixed with 150 μL of recipient bacteria, and 5 μL of 2M $CaCl_2$. The mixture was incubated with shaking at 37 ºC for exactly 20 min. 5 mL of 20 mM sodium citrate was added, the culture was centrifuged to pellet the bacteria, the pellet washed twice with 20 mM sodium citrate, then incubated for 1.75 h in 500 μL of 20 mM sodium citrate. Finally, the mixture was plated on TSA supplemented with the appropriate donor antibiotic and sodium citrate and these plates were incubated for 48 hours at 37 ºC.

Construction of clfB deletion and complementation mutants. The *clfB* gene was replaced

with an erythromycin resistance cassette in *S. aureus* strain RN4220 by homologous recombination using the pMAD vector (kindly provided by Michel Débarbouillé and Maryvonne Arnaud, Pasteur Institute, Paris, France) as previously described by Arnaud et al. (Arnaud, Chastanet et al., 2004). Genomic DNA from strain SA113 was used as a template for PCR using primers *clfB*Soe1 and *clfB*Soe4 and the PCR product was cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA). The resulting construct was amplified using primers *clfB*Soe2 and *clfB*Soe3, digested with XhoI, and ligated to an erythromycin (*erm*) resistance cassette excised from plasmid pSC57 with XhoI. The resulting plasmid was digested with BamHI and the region surrounding *clfB* gene and the intervening *erm* cassette were ligated to pMAD. This construct was electroporated into strain RN4220 (Lee, 1995). Once gene replacements in RN4220 were confirmed, the mutations were then transduced into *S. aureus* strains 10833 and Newman using phage 80α (Kasatiya and Baldwin, 1967) and transductants were selected on TSA plates containing 10 mg erm/mL.

For complementation of the deletion, *clfB* was cloned into the isopropyl-β-$_{D}$-thiogalactopyranoside (IPTG)-inducible vector pCL15 (Luong and Lee, 2006). Primers *clfB*-pCL15Fwd and *clfB*-pCL15Rev were used to amplify the *clfB* gene from strain SA113 genomic DNA. The PCR product was digested with BamHI and EcoRI and ligated to pCL15 to produce *clfB*-pCL15. Plasmids were purified and transformed into RN4220 and selected on TSA containing chloramphenicol (cm) before the constructs were transduced into strains 10833 and Newman by phage 80α. Strains harboring *clfB*-pCL15 or pCL15 empty vector were cultured in the presence of 10 mg cm/mL and

induced in the presence of 1 mM IPTG.

Construction of aur deletion and complementation mutants. The *aur* transposon insertion mutations within strains 10833 and Newman were generated by phage transducing the mutation from the NARSA NE163 strain essentially as described above (Kasatiya and Baldwin et al., 1967). The resultant transductants were selected on TSA plates containing 10 mg erm/mL.

For complementation of the deletion, *aur* from 10833 or Newman was cloned into the isopropyl-β-$_D$-thiogalactopyranoside (IPTG)-inducible vector pCL15 (Luong and Lee, 2006). Primers *aur*-pCL15Fwd and *aur*-pCL15Rev were used to amplify the *aur* gene from 10833 or Newman genomic DNA. The PCR product was digested with BamHI and KpnI and ligated to pCL15 to produce Newman-*aur* pCL15 or 10833-*aur* pCL15. Plasmids were purified and transformed into RN4220 and selected on TSA containing chloramphenicol (cm) before the constructs were transduced into the *aur* mutant strains of 10833 and Newman by phage 80α. Strains harboring *aur*-pCL15 were cultured in the presence of 10 mg cm/mL and 1 mM IPTG

Antibody mediated inhibition of aggregation and clumping. Overnight cultures of *S. aureus* strains were diluted and added to test tubes containing TSBG or TSBG + 12.5 mM EGTA and 4.0 µg/mL α-ClfB antibody or rabbit pre-immune sera or the absence of any added antibody. The tubes were additionally supplemented with 1 mM IPTG for induction of complementation constructs. The tubes were incubated at 4 °C for 2 h to

promote antibody binding and thereafter moved to 37 °C for 18 h without shaking. Tubes were lightly vortexed and visually inspected for clumping and imaged using a Nikon CoolPix S80 digital camera (Nikon).

Antibody mediated inhibition of biofilm formation. Overnight cultures of each strain of *S. aureus* were diluted to a final OD_{600nm} of 0.015 and inoculated in TSBG or TSBG + 12.5 mM EGTA and treated with varying concentrations of either α-ClfB antibody or rabbit pre-immune sera (0.5 – 16.0 μg/mL) or in the absence of any added antibody and added to wells of a 96-well polystyrene plate (Cellstar, Greiner Bio-one, Monroe, NC). The plate was incubated at 4 °C for 2 h to promote antibody binding and thereafter moved to 37 °C for 18 h without shaking. Non-adherent cells were removed by washing with water and adherent bacteria were stained with safranin. Biofilms were imaged using a flatbed HP color LaserJet 2820 scanner (Hewlett-Packard Company) and quantified by dissolving the stain with 200 μL 33% (v/v) acetic acid and determining the OD_{415nm} using a 96-well plate spectrophotometer (BioTek 800 Plate reader, Winooski, VT). The percent inhibition of biofilm formation was determined using the formula: $[1-(Biofilm_{415nm\ (with\ antiserum)} / Biofilm_{415nm\ (without\ antiserum)}] \times 100$ (Hussain, Herrmann et al., 1997). Growth of the bacterial culture was also assessed to ensure high concentrations of the antibody did not interfere with growth. Therefore, specific wells targeted for growth assessment were resuspended by pipetting and read at OD_{595nm}.

Sequence analysis of clfB promoter and aur from 10833 and Newman. Genomic DNA

was isolated from 10833 and Newman using the DNeasy tissue kit (Qiagen). The *clfB* gene and 0.4 kb 5'-proximal to the gene were amplified using primers *clfB*PROFWD and *clfB*PROREV and were sequenced (Eurofins MWG Operon, Huntsville, AL).

The *aur* gene was PCR amplified using a pair of external primers (*aur*-Seq-ExtFwd and *aur*-Seq-ExtRev) and a pair of internal primers (*aur*-Seq-IntFwd and *aur*-Seq-IntRev). The external primers covered 0.35 kb 5'-proximal and 0.15 kb 3' of the gene while the internal primers were generated to provide a 0.095 kb overlap during the amplification. The PCR amplicons were sequenced at the VCU Nucleic Acids Core Facility under the supervision of Dr. Gregory Buck. The sequence analysis and alignment was performed using Serial Cloner 2.1 and sequence translation was done using the StartORF 1.0.

All PCR reaction mixtures contained 2.5 mM dNTPs, 5X Phire Buffer, 1000 pM forward and reverse primers, F-120S Phire Hot start polymerase (Finnzymes, Espoo, Finland), nuclease free water, and gDNA. Conditions for the all the PCR reactions were as follow, one initial cycle at 94 °C for 2 min, followed by 35 cycles of- 94 °C for 30 s to denature, 54 °C for 30 s to hybridize, and 72 °C for 30 s for extension. One final extension at 72 °C for 5 min after the final cycle and thereafter reactions were cooled to 4 °C before being sent off for sequencing.

Poly-N-acetyl glucosamine (PNAG) blot. Overnight cultures of wildtype SA113, MN8 and their isogenic *ica* operon deletion mutants were grown in TSBG. Strain 10833 was grown overnight in either TSBG or media with 12.5 mM EGTA. Cultures were

centrifuged to pellets and were standardized before being resuspended in 0.5 M EDTA (Surface-associated fraction). The supernatants from the pellets (Secreted fraction) and the Surface-associated fractions were boiled for 5 min then centrifuged to pellet cell debris. The supernatant was transferred to a new tube, 20 µL of proteinase K was added to each sample and was heated at 65 ºC for 1 h. Samples were boiled for 5 minutes to inactive the proteinase K. Samples were briefly sonicated for 3-5 seconds and dilutions made in 1X PBS. A piece of nitrocellulose blotting membrane (BioTrace NT, Pall Corporation, Ann Arbor, MI) was cut to fit inside the blot apparatus (7.5 cm wide by 11.5 cm long). The cover paper was removed from the nitrocellulose and the membrane was soaked in 50 mL of 5% methanol for 1-2 minutes. The nitrocellulose membrane was rinsed in 50 mL of 1X PBS; the nitrocellulose membrane was placed within the blotting apparatus and care was used to ensure that the instrument gasket had produced a tight seal. 200 µL of stock or diluted PNAG samples from each fraction were loaded into individual wells of the blotting apparatus and the machine was allowed to run for 20 minutes or until all the sample was soaked up by the membrane. The membrane was removed carefully and allowed to dry overnight between pieces of kimwipe.

The dried nitrocellulose membrane was soaked in 25 mL of 5% bovine serum albumin (BSA) solution, diluted in 1X PBS, for 1 h at room temperature on a rocker. The membrane was probed with 15 mL of primary antibody solution containing 3 µL of goat α-PNAG antibody (1:5000 dilution), 1 ml 5% BSA solution, and 14 mL of 1X PBS for 3 h at room temperature on a rocker. The membrane was washed 3X with 15 mL of 1X PBS for 7 min each. After the wash steps, the membrane was probed with l5 mL of

secondary antibody solution containing 1.5 µL of rabbit α-goat-HRP labeled antibody (1:10,000 dilution) and 15 mL of 1X PBS for 1 h at room temperature on a rocker. The membrane was again washed 3X with 15 ml of 1X PBS for 7 min each. The blot was incubated in ECL™ Plus chemiluminescence developing reagent (GE Healthcare, Piscataway, NJ). The blot was then wrapped in saran wrap, exposed to X-ray film, and developed in an X-O-Mat.

Western blot analysis. *S. aureus* strains 10833, Newman, USA 300 JE2, and their isogenic chromosomal or tansposon insertion mutants were grown stationarily for 18 h in tubes containing TSBG or added 12.5 mM EGTA. The bacteria were collected by centrifugation of the media at 4000 rpm at 4 ºC for 15 min. Protein amount was standardized by pellet weight after centrifugation to collect the bacteria. Total bacterial surface protein was extracted using 0.5 µg/mL of lysostaphin treatment, diluted in 1X TE buffer and incubation at 37 ºC for 45 min. The tubes were briefly sonicated for 3-5 s and centrifuged at 14,000 rpm at 4 ºC for 10 min. The supernatants were collected and equal volumes of supernatants were added to 4X NuPAGE LDS loading buffer to achieve a 1X concentration. The samples were boiled for 10 min prior to gel loading. Protein samples were loaded onto a NuPAGE pre-cast Novex Bis-Tris 4-12% gradient gel (Invitrogen, Carlsbad, CA). Gels were run in 1X MES SDS running buffer (Invitrogen) for 1.75 h at 200 V.

Following electrophoresis, the polyacrylamide gel was rinsed in deionized water. A 0.45 µm PVDF membrane (Invitrogen) was wetted in 100% methanol then incubated in 1X

NuPAGE transfer buffer. Sponges were wetted in the same transfer buffer and used to surround the gel and membrane to ensure the system remained saturated with transfer buffer at all times. The transfer was performed at 40 V for 2 h following which the membrane was blocked overnight in 25 mL of 5% skim milk (Difco, Franklin Lakes, NJ) diluted in 1X PBS, at 4 ºC overnight on a rocker. The rocker was removed from 4 ºC cold room and allowed to equilibrate back to room temperature for about 1.5 h before the membrane was incubated for 2 h at room temperature with shaking in a 1:10,000 dilution of α-ClfB antibody (4.0 µg/mL) in 1X PBS, 0.15% skim milk and 0.05% Tween-20 (Fisher Scientific, Pittsburgh, PA). The membrane was washed 4 times for 10 minutes each in 1X PBS-t (supplemented 0.05% Tween-20) followed by a 1 h incubation in a 1:15,000 dilution of goat α-rabbit-HRP conjugate secondary antibody (Invitrogen) in 1X PBS-t. The membrane was washed four times for 10 min each in 1X PBS-t. The blot was incubated in ECL™ Plus chemiluminescence developing reagent (GE Healthcare, Piscataway, NJ). The blot was then wrapped in saran wrap, exposed to X-ray film, and developed in an X-O-Mat.

CHAPTER 3

A low molecular weight component of serum inhibits biofilm formation in *Staphylococcus aureus*

Introduction

Staphylococcus aureus is one of the leading causes of nosocomial infections. It is an opportunistic pathogen that can infect a variety of different human tissues leading to various clinical manifestations ranging in severity from minor purulent infections like folliculitis to more severe, potentially fatal, conditions like endocarditis and osteomyelitis (Lowy, 1998). *S. aureus* has a large arsenal of virulence factors that allow the bacteria to evade the host immune system, adhere to host tissue, and acquire nutrients from the host (Foster, 2005). In addition, *S. aureus* has the capacity to form biofilms, now recognized as an important virulence factor particularly in the pathogenesis of medical device-related infections such as intravenous catheter infections.

A biofilm is a community of microorganisms that adheres to a surface and is encased within an extracellular polymeric matrix. The staphylococci account for more than half of prosthetic device-related infections and biofilm formation is a key component to the pathogenesis of such infections (Zimmerli and Ochsner, 2003). The two major steps associated with this complex developmental process is adherence of the bacteria to a surface followed by cell-to-cell adhesion (aggregation). In *S. aureus*, adherence is

mediated by surface-associated adhesins including, but not limited to, the *S. aureus* surface protein G (SasG) (Kuroda, Ito et al., 2007) and fibronectin-binding proteins (FnbA and FnbB) (O'Neill, Pozzi et al., 2008) while aggregation is brought about, under certain conditions, by the synthesis of β-1,6 poly-*N*-acetylglucosamine (PNAG) also referred to as polysaccharide intercellular adhesion (PIA). First identified in *S. epidermidis* and now known to be functionally conserved within *S. aureus*, the polysaccharide is synthesized by proteins encoded by the *icaADBC* operon (Heilmann, Schweitzer et al., 1996; Cramton, Gerke et al., 1999).

Transcription of *icaADBC* and subsequent polysaccharide production and biofilm formation, is negatively regulated by the product of the *icaR* gene, transcribed divergently from *icaADBC* operon. Conversely, biofilm formation is positively regulated by the global transcriptional regulator SarA and the carbon catabolite protein CcpA (Conlon, Humphreys et al., 2002; Jefferson, Pier et al., 2004; Seidl, Müller et al., 2009; Seidl, Goerke et al., 2008; Valle, Toledo-Arana et al., 2003; Beenken, Blevins et al., 2003). The alternative sigma factor (σ^B) is also a positive regulator of biofilm formation but it is not clear how it exerts this function (Rachid, Ohlsen et al., 2000). Together these intra- and intercellular components, and others, are coordinated to orchestrate the process of biofilm formation. In addition to these cellular components, extracellular factors play a key role as well, as they cue the bacterial cell to conditions under which the biofilm mode of growth is preferable. External cues that inform bacteria that they are *in vivo* and affect expression biofilm-related genes are poorly understood. Because *S. aureus* can form biofilms on intravenous catheters and other inserted medical devices that come into

contact with blood, we hypothesized that serum would be an external cue to trigger biofilm formation. However, we found the opposite to be true; serum inhibited the biofilm phenotype under certain conditions, and this inhibition occurred at least in part, at the level of gene expression.

Results

Effect of serum on biofilm formation

Biofilm formation plays a key role in the pathogenesis of medical device-related infections and in the case of intravenous catheter-related infections and other device-related infections, *S. aureus* is exposed to blood. Furthermore, pre-coating polystyrene with serum is frequently used as a method to augment biofilm formation (Speziale, Pietrocola et al., 2009). We therefore hypothesized that biofilm formation would be induced in serum. To test this hypothesis, the strong biofilm producing type strain SA113 (**Figure 4A**), and clinical isolate Newman (**Figure 4B**), a strong-coagulase-producing isolate from a case of osteomyelitis (Duthie and Lorenz, 1952; Baba, Bae et al., 2008) were grown in TSBG alone or media supplemented with human serum, fetal bovine serum or PBS. Both strains were observed to exhibit similar biofilm trends. While TSBG and TSBG containing 10% or 50% PBS fostered the elaboration of a thick biofilm, in either strain, addition of 10% or 50% human serum (HS), surprisingly, inhibited biofilm formation. This inhibitory effect was not limited to human serum as both 10% and 50% fetal bovine serum (FBS) revealed a similar ablated phenotype (**Figure 4A, B** red bars). In order to confirm inhibition of biofilm formation was not due to growth inhibition, we

measured growth under the same conditions. The optical densities of the cultures after 18 h revealed that the presence of any concentration of human or fetal bovine serum supported planktonic growth (**Figure 4A, B** blue bars).

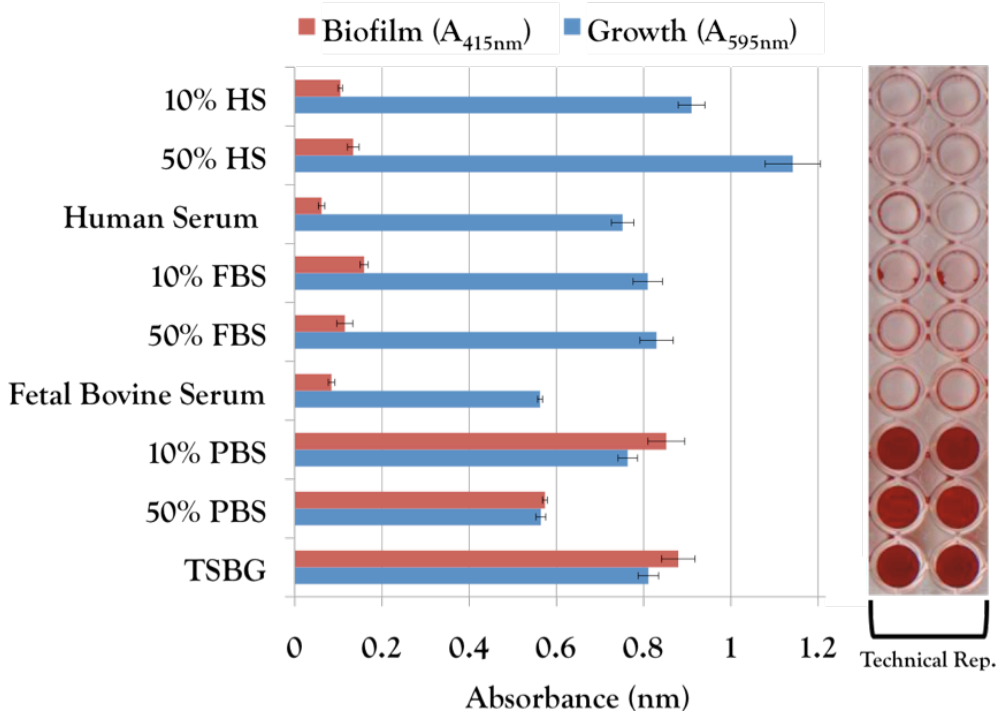
Characterization of the inhibitory component/s

To obtain a crude estimation of the molecular weight of the component(s) responsible for this novel inhibitory activity of serum observed in **Figures 4A** and **4B**, components with approximate molecular weights >3000 Da was removed using a centrifugal concentrator. Large serum proteins such as lactoferrin, apo-transferrin, and albumin have been shown previously to inhibit bacterial and *S. aureus* aggregation and biofilm formation (Ardehali, Shi et al., 2002; Ardehali, Shi et al., 2003; Singh, Parsek et al., 2002; Hammond, Dertien et al., 2008), but a role for a small molecular weight component/s has never been established. Therefore, the low (<3000 Da) molecular weight fraction (LMWF) of fetal bovine (**Figure 5**) and human serum (**Figure 6**) were assessed for inhibition of biofilm formation within strains SA113 and Newman.

Both whole FBS and human serum, retained the ability to effectively inhibit biofilm formation compared to control TSBG alone or with supplemented PBS in either strain tested (**Figure 5, 6**). Surprisingly, the LMWF of either human or fetal bovine serum continued to inhibit biofilm formation similar to results observed with whole FBS or human serum. To further investigate the nature of the small molecular weight component in the LMWF, we heated the <3000 MW fraction to 100 °C for 5 min (HI LMWF). Heating did not inactivate the inhibitory activity. To eliminate the possibility that a heat

Figure 4. Serum inhibits *S. aureus* biofilm formation. Overnight cultures of SA113 (**A**) and Newman (**B**) grown in LB were diluted 1:200 and inoculated in: TSBG, TSBG + 50% or 10% PBS, Human Serum (HS), TSBG + 50 or 10% HS, Fetal Bovine Serum (FBS), TSBG + 50% or 10% FBS. Biofilms were allowed to form in 96-well polystyrene plates at 37 °C for 18 h. Non-adherent cells were removed by washing with 1X PBS and the adherent bacteria were dried and stained with safranin. Scanned representative images of the bacteria that formed biofilms are shown on the right and quantitative representation of growth versus biofilm formation under the respective conditions is shown on the left. Growth (blue bars) was determined by resuspending the bacteria and measuring $A_{595\ nm}$. Biofilm formation (red bars) was assessed by solubilizing the safranin in 33% (v/v) acetic acid and measuring at $A_{415\ nm}$. Each condition was tested 5 times within each experiment and experiments were performed a minimum of three times on separate days with similar results and shown here is a representative image.

A.



B.

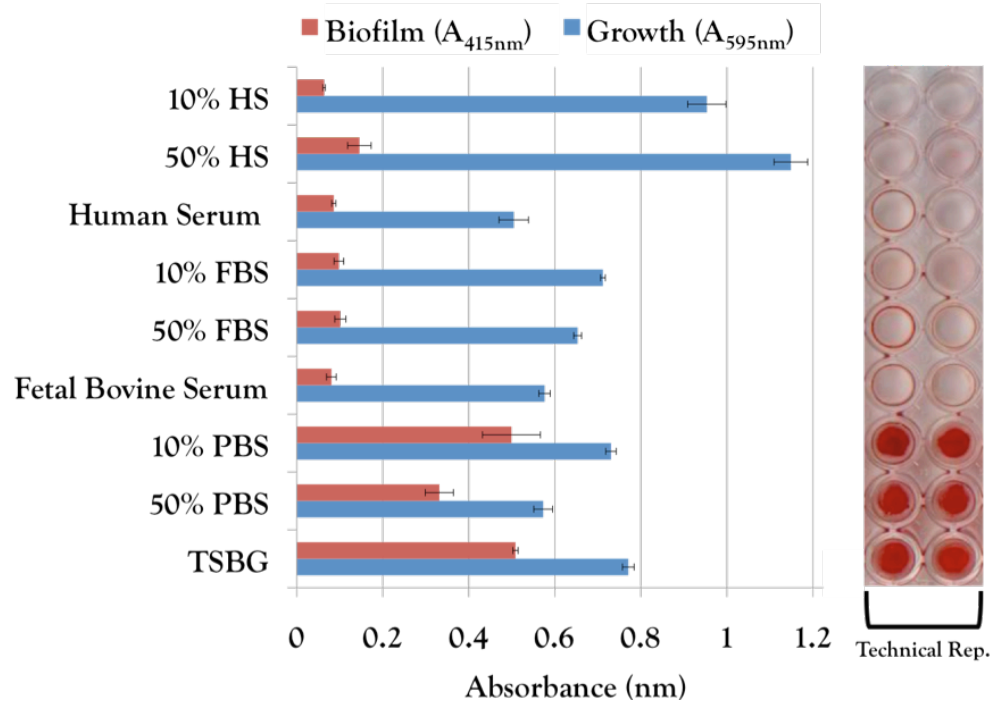
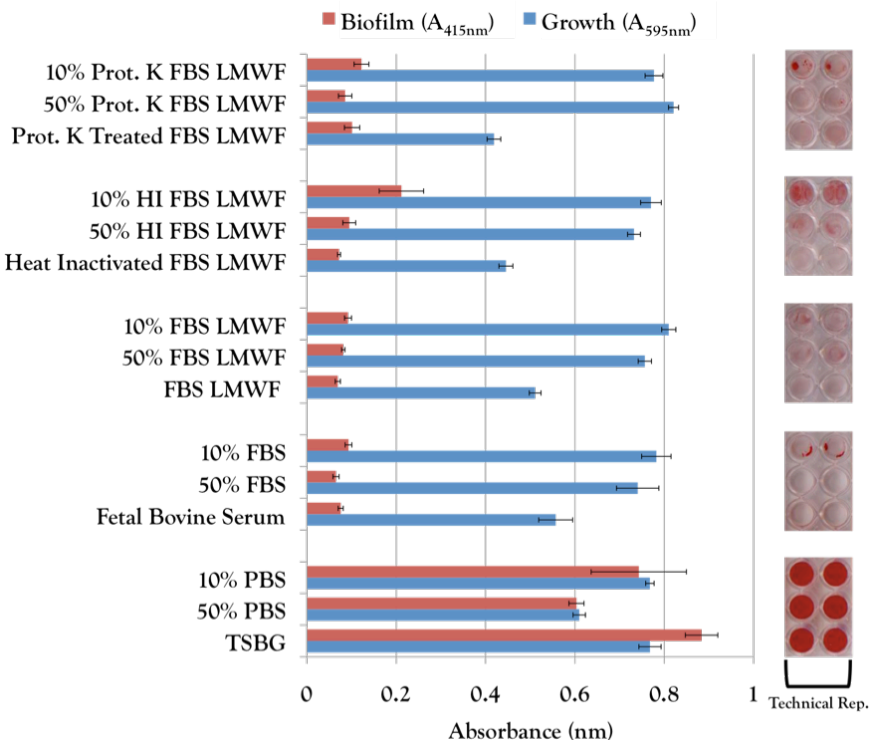


Figure 5. The low molecular weight fraction (LMWF) of fetal bovine serum inhibits biofilm formation. Strains SA113 (**A**) and Newman (**B**) were grown for biofilms in 96-well plates in TSBG, TSBG + 50% or 10% 1X PBS, Fetal Bovine Serum (FBS), TSBG + 50% or 10% FBS, <3,000 Da component of fetal bovine serum (FBS LMWF), TSBG + 50% or 10% FBS LMWF, Heat Inactivated (HI) FBS LWMF, TSBG + 50% or 10% HI FBS LMWF, and Proteinase K (Prot. K) treated FBS LMWF, TSBG + 50% or 10% Prot. K-treated FBS LMWF. >3,000 Da fraction of serum was removed by filtration. Biofilms were imaged and thereafter read at $A_{415\ nm}$ (red bars) to determine biofilm formation under the respective conditions. Growth was determined within the individual conditions prior to biofilm washing and staining and measured at $A_{595\ nm}$ (blue bars). Results represent the data from 3 independent experiments.

A.



B.

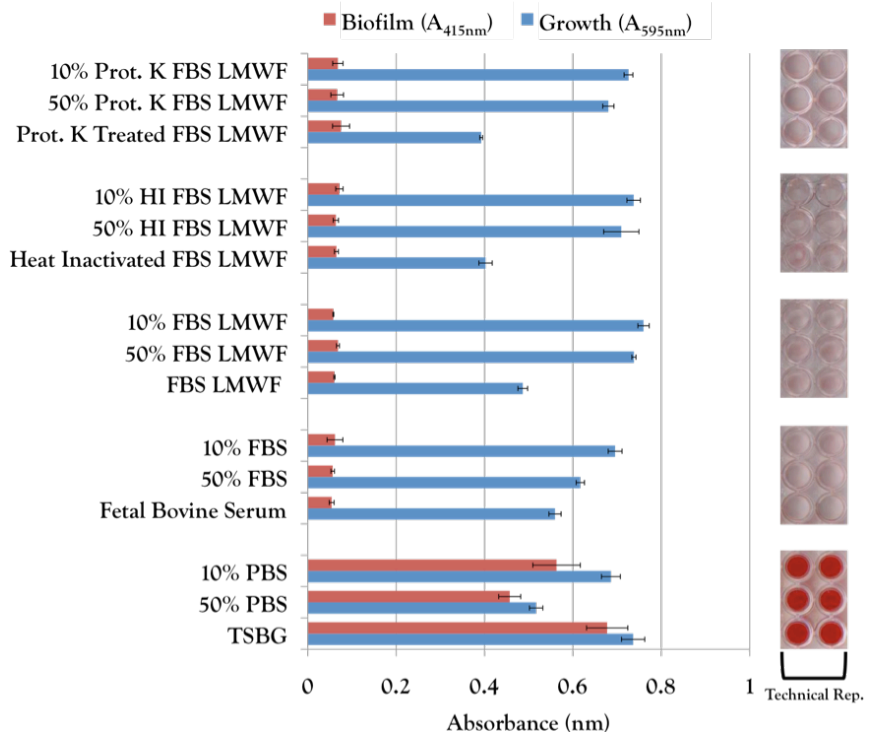
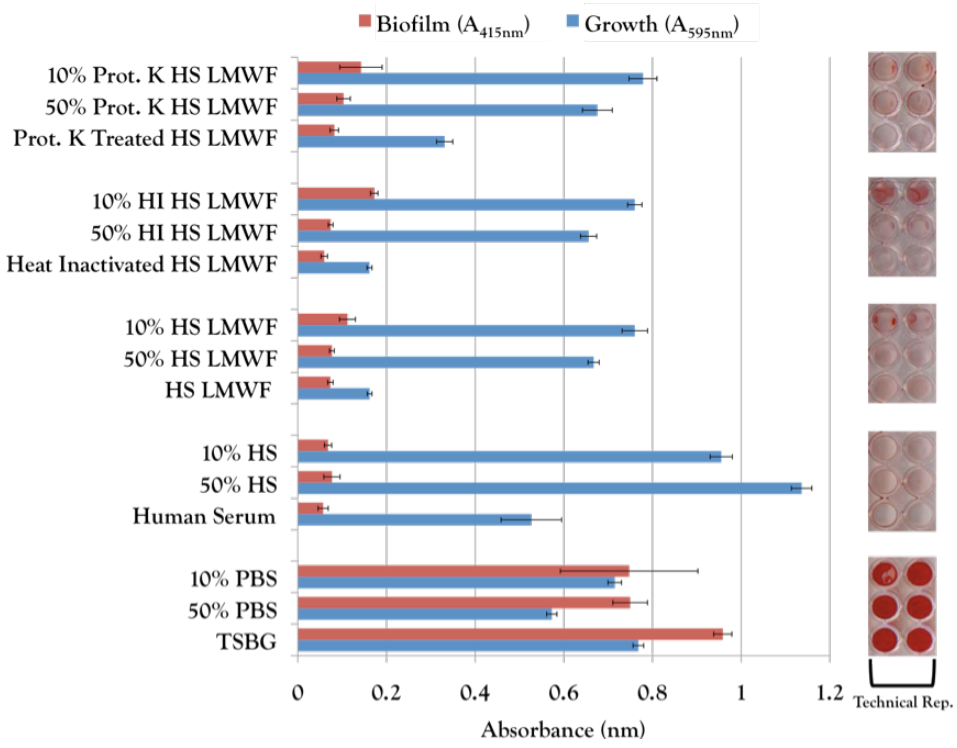
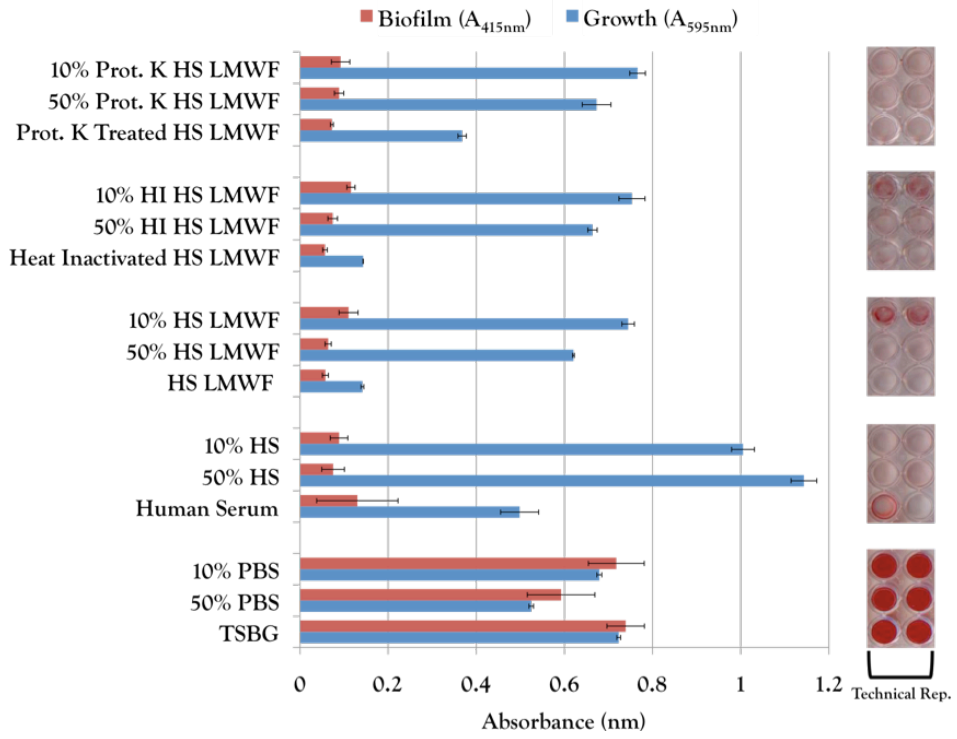


Figure 6. The low molecular weight fraction (LMWF) of human serum inhibits biofilm formation. Strains SA113 (**A**) and Newman (**B**) were grown for biofilms in 96-well plates in TSBG, TSBG + 50% or 10% 1X PBS, Human Serum (HS), TSBG + 50% or 10% HS, <3,000 Da component of human serum (HS LMWF), TSBG + 50% or 10% HS LMWF, Heat Inactivated (HI) HS LWMF, TSBG + 50% or 10% HI HS LMWF, and Proteinase K (Prot. K) treated HS LMWF, TSBG + 50% or 10% Prot. K-treated HS LMWF. >3,000 Da fraction of serum was removed by filtration. Biofilms were imaged and thereafter read at $A_{415\ nm}$ (red bars) to determine biofilm formation under the respective conditions. Growth was determined within the individual conditions prior to biofilm washing and staining and measured at $A_{595\ nm}$ (blue bars). Results represent the data from 3 independent experiments.

A.



B.

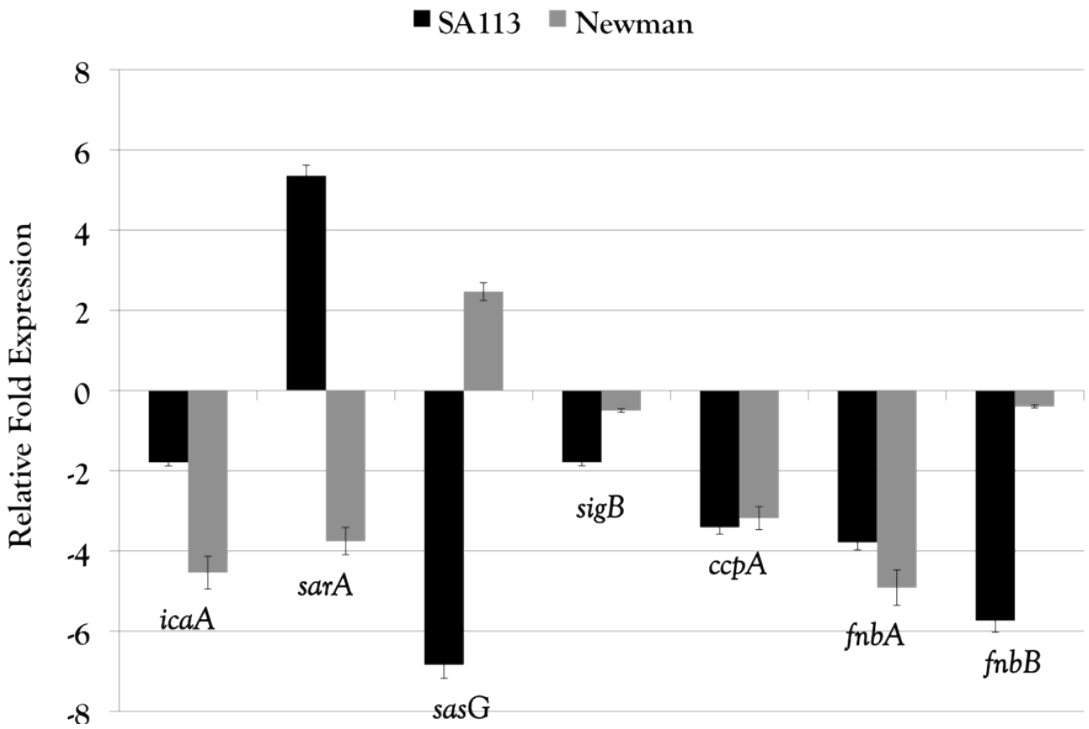


stable protein was responsible for biofilm inhibition, Proteinase K was used to degrade proteins in the FBS flow-through (Prot. K treated LMWF) but this did not affect the ability of this low molecular weight fraction to inhibit biofilm formation. The results were similar for FBS or human serum in both strains- SA113 and Newman. Together these results suggest that the compound in serum that inhibits *S. aureus* biofilm formation is not proteinaceous. We found in a previous study that basic conditions can inhibit biofilm formation. Because the pH of serum is slightly basic, we buffered the <3000 Da fraction to pH 7.0 to determine whether the effect was due to high pH; however, this did not affect the biofilm-inhibiting activity (Thompson, Abraham et al., 2010).

Serum components affect expression of biofilm-related genes

To determine whether inhibition of biofilm formation occurred at the level of gene expression, total RNA was isolated from cells grown in TSBG or the LMWF of human serum and expression levels of specific genes that have been previously implicated in biofilm formation or regulation were determined by quantitative realtime RT-PCR. The individual genes were normalized to the 16S rRNA and the relative fold expression of each gene is shown with respect to the TSBG control (**Figure 7**). In comparison to TSBG, expression of the *icaA*, *ccpA*, and *fnbA* and *fnbB* genes was significantly lower (by Student's *t*-test $P<0.002$) within the serum fraction. IcaA (Intercellular Adhesin protein A) is a glucosyltransferase, necessary for the synthesis of PNAG/PIA, the primary component of *S. aureus* biofilms. Interestingly, there were strain-variable differences in the expression of other biofilm-related genes. The serum fraction led to a drastic decrease

Figure 7. Transcript levels of biofilm associated genes under various media conditions. Total RNA was isolated from strains SA113 (black bars) and Newman (grey bars) grown in TSBG or LMWF of human serum during mid-log phase of growth. The transcript levels of specific genes that have been previously implicated in biofilm formation were analyzed by realtime RT-PCR. Individual genes were normalized to 16S rRNA and relative fold expression is shown with respect to TSBG. Each gene was assessed in triplicate and the experiment itself was performed in biologic duplicate.



in *sasG* transcript levels ($P<0.0005$) in strain SA113 but actually lead to a slight increase in Newman. SasG is a cell walled anchored protein that functions as an adhesin, facilitating the initial steps associated with intercellular bacterial binding to the substrate during biofilm formation (Kuroda, Ito et al., 2008; Corrigan, Rigby et al., 2007). Furthermore, the level of *sarA* transcript increased approximately 5-fold in SA113 ($P<0.001$) but was repressed in strain Newman and levels of the *sigB* and *fnbB* transcripts were inhibited by the serum fraction in SA113 but remained unchanged in Newman ($P>0.05$).

Discussion

Tenacious biofilms of *S. aureus* play an important role in various host infections including medical device related infections. Additionally, biofilms formed by this bacterium plays a key role in the pathogenesis of catheter-related infections. Biofilm development within *S. aureus*, is facilitated through the coordinated efforts of a variety of genes that can directly or indirectly influence the process of biofilm formation. The ability to establish a biofilm is an important virulence factor for this pathogen, and yet, the regulation of this process *in vivo* is not well understood. Stressors present in the environment of a bacterial cell strongly influence its homeostatic and adaptive physiology. Stress responses are often facilitated by global changes in transcriptional or post-translational gene expression. Certain stressors including increased temperature, changes in the availability of glucose, exposure to toxic chemicals such as ethanol and

antibiotics can induce global changes in gene expression that initiate the switch to a sessile, biofilm mode of growth.

In order to investigate whether or not serum is conducive to the process of biofilm formation, we grew two different *S. aureus* strains, SA113 and Newman, in serum and analyzed biofilm thickness and expression of biofilm-related genes. Whereas serum supported planktonic bacterial growth, it was a potent inhibitor of biofilm formation in either strain tested (**Figure 4**). These findings were not completely surprisingly since previous studies have directly implicated large molecular weight components like lactoferrin, apo-transferrin, and albumin to inhibit aggregation and biofilm formation (Ardehali, Shi et al., 2002; Ardehali, Shi et al., 2003; Singh, Parsek et al., 2002; Hammond, Dertien et al., 2008). However, a role for a small molecular weight component/s within serum has never been established. We found by filtration-mediated size separation a low molecular weight component of serum (<30000 Da) that prevented biofilm establishment (**Figure 5, 6**). Interestingly, serial dilutions of this fraction up to 10% continued to inhibit formation of any detectable biofilm. This suggested that the inhibitory factor is quite effective at inhibiting biofilm formation.

To further tease apart the nature of this unique component/s we heat inactivated (HI) this fraction by boiling the LMWF serum to 100 °C for 5 min. 56 °C is typically used to inactivate the components of the innate immune system including complement. However, heating this fraction at higher temperatures we performed dual service of inactivating the smaller components of the innate immune system but additionally, inactivating other small heat-stable components that may occur within this fraction. Heat

inactivation of human or fetal bovine serum continued to inhibit biofilms from forming in either strain. Thus we deduced the component to be heat stable.

Antimicrobial peptides (AMPs) are an important arm of the innate immune system that target and kill *S. aureus*. These small cationic peptides, which are found in virtually all higher organisms, have recently become attractive drug candidates, due to their natural production in and protection of many host tissues and cell types (Li, Cha et al., 2007; Zasloff, 2002; Hancock and Diamond, 2000). AMPs are highly effective against most bacteria, regardless of Gram type, because the external charge on the bacterial envelope is negative, which differentiates them from eukaryotic cells, which are typically "seen" as neutral. AMPs act by binding to the bacterial membrane via charge interactions and form pores within the bacterial membrane (Herbert, Bera et al., 2007; Kraus and Peschel, 2008). Therefore, to rid the serum components of such AMPs, the serum LMWFs were treated with proteinase K and thereafter additionally heated to 100 °C to inactivate the proteinase. Biofilm formation within proteinase K treated fractions was also inhibited revealing the component to be protease-resistant or non-proteinaceous.

Identifying the serum component to be heat-stable and protease-resistant we explored the transcriptional expression levels of various genes that have been implicated in biofilm formation or its regulation, when strains were grown within the LMWF of serum (**Figure 7**). The serum component resulted in a significant decrease in the transcription of the intercellular adhesin gene *icaA* and the fibronectin binding protein gene *fnbA*. Transcription of other biofilm-related genes were affected in a strain-

dependent manner. These results reveal that serum inhibits biofilm formation despite the fact that biofilms form on intravenous catheters. This may suggest that *in vivo*, biofilm formation is "selected for" by the force of blood flow and/or immune pressure rather than "induced" by serum.

These findings indicate that the response of different strains to serum varies and it is possible that other strains that we did not test, could establish biofilms in the presence of serum. This is not surprising, as biofilm formation in different strains depends upon different proteins and polysaccharides, and can even depend on extracellular DNA (Mack, Fischer et al., 1996; Maira-Litràn, Kropec et al.,2002; Corrigan, Rigby et al., 2007; Arciola, Campoccia et al. 2005; O'Neill, Humphreys et al. 2009; Rice, Mann et al., 2007). These findings reveal- inhibition of biofilm formation by the LMWF of FBS or human serum, at least in part, is due to inhibition of expression of biofilm-related genes. Therefore, biofilm formation *in vivo* may be "selected for" (possibly by immune pressure and sheer forces) rather than "induced" at the level of transcription. It is possible that *S. aureus* exhibits the planktonic mode of growth *in vivo* to maximize its growth potential in a nutrient-rich environment. The few bacteria that happen to adhere to the device would have a selective advantage over the planktonic population, which would be killed by immune defenses. Serum-mediated inhibition of biofilm formation may represent a previously uncharacterized aspect of host innate immunity that targets the expression of a key bacterial virulence factor: the ability to establish a resistant biofilm.

CHAPTER 4

The effect of Catheter Lock Solutions on *Staphylococcus aureus* biofilm formation; A strain dependent phenomenon

Introduction

Staphylococcus aureus is a leading cause of nosocomial infections and of bloodstream infections (BSIs) associated with contaminated intravenous catheters. Despite recent reductions in rates of central-line associated BSIs, catheter-associated infections remain a leading cause of healthcare-associated infection (CDC, 2011). Catheter-associated BSIs increase mortality rates, prolong patient stay, and increase healthcare costs (Hugonnet, Sax et al., 2004). An important virulence factor in such infections, especially *S. aureus*, is the bacterium's ability to adhere to and form a biofilm on the surface of the catheter.

Bacterial biofilms are communities in which intercellular bonds enforced by polymeric compounds, and intercellular communication mediated by signaling molecules, result in a bastion that is sheltered from sheer forces, antimicrobial agents, and host immune components. Consequently, biofilm-related infections are very difficult to treat therapeutically, and while antibiotics may eradicate the BSIs and temporarily relieve the clinical signs of the infection, the biofilms often persist and act as a nidus for relapsing infection (Hall-Stoodley, Costerton et al., 2004). Recent guidelines for

prevention and treatment of catheter-related infections propose the use of intraluminal antimicrobial lock solutions or catheter lock solutions (CLS) (Mermel, Allon et al., 2009; O'Grady, Alexander et al., 2002; Raad and Hanna, 2002).

Heparin has traditionally been used to lock catheter lumens during interdialytic periods. The use of heparin in lock solutions is safe and well tolerated, though it does not help to prevent or treat central venous catheter (CVC)-related infections (Bestul and Vandenbussche, 2005). This finding might be related in part to the fact that heparin alone does not have any innate antimicrobial activity (Capdevila, Gavaldà et al., 2001). However, recently, the use of heparin in lock solutions has been associated with some risk factors including heparin-induced thrombocytopenia and thrombosis (Arepally and Cines, 1998). Moreover, several investigators recently reported that heparin augments *S. aureus* biofilm formation on catheter surfaces (Shanks, Sargent et al., 2006; Shanks, Donegan et al., 2005) possibly mediated by the production of heparin-binding proteins by the bacteria (Fallgren, Utt et al., 2001; Liang, Ascencio et al., 1992). Thus, heparin may actually contribute to the incidence of catheter-related infections.

Therefore, as heparin alternatives, chelators such as trisodium citrate (NaCi), Ethylenediaminetetraacetic acid (EDTA), and Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) have come into use as critical components of CLS. These compounds have significant advantages providing three necessary and important functions: an anticoagulant effect, an antibiofilm effect, and a synergistic antimicrobial effect (Banin, Brady et al., 2006; Percival, Kite et al., 2005; Shanks, Sargent et al., 2006). Metallic cations such as calcium (Ca^{2+}), and magnesium (Mg^{2+}) play an important role in

microbial adherence, biofilm formation and bacterial growth. These divalent cations can stimulate cell-cell adhesion and aggregation through their interactions with cell wall teichoic acids (Dunne and Burd, 1992; Sarkisova, Patrauchan et al., 2005). Therefore removal of free cations from the milieu, via these chemical chelators, reduces intercellular adhesion and biofilm formation. Furthermore, chelating agents can reduce biofilm formation by inhibiting the production of the staphylococcal polysaccharide intercellular adhesin or poly-N-acetylglucosamine (PIA or PNAG) encoded by the *ica* operon (Juda, Paprota et al., 2008; Ozerdem Akpolat, Elci et al., 2003). However, *S. aureus* has many components, in addition to PNAG, that can contribute to adhesion and biofilm formation.

The surface of *S. aureus* is coated with a variety of adhesins that are capable of binding different host proteins present in plasma and/or the extracellular matrix. In some cases these adhesins can also mediate bonds between bacterial cells (Corrigan, Rigby et al., 2007; Cucarella, Tormo et al., 2002). There are at least 28 different *S. aureus* proteins that promote the binding to 18 different host proteins (Clarke and Foster, 2006). Adhesins that mediate such binding to host extracellular matrix proteins are termed MSCRAMMs. *S. aureus* can express 4 distinct fibrinogen-binding MSCRAMMs: clumping factors A and B (ClfA and ClfB) (Ni Eidhin, Perkins et al., 1998) and the fibronectin binding proteins FnbA and FnbB, which bind to fibronectin and fibrinogen (Wann, Gurusiddappa et al., 2000). Insertion of indwelling devices including artificial heart valves, pacemakers, and catheters causes them to be immediately coated with blood components including

fibrinogen and fibronectin and subsequently serve as efficient substrates for *S. aureus* binding.

The effects of NaCi and other chelators on biofilm formation have not been fully defined and we therefore designed this study to examine their effects. NaCi at low concentrations has been shown to positively affect biofilm formation, not through its chelating activity, but rather by acting as a tricarboxylic acid cycle intermediate (Shanks, Meehl et al., 2008; Shanks, Sargent et al., 2006). Therefore we also investigated the effect of EGTA. This study aimed to highlight the differences that exist between *S. aureus* strains that vary dramatically in their ability to form biofilms in the presence of chelating agents. Of clinical relevance, these results suggest that chelating agents in catheter lock solutions may actually augment, rather than inhibit biofilm formation in certain strains of *S. aureus* and suggest that a method to monitor their response to the solutions may be necessary.

Results

Chelators exert different effects on biofilm formation in different strains

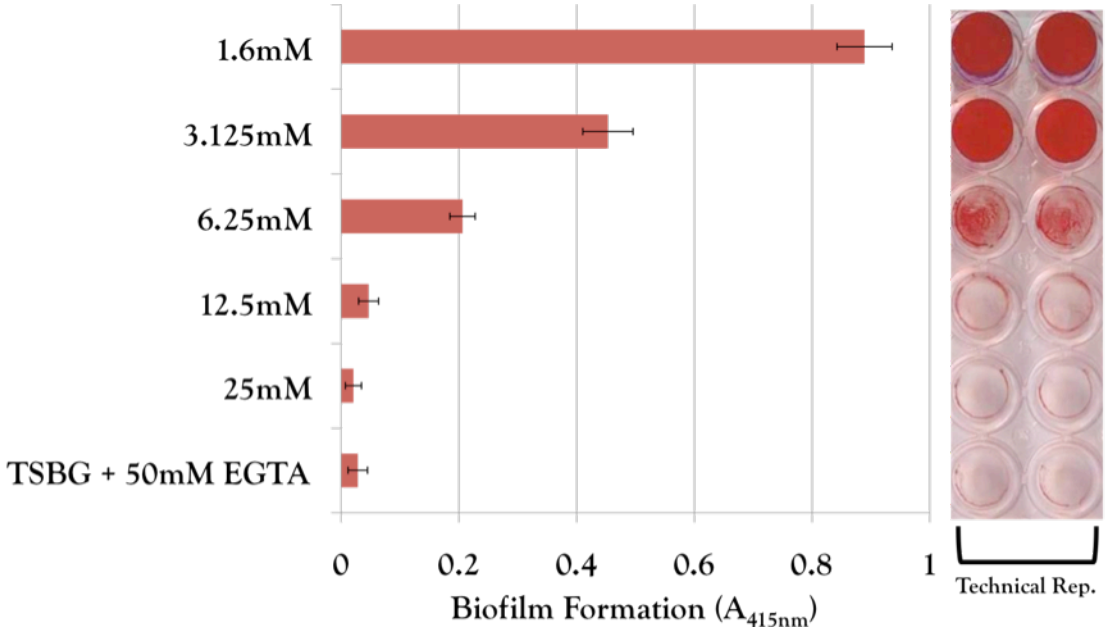
To gain some perspective on the effect of chelators (EGTA and NaCi) on *S. aureus* biofilm formation, assays were performed using various laboratory strains. Strain SA113 (ATCC 35556), derived from laboratory strain NCTC8325, is a proficient biofilm producing type strain. To determine the minimum biofilm inhibition concentration, bacteria were inoculated in TSBG with varying concentrations of EGTA ranging from 50 – 1.6 mM (**Figure 8A**). Biofilm formation was observed to decrease in a concentration

dependent manner with complete ablation of any detectable biofilm by 12.5 mM. To confirm that this chelator concentration was specifically inhibiting biofilm formation and not just preventing growth, we analyzed both planktonic growth and biofilm formation (**Figure 8B**). We found that 12.5 mM EGTA and 12.5 mM NaCi were sub-inhibitory and allowed planktonic growth to occur (**Figure 8B**, blue bars) but completely ablated biofilm formation (**Figure 8B**, red bars $P<0.0001$). Using the QuantiChrom™ Calcium Assay kit we quantified free calcium levels within TSBG and TSBG containing EGTA. The kit contains a phenolsulphonephthalein dye, which when complexed with free Ca^{2+} forms a very stable blue color. The intensity of the color, as measured by a spectrophotometer at $A_{612\ nm}$, is directly proportional to the calcium concentration in the sample. TSBG was observed to contain 4.2 mM Ca^{2+} while free Ca^{2+} was not detectable by the assay with TSBG containing 12.5 mM EGTA. Postulating a role for free Ca^{2+} in supporting biofilm formation within strain SA113, the addition of Ca^{2+} individually or in concert with either EGTA or NaCi restored biofilm formation (**Figure 8B**).

While biofilm assays performed above confirmed the inhibitory effects of EGTA and NaCi on SA113 biofilms we reconfirmed these results using a Live/Dead BacLight™ stain kit (**Figure 9)**. SA113 were grown for biofilms within 6-well plates. Plates were incubated for 18 h at 37 °C and thereafter washed away for non-adherent bacteria and stained with SYTO® 9 and propidium iodide before confocal microscopy was performed. Green-fluorescent SYTO® 9 stain when used alone labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, masking SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact

Figure 8. 12.5 mM EGTA or NaCi effectively inhibits SA113 biofilm formation. (**A**) Strain SA113 was grown in TSBG supplemented with increasing concentrations of EGTA ranging from 50 – 1.6 mM in a 96-well microtiter plates and the plate was incubated for 18 h at 37 °C. The wells were washed for non-adherent bacteria, stained with safranin, imaged and quantified at A_{415nm}. (**B**) Strain SA113 was grown in TSBG alone or supplemented with 12.5 mM $CaCl_2$, 12.5 mM EGTA, 12.5 mM NaCi, 12.5 mM $CaCl_2$ + 12.5 mM EGTA, or 12.5mM $CaCl_2$ + 12.5mM NaCi in 96-well microtiter plates. The wells were either resuspended to determine growth (A_{595}) or washed to remove non-adherent bacteria and stained with safranin to gauge biofilm formation. Plates were scanned using a flatbed scanner (right) and shown here to represent technical duplicates from one representative plate. Safranin stain was dissolved in acetic acid and quantified at A_{415nm}. Quantification of growth at A_{595} (blue bars) versus biofilm formation at A_{415nm} (red bars) under the respective conditions within the respective media condition is shown. $^{**}P<0.0001$ as determined by Student's t-test.

A.



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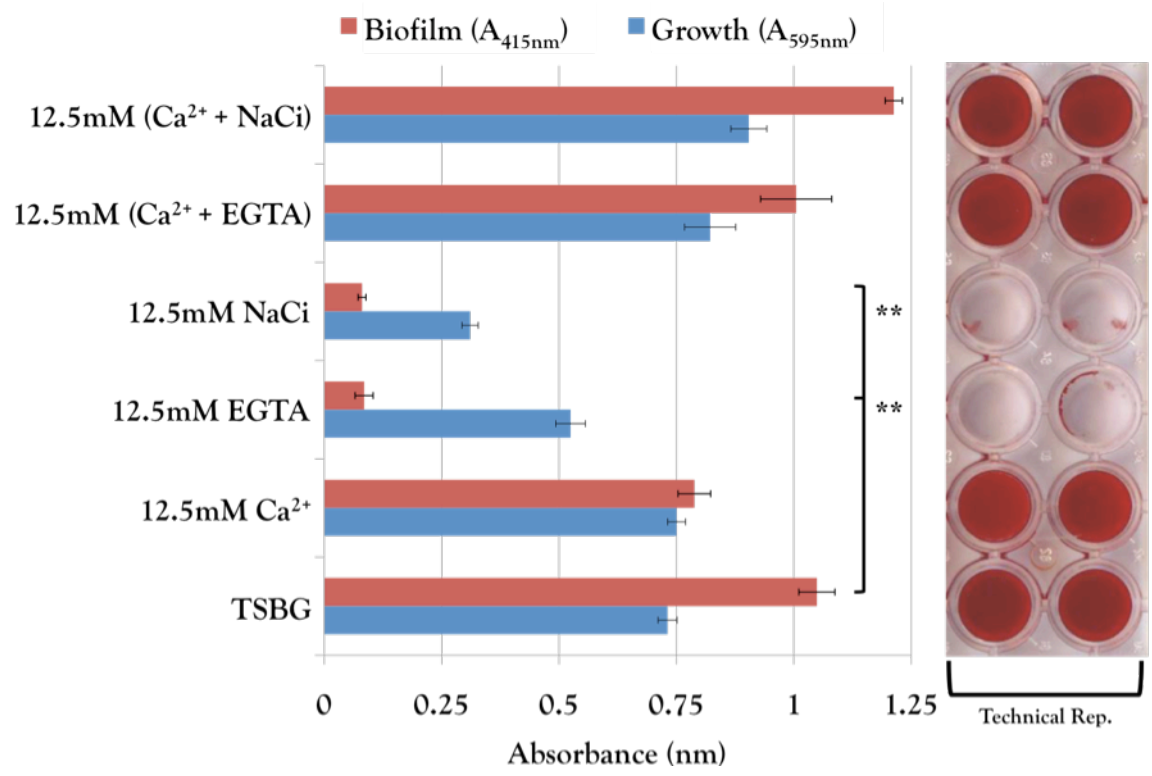
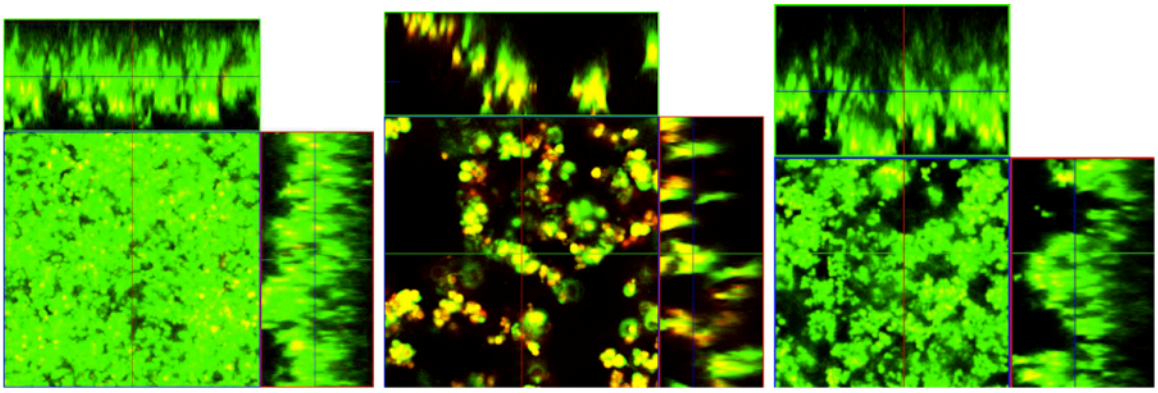


Figure 9. Confocal microscopy confirms an ablated biofilm phenotype within EGTA. SA113 was grown for biofilms within 6 well plates in TSBG + 12.5 mM Ca^{2+} (calcium replete) or 12.5 mM EGTA (calcium deplete) or the two in concert with one another- TSBG + 12.5 mM Ca^{2+} + 12.5 mM EGTA (calcium reconstituted) and stained with Live/Dead BacLight™ stain and analyzed by confocal laser scanning microscopy. The large panels are a top-down view of the biofilm. The right panels are a side view of an x-axis section and the top panels are a side view of the y-axis section. Green color indicates viable bacteria while the red color or yellow (red and green co-localized) indicates dead bacteria.



Ca^{2+} Replete
(+12.5mM Ca^{2+})

Ca^{2+} Deplete
(+12.5mM EGTA)

Ca^{2+} Reconstituted
+12.5mM (Ca^{2+} + EGTA)

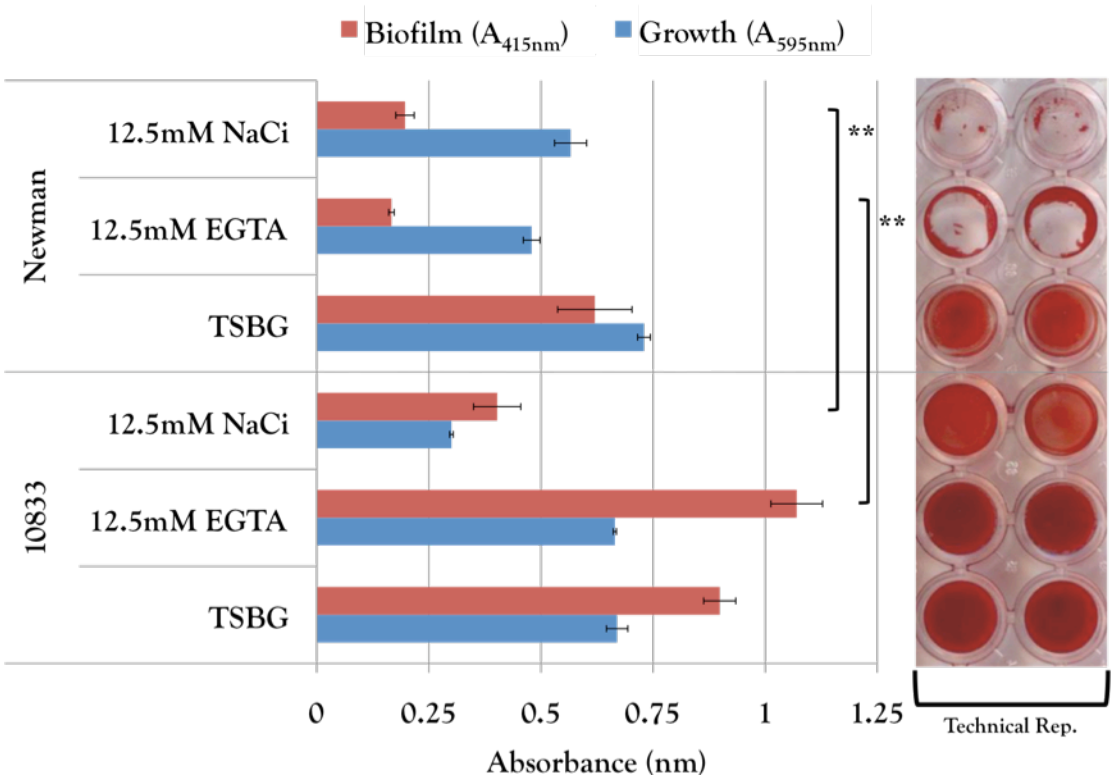
membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. We observed under Ca^{2+} replete or when reconstituted with EGTA, robust biofilms were established with a high density of green fluorescence throughout the architecture. In contrast SA113 under Ca^{2+} deplete conditions were strongly inhibited for biofilms and the few cells that remained attached were non viable.

To extend our findings, we analyzed biofilm formation under similar chelated conditions using Newman, which apart from SA113 was previously observed (Chapter 3) to repress biofilm formation within serum, and compare it to its genetically related variant- strain 10833 (**Figure 10**). The biofilm phenotype was significantly inhibited by EGTA and NaCi in Newman (**Figure 10**), similar to what was observed with strain SA113 (**Figure 8B, 9**). However, in strain 10833, EGTA and NaCi actually increased biofilm formation relative to the diminished or ablated biofilms observed with Newman (**Figure 10**, $P<0.0001$) or SA113 (**Figure 8B, 9**).

Increased clumping within *S. aureus* strain 10833

Strains 10833 (ATCC 25904) and Newman, a throat swab isolate, are two closely related strains, as previously determined by MLST and *spa* typing (Duthie and Lorenz, 1952; Grundmeier, Hussain et al., 2004; Kropec, Maira-Litran et al., 2005; personal communication). We hypothesized that because they are genetically similar, the diametrically opposed phenotypes expressed by these two strains in the presence of chelators could have a relatively straightforward molecular basis. Strong clumping factor activity of 10833 has been previously documented (Duthie and Lorenz, 1952). We

Figure 10. Different strains of *S. aureus* exert diametric effects for biofilm formation within chelating agents. Strains 10833 and Newman were grown for biofilms in TSBG alone or supplemented with 12.5 mM EGTA or 12.5 mM NaCi within 96-well polystyrene microtiter plates. Plates were incubated statically at 37 °C for 18 h. Thereafter, the wells were washed for their media and non-adherent cells, and stained for any detectable biofilms using safranin. Biofilms were imaged and then quantified using 33% acetic acid and measured at A_{415nm} (red bars). Growth, within the individual media conditions, was also assessed by resuspending the cells, prior to washing, and quantified at A_{595nm} (blue bars). Results are and accumulation of three biologic replicates each with five technical replicates. $^{**}P<0.0001$ as determined by Student's t-test.



therefore compared the clumping activity of the two strains in the absence and presence of 12.5 mM EGTA (**Figure 11**). When diluted cultures of bacteria were inoculated into media and incubated overnight, 10833, within the EGTA chelated media aggregated and fell out of solution (clumped). Newman, on the other hand, did not exhibit any significant clumping and continued to remain dispersed throughout the volume of the liquid. Neither strain was observed to show any signs of clumping when cultured with TSBG alone.

To further elucidate the increased clumping effect displayed by 10833 we performed scanning electron microscopy (SEM) on biofilms produced by 10833 in TSBG or added 12.5 mM EGTA (**Figure 12**). Biofilms produced by this strain, in either media condition, were grown on clear glass coverslips, washed to remove non-adherent bacteria and negatively stained with phosphotungstic acid before scanning electron microscopy was performed. Microscopy clearly showed a disparity between the biofilms produced by this strain, with a significant amount of an extracellular surface-associated material when this strain is grown in EGTA (Red arrows) versus that in TSBG alone.

10833 displays increased adherence to catheters in the presence of EGTA

To test the hypothesis that chelators like EGTA may not effectively inhibit biofilm formation on catheters by all *S. aureus* strains, we quantified adherence to catheter tubing. For this purpose, bacteria (10833 or Newman) were diluted in test tubes with either TSBG or media supplemented with 12.5 mM EGTA and a small piece of catheter tubing was added to each tube and incubated for 18 h at 37 °C without shaking. The catheters were sterilely removed, washed for non-adherent bacteria, sonicated and

Figure 11. 10833 exhibits a clumping phenotype within chelated media. Strains 10833 and Newman were incubated, without shaking, in TSBG alone or supplemented with 12.5 mM EGTA at 37 °C for 18 h. The tubes were then briefly vortexed and then imaged. The red circle highlights the observed aggregation and clumping.

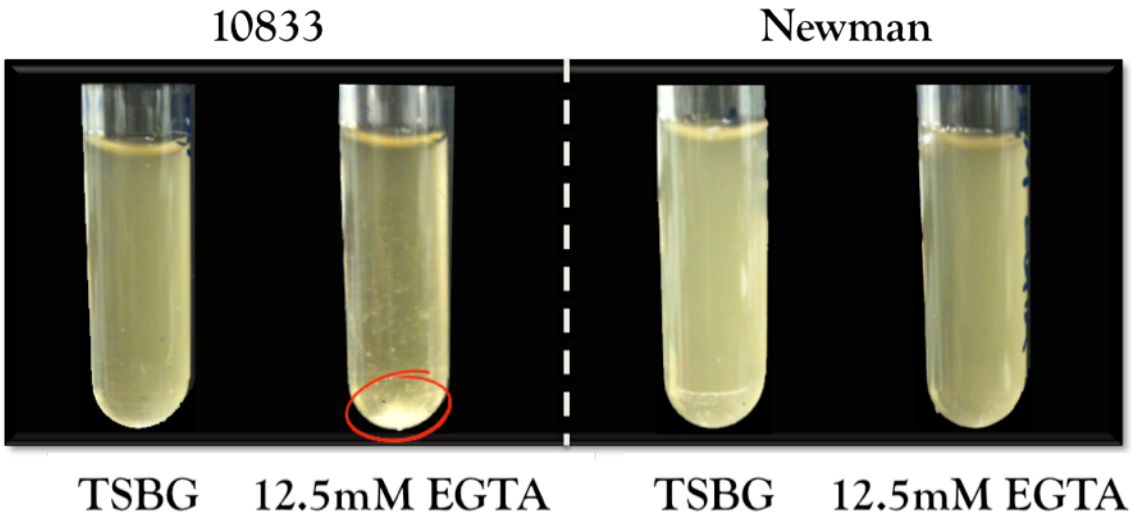
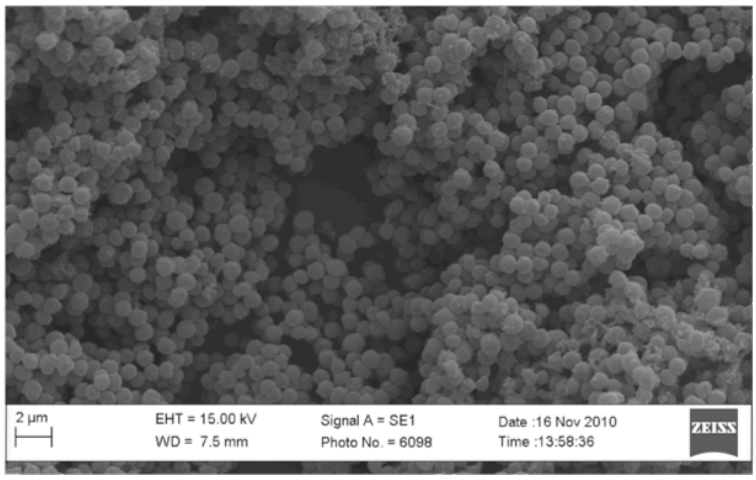
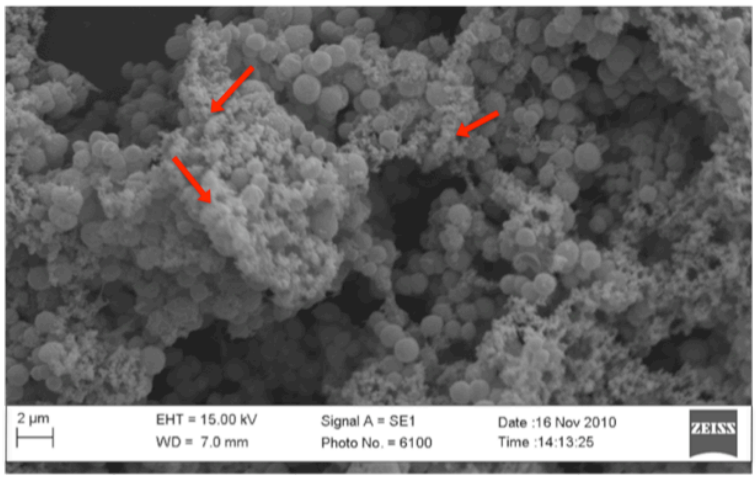


Figure 12. Scanning electron microscopy of 10833 biofilms. Overnight cultures of strain 10833 were grown for biofilms on clear glass coverslips placed within 6-well plates. Biofilms were grown in TSBG or TSBG + 12.5 mM EGTA and allowed to incubate at 37 °C for 18 h. The biofilms produced on the coverslips were carefully washed for non-adherent bacteria and then negatively stained with phosphotungstic acid. Scanning electron microscopy revealed large clumps of adherent bacteria in 12.5 mM EGTA. Associated with these aggregations was a significant amount of a surface-associated extracellular material (red arrows) that was not as pronounced with biofilms produced within TSBG alone. These micrographs are single image representations found throughout the well. (Magnification 7000X).

TSBG



12.5mM EGTA

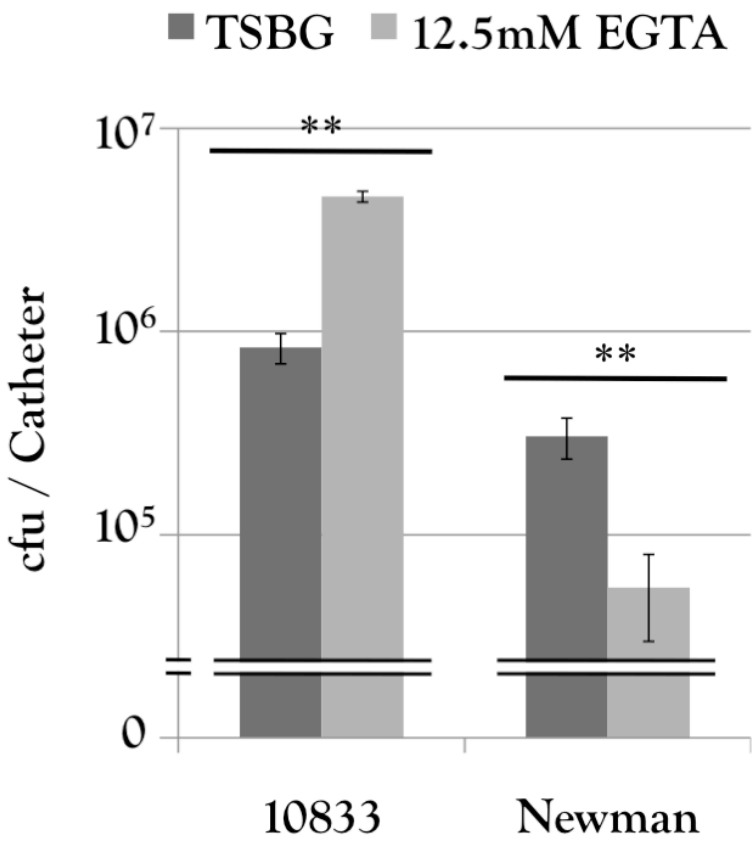


plated for cfu counts on TSA plates (**Figure 13)**. Adherence of strain Newman to catheter tubing was inhibited by EGTA; however, a 6-fold increase in adherence was observed for 10833 in the presence of 12.5 mM EGTA relative to the TSBG control. The high concentration of EGTA did not decrease cell viability, therefore, this assay additionally confirmed the viability of 10833 in EGTA.

Increase in biofilm formation by 10833 in EGTA is not due to an increase in PNAG

S. aureus biofilms can be composed of various components including polysaccharides like PNAG (O'Gara, 2007), proteinaceous adhesins (Corrigan, Rigby et al., 2007; Rohde, Burandt et al., 2007) and/or eDNA (Rice, Mann et al., 2007). Each of these components may be present in varying amounts or absent completely, depending on the strain background and the growth conditions. The *ica* locus of *S. aureus* encodes the exopolysaccharide PNAG that has been previously shown to contribute to biofilm formation within this species (Cramton, Gerke et al., 1999). Strains likes SA113 and MN8, a clinical isolate from a nonmenstrual toxic shock syndrome case (Schlievert and Blomster, 1983), produce copious amounts of PNAG that aid in biofilm formation (Cramton, Gerke et al., 1999; Jefferson, Pier et al., 2004). Therefore, we wanted to investigate the relative contribution of PNAG within 10833 biofilms established in the chelating conditions (**Figure 14A**). Standardized amounts of overnight cultures of bacteria were centrifuged to pellets (Surface-associated fraction) and the cells were resuspended in 0.5 M EDTA. The supernatant from the pelleted cells (Secreted fraction) and the Surface-associated fraction were boiled, diluted in 1X PBS, and blotted onto a

Figure 13. 10833 exhibited greater adherence to catheter tubing in the presence of EGTA. Bacteria were diluted in test tubes with either TSBG (dark grey bars) or TSBG + 12.5 mM EGTA (light grey bars) along with a ~2 cm piece of vialon based catheter tubing. The tubes were incubated for 18 h at 37 °C without shaking. The catheters were sterilely removed, washed to remove non-adherent bacteria, sonicated and plated for cfu counts on TSA plates. $^{**}P<0.0001$ as determined by Student's t-test.



nitrocellulose membrane. The membranes were then dried, blocked with BSA, and probed with α-PNAG antibody followed by a α-goat HRP labeled secondary antibody (**Figure 14A**). Strain 10833 produced only a small amount of PNAG when grown in TSBG, however, the strain showed very little, if any, detectable polysaccharide production when grown in EGTA.

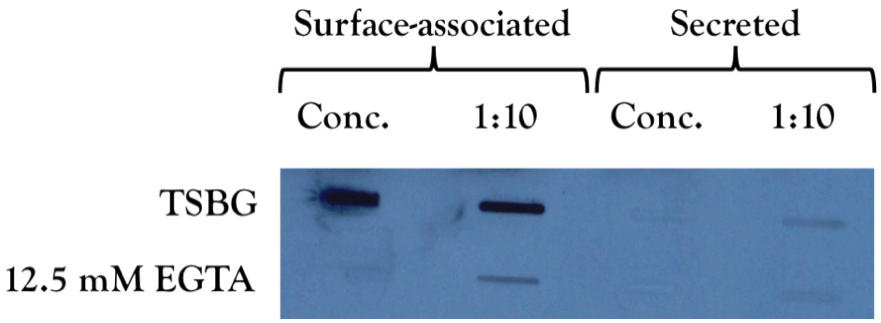
We confirmed these results by assessing the transcript levels of the *icaA* and *icaR* genes by isolating total RNA from 10833 grown in TSBG or added 12.5 mM EGTA or 12.5 mM NaCi and performing quantitative realtime RT-PCR analysis (**Figure 14B**). The *icaA* gene is the first gene of the intercellular adhesin *icaADBC* operon and functions as an *N*-acetylglucosaminyltransferase and, using UDP-*N*-acetylglucosamine as its substrate, facilitates the synthesis of 10-20-mers of *N*-acetylglucosamine (Gerke, Kraft et al., 1998). IcaR is as a *trans*-acting transcriptional repressor of the *ica* operon and is located immediately upstream of *icaADBC* operon. **Figure 14B** shows an approximately 3-fold increase in expression of *icaR* when 10833 was cultured in either 12.5 mM EGTA or NaCi. In addition, there was a simultaneous repression of the *icaA* transcript with each of these chelating conditions. This would suggest that the increase in biofilm formation by 10833 in EGTA or NaCi is not due to the production of PNAG. Identifying neither eDNA nor PNAG to play a role within 10833 biofilms within chelating agents would suggest a role for proteinaceous adhesins mediating this phenotype.

Chelators lead to an increase in the level of Clumping factor B transcript

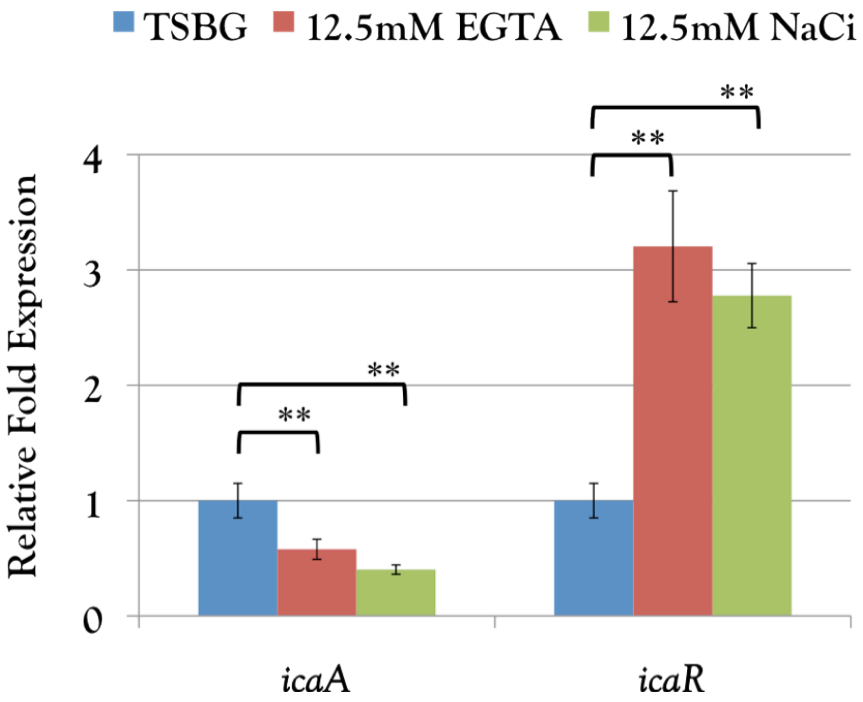
The two primary staphylococcal factors associated with clumping are clumping

Figure 14. 10833 biofilms in EGTA is not due to increased PNAG production. (**A**) Overnight cultures of wildtype strain 10833 was grown overnight in either TSBG or media with 12.5 mM EGTA. Cultures were centrifuged to pellets and resuspended in 0.5 M EDTA (Surface-associated). The supernatants from the pellets (Secreted) and the Surface-associated fractions were boiled to extract PNAG, diluted 1:10 within 1X PBS, and blotted onto a nitrocellulose membrane. The membranes were probed with an α-PNAG antibody and an HRP-labeled secondary antibody before being analyzed by x-ray film. (**B**) RNA was extracted from 10833 grown in TSBG, TSBG + 12.5 mM EGTA or TSBG + 12.5mM NaCi during mid-log phase of growth. Transcript levels for the *icaA* and *icaR* genes were analyzed by quantitative realtime RT-PCR. The individual genes were normalized to 16S rRNA and the relative fold expression with respect to TSBG alone is shown. $^{**}P<0.0001$ as determined by Student's t-test.

A.



B.

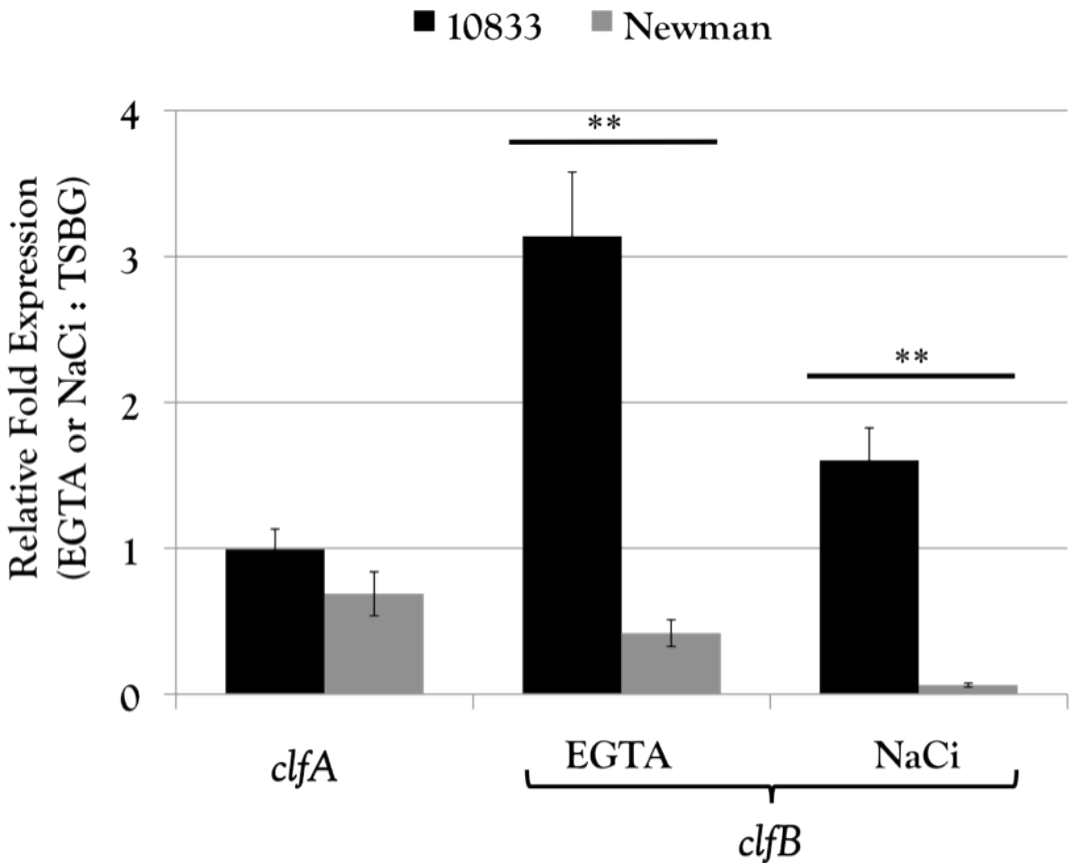


factors A (ClfA) and B (ClfB). Previous reports suggest that ClfA and ClfB-mediated clumping requires the presence of fibrinogen (Ni Eidhin, Perkins et al., 1998); however, we hypothesized that in the presence of a chelating agent these factors could induce clumping in the absence of fibrinogen. We therefore investigated the relative expression of the clumping factor genes *clfA* and *clfB* when bacterial cultures were incubated in TSBG containing 12.5 mM EGTA or 12.5 mM NaCi relative to TSBG alone (**Figure 15**). The levels of *clfA* transcript were unchanged by EGTA in either strain. However, in Newman, *clfB* transcript levels were lower in the presence of EGTA but were increased 3-fold in the presence of EGTA in strain 10833. Additionally, we observed a 1.5-fold increase in *clfB* transcript levels in 10833 in 12.5 mM NaCi, whereas *clfB* transcript levels were severely decreased for Newman cultures grown in NaCi. In sum, the expression of *clfB* was negatively regulated in the presence of EGTA or NaCi in Newman but positively regulated by the chelators in 10833.

Gene sequence and transcriptional start site is conserved between 10833 and Newman.

We next sought to determine whether the difference in *clfB* expression in strains 10833 and Newman was due to a mutation in the gene or promoter. For this purpose, we sequenced the *clfB* gene and the upstream region (**Figure 16**). The *clfB* gene from 10833 was identical to Newman except for the presence of additional SD repeats, only lengthening the protein, and the upstream promoter regions were 100% identical to the previously published Newman sequence (Baba, Bae et al., 2008). We also determined the

Figure 15. Expression of clumping factor B (*clfB*) in the presence of NaCi and EGTA. Total RNA was isolated from strains 10833 and Newman that were grown in TSBG, TSBG + 12.5 mM EGTA, and TSBG + 12.5 mM NaCi during mid-log phase of growth. Transcript levels of the clumping factor gene *clfB* were analyzed by realtime RT-PCR. Individual genes were normalized to 16S rRNA and the relative fold difference is shown with respect to TSBG. Black bars represent relative gene expression by strain 10833 while the grey bars are representative of Newman. $^{**}P<0.0001$ as determined by Student's t-test.



transcriptional start site using 5'-RACE analysis and observed transcription started at the same nucleotide in both strains.

Clumping factor B, *clfB* is required for EGTA-induced biofilm formation in 10833

To further investigate the role of clumping factor B in the differential effect of EGTA on biofilm formation in strains Newman and 10833, isogenic *clfB* deletion mutants were made and biofilm assays were performed (**Figure 17**). Deletion of *clfB* in strain 10833 abrogated biofilm formation significantly (**Figure 17B**, $P<0.0001$) when the strains were grown in 12.5 mM EGTA but did not significantly affect biofilm formation in the absence of EGTA. Quantitative realtime RT-PCR demonstrated that in strains complemented *in trans* with *clfB*-pCL15, 1 mM IPTG restored *clfB* transcript levels to that of the parental strains (**Figure 17A**). Complementation of *clfB* gene expression restored biofilm formation, of 10833 in EGTA, to that of wildtype (**Figure 17C**, black arrows). In strain Newman, biofilm formation in TSBG was unaffected by the deletion, but the weak biofilm formed in the presence of EGTA was further reduced by the *clfB* mutation (**Figure 17B**, $P<0.0001$). Complementation of the gene restored biofilm levels to that observed with the wildtype strain in the presence of EGTA. Together these results suggest that biofilms formed in the presence of EGTA are ClfB-dependent and that ClfB results in the production of a thicker biofilm in 10833 than in Newman.

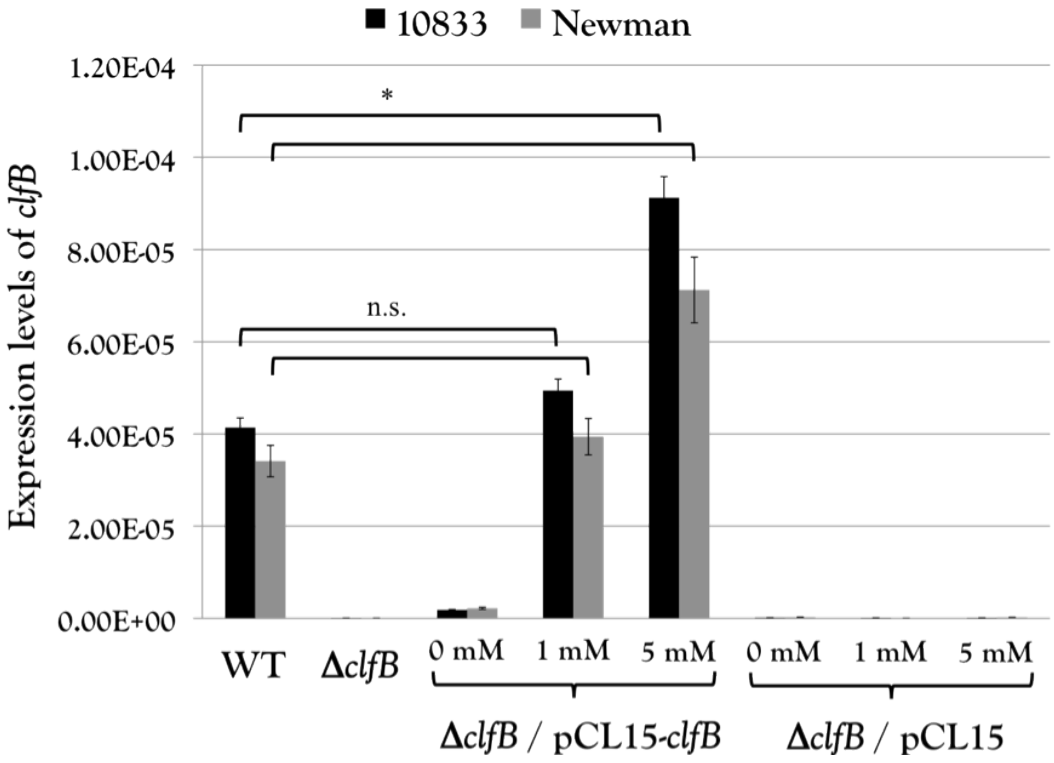
ClfB is required for increased biofilm formation in EGTA

The data thus far has pointed towards ClfB playing a critical role in contributing

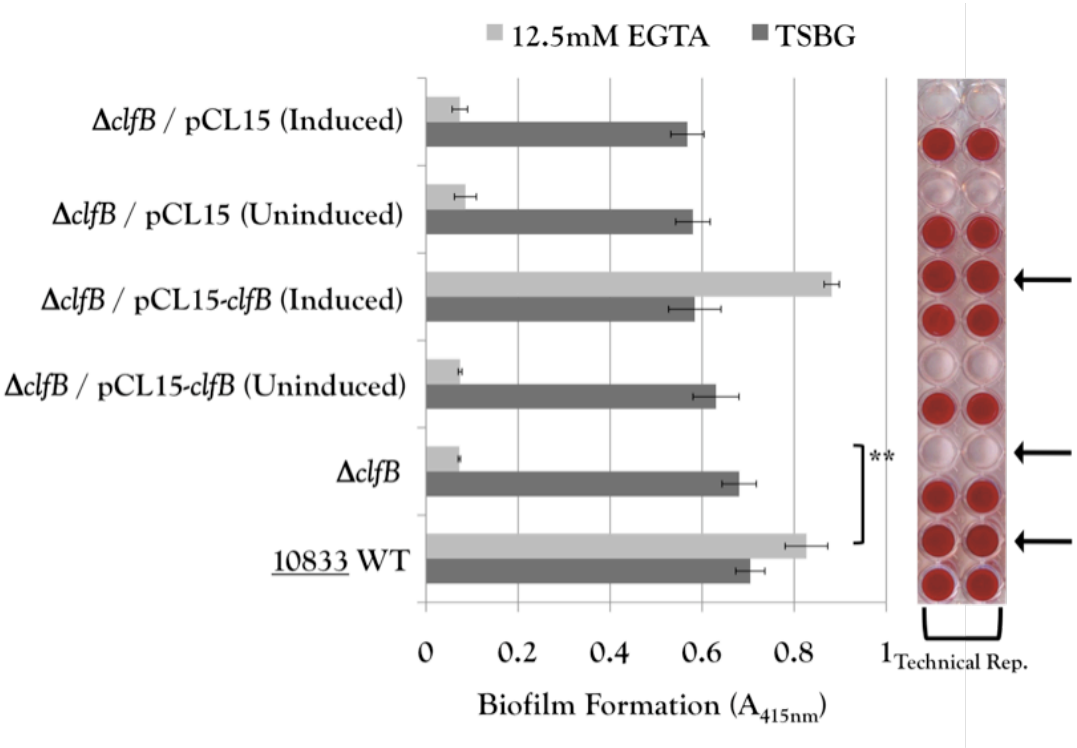
Figure 16. Transcriptional start sites for *clfB* are identical in 10833 and Newman. Schematic representation of the *clfB* gene displaying transcriptional start site (+1) for strains 10833 and Newman as determined by 5'-RACE analysis. The translational start site is underlined. The predicted -10 and -35 promoter-binding sites as determined bioinformatically using the SoftBerry promoter analysis program, are also shown.

Figure 16. Deletion of *clfB* abrogates biofilm formation in the presence of EGTA. (**A**). Overnight cultures of strains 10833, Newman, and their respective isogenic and complementation mutants were diluted in fresh TSBG and total RNA was isolated from cultures grown until mid-logarithmic phase. Quantitative realtime RT-PCR analysis was performed to determine the concentration of IPTG (0 mM, 1 mM, or 5 mM) required to restore *clfB* transcripts levels, within the complementation mutants, to that of wildtype. *S. aureus* strain 10833 (**B**), Newman (**C**) and their isogenic *clfB* deletion and complementation mutant derivatives were grown in 96-well polystyrene plates in TSBG (dark grey bars) or TSBG + 12.5 mM EGTA (light grey bars) and assessed for biofilm formation. Biofilms were stained for visualization (right) and quantified at A_{415nm} (left). The complementation mutants were induced (I) with 1 mM IPTG or remained uninduced (U). The black arrows point towards the visual differences between the WT, deletion mutant, and the induced complemented strain. **P*<0.001, ***P*<0.0001 as determined by Student's t-test.

A.



B.



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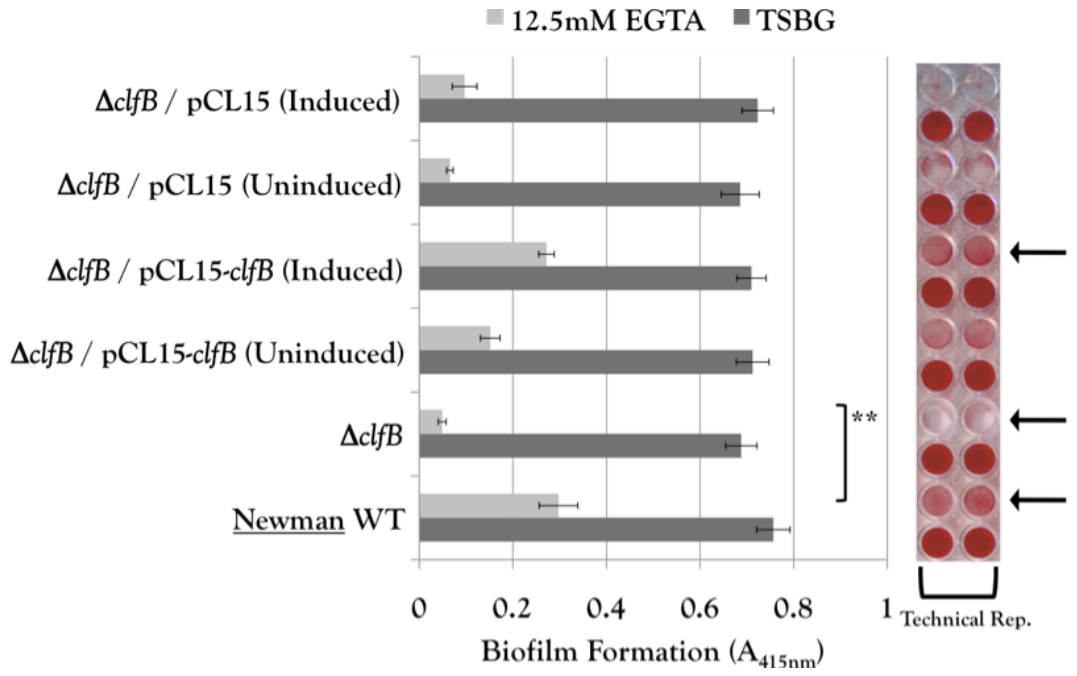


to the increased biofilms observed by lab strains within chelating agents like EGTA and NaCi. *S. aureus* expresses a large number of MSCRAMM proteins that can bind specifically to different components of the host extracellular matrix. Among these proteins, four are known to bind fibrinogen. Apart from the fibrinogen-binding clumping factor proteins ClfA and ClfB, recently, the fibronectin binding protein FnbA has also been implicated in a similar adherent capacity (Wann, Gurusiddappa et al., 2000). **Figure 18A** schematically describes the high degree of structural and organization similarity that exists between ClfA, ClfB and FnbA. The 'A' or ligand-binding domain of ClfA and ClfB facilitates fibrinogen binding (Perkins, Walsh et al., 2001) and, we hypothesize this domain within ClfB to contribute to intercellular aggregation and biofilm formation observed within strains like 10833. Therefore, to rule out the possibility these other MSCRAMMs were playing a role with increased biofilm formation in chelated media we acquired a series of transposon insertion mutants within these genes.

In an effort to enhance the research capabilities of the staphylococcal research community, the Center for Staphylococcal Research (CSR) at the University of Nebraska Medical Center has generated a collection of sequence-defined transposon (Tn) insertion mutants within the non-essential genes of *S. aureus* strain USA300 JE2. This strain was derived from a well-characterized CA-MRSA strain isolated from the Los Angeles County jail- USA300 LAC (Kennedy, Otto et al., 2008). Although the genome of this strain has not been sequenced, only 11 SNPs (single nucleotide polymorphisms) were detected between this strain and the sequenced strain USA300 FPR3757 (Kennedy, Otto et al., 2008). This Nebraska Tn Mutant Library was constructed using *bursa aurealis*, a

mariner-based transposon (Bae, Glass et al., 2008). The mutants that revealed a transposon insertion within the first third of a gene were robotically re-arrayed into a 96-well format and then resequenced to verify the identify of each mutant.

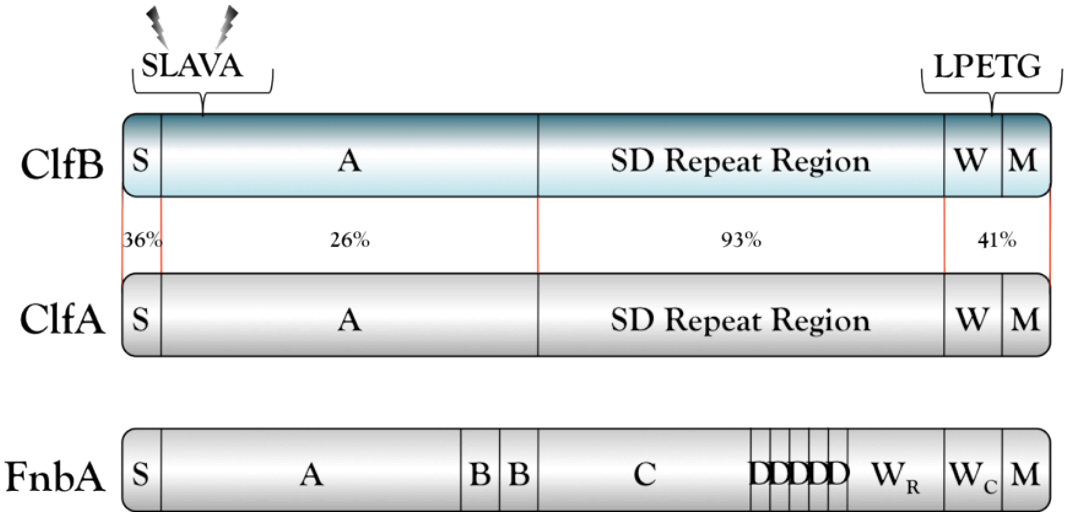
Using standard biofilm assays, we compared the wildtype strain USA300 JE2 to a series of transposon insertion mutants within genes including *clfB* (NE391), *clfA* (NE543), *fnbA* (NE186), and another undefined putative fibrinogen/fibronectin binding protein gene (NE551) (**Figure 18B**). This assay revealed two very interesting findings- The wildtype USA300 JE2 strain, similar to 10833, showed a severe increase in the biofilm phenotype when grown under the chelating condition (EGTA). Secondly, the NE391 (Δ*clfB*) strain failed to form a biofilm in EGTA. All other mutants revealed high biofilm mass similar to the wildtype when cultured within EGTA. This directly implicates ClfB as the sole determinant associated with increased biofilms under chelating agents. Additionally, the use of this *clfB* transposon mutant serves as independent confirmation of our own chromosomal deletion phenotype, generated within 10833 and Newman, that reveal loss and further reduction of biofilms compared to their respective wildtype strains when grown under similar chelating conditions.

Effect of chelators on biofilm formation in cardiac device-associated isolates

To identify whether this phenomenon; chelator-mediated augmentation of biofilm formation within strains like 10833 and USA300 JE2, was a common occurrence with clinical strains, we next tested a panel of clinical isolates from the bloodstream of patients with cardiac device-associated infections (provided by Dr. Vance Fowler, Duke

Figure 18. ClfB promotes biofilm formation within chelated media. (A) Schematic representation and comparison between the ClfB, ClfA and FnbA MSCRAMMs. S: signal sequence; A: unique N-terminal ligand binding domain, binds fibrinogen (ClfA and ClfB); B, C, and D: homologous repeats of unknown function; SD Repeat Region: serine-aspartate dipeptide repeat region; W, wall-spanning region (W_R: Octapeptide repeat, W_C: Conserved repeat); M: membrane-spanning domain. The LPETG motif, is involved in anchoring the proteins to the cell wall peptidoglycan (all three proteins) and the SLAVA motif is a putative metalloprotease cleavage site (only ClfA and ClfB). The percentage residue identity between the respective regions of ClfA and ClfB proteins is shown. (**B**) *S. aureus* strain USA300 JE2 and the transposon insertion mutants NE391 (Δ*clfB*), NE543 (Δ*clfA*), NE186 (Δ*fnbA*), and NE551 (undefined fibrinogen/fibronectin binding protein) were grown in TSBG (dark grey bars) or TSBG + 12.5 mM EGTA (light grey bars) within individual wells of a 96-well polystyrene plate. Biofilms were stained for visualization (right) and quantified at A_{415nm} (left). $^{**}P<0.0001$ as determined by Student's t-test.

A.



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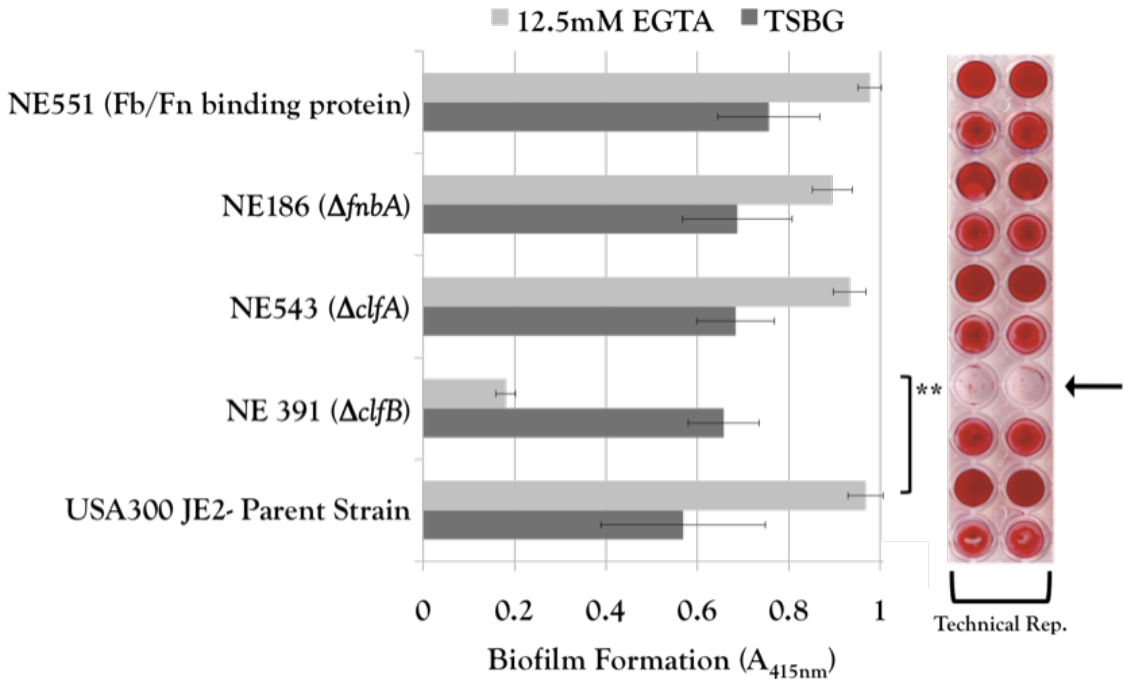


University, Durham, NC) (**Table 3**). Out of 27 isolates, biofilm formation was inhibited (by at least 25%) by both chelators in 5, was not significantly affected by the chelators in 3, and was augmented by both chelators in 8. The remaining strains exhibited variable responses to NaCi and EGTA. These results demonstrated that, while sub-inhibitory concentrations of EGTA and NaCi inhibit the biofilm phenotype in certain strains of *S. aureus*, they actually stimulate the biofilm phenotype in others.

To further add weight to our growing hypothesis that ClfB was playing a dominant effect in strains that exhibited augmented biofilm phenotypes within chelated media, we phage transduced the *clfB* chromosomal deletion into two clinical isolates, VF Strains 19 and 24, that were observed to show increased biofilms within EGTA and NaCi and performed biofilm assays (**Table 3**, **Figure 19**). Deletion of *clfB* from Strain 19 showed a significant decrease, 73% and 46% in EGTA and NaCi respectively ($P<0.0001$), in relation to the wildtype controls. Likewise, *clfB* mutants of Strain 24 showed similar trends- 98% and 48% decrease in biofilms in EGTA and NaCi ($P<0.0001$). We therefore concluded that, there exist, naturally occurring, clinical strains of *S. aureus* that are refractory to the inhibitory effects of such chelating agents and augment biofilm formation within sub-inhibitory concentrations of these chelators. Furthermore, ClfB appears to be the mediator of this effect, at least in the strains that we tested in this study. Resistance of biofilm formation to the chelating agents could potentially account for the rapidly increasing numbers attributing *S. aureus* as one

Table 3. Effect of 12.5 mM EGTA or 12.5 mM NaCi on biofilm formation in 27 cardiac device-associated isolates

VF Strain #	% Biofilm in 12.5 mM EGTA relative to TSBG	% Biofilm in 12.5 mM NaCi relative to TSBG
Strain 1^{DOWN}	*29.78 ± 0.07*	*31.75 ± 0.04*
Strain 2^{DOWN}	*21.06 ± 0.17*	*74.33 ± 0.04*
Strain 3^{UP}	**125.23 ± 0.06**	**163.48 ± *0.04***
Strain 4	59.25 ± 0.23	173.68 ± 0.07
Strain 5^{UP}	**161.67 ± 0.05**	**174.34 ± *0.06***
Strain 6	101.70 ± 0.03	82.23 ± 0.04
Strain 7	103.96 ± 0.04	79.01 ± 0.03
Strain 8	132.61 ± 0.05	63.27 ± 0.06
Strain 9^{UP}	**130.58 ± 0.03**	**140.28 ± *0.06***
Strain 10^{UP}	**126.37 ± 0.04**	**145.30 ± *0.04***
Strain 11	134.86 ± 0.06	80.62 ± 0.06
Strain 12^{DOWN}	*6.95 ± 0.01*	*32.27 ± 0.02*
Strain 13	54.26 ± 0.14	92.15 ± 0.10
Strain 14	117.36 ± 0.04	91.35 ± 0.06
Strain 15	209.29 ± 0.10	105.01 ± 0.05
Strain 16	125.52 ± 0.03	90.60 ± 0.04
Strain 17	127.56 ± 0.06	97.27 ± 0.07

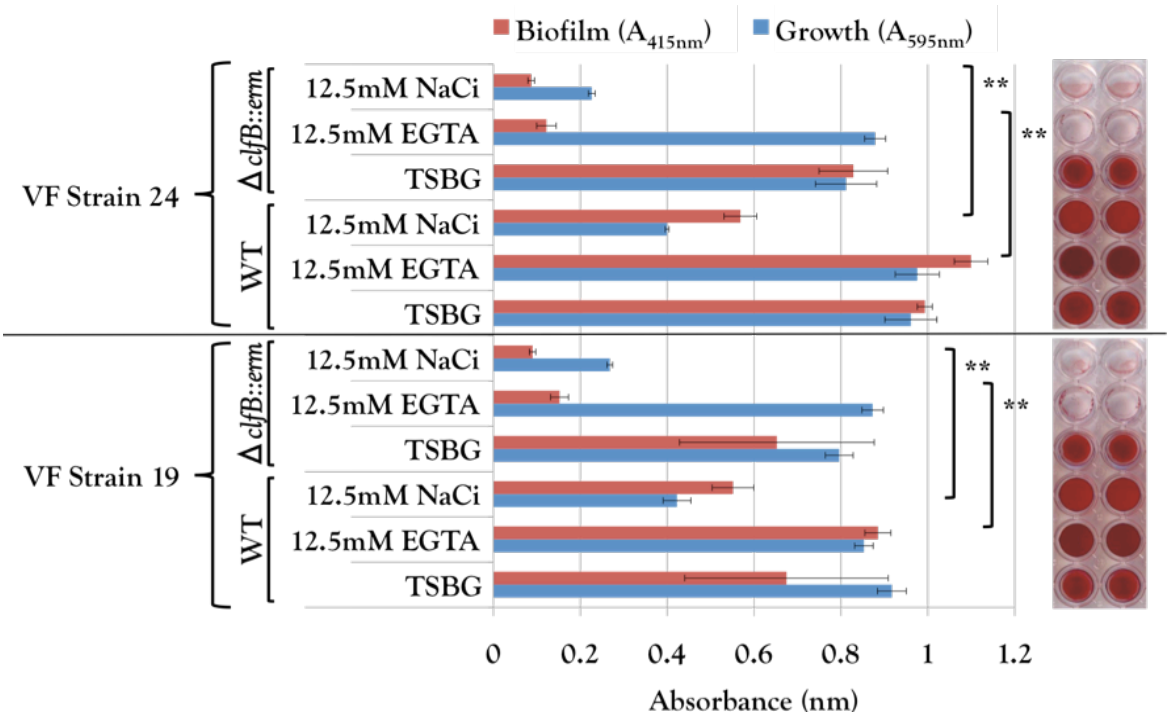
Strain 18	113.92 ± 0.02	60.38 ± 0.04
Strain 19^{UP}	**126.88 ± *0.06***	**136.82 ± *0.07***
Strain 20	203.33 ± 0.07	106.29 ± 0.03
Strain 21 ^{DOWN}	*16.49 ± 0.08*	*45.24 ± 0.03*
Strain 22 ^{DOWN}	*7.92 ± 0.00*	*30.65 ± 0.02*
Strain 23^{UP}	**432.11 ± *0.16***	**243.17 ± *0.06***
Strain 24 ^{UP}	**314.24 ± *0.08***	**140.09 ± *0.05***
Strain 25 ^{UP}	**757.19 ± *0.06***	**162.41 ± *0.02***
Strain 26	73.16 ± 0.01	180.31 ± 0.01
Strain 27	115.06 ± 0.07	126.01 ± 0.07

^{UP} **Strains**- observed to showed significant increase (≥ 25% relative to TSBG) in biofilm formation within EGTA and NaCi relative to their TSBG control (P<0.0001)

^{DOWN} *Strains*- significantly decreased in biofilm formation in EGTA and NaCi (P<0.0001)

Underlined VF Strains 19 and 24 were used to assess the role for ClfB in promoting increased biofilms within the two chelating conditions (**Figure 19**)

Figure 19. Biofilms produced by VF Strains 19 and 24 in EGTA and NaCi are ClfB-dependent. Two clinical isolates VF Strain 19 and Strain 24, identified to exhibit increased biofilm within EGTA and NaCi (**Table 3**) were compared to their, phage transduced, *clfB* deletion mutants for growth (A_{595nm}, Blue bars) and biofilm formation (A_{415nm}, Red bars) within TSBG, TSBG + 12.5 mM EGTA, and TSBG + 12.5mM NaCi. Bacteria were inoculated into wells of a 96-well polystyrene plate and incubated at 37 °C for 18 h. Growth was assessed prior to biofilm staining. Biofilms were washed, stained for visualization with safranin (right) and quantified at A_{415nm} (left). $^{**}P<0.0001$ as determined by Student's t-test.



of the leading causes of catheter-related BSIs. These results demonstrate prevalence of a subset of strains that exhibit chelator-mediated biofilm formation and this novel phenomenon seems to be dependent on ClfB as the primary molecular determinant of these biofilms.

α-ClfB antibody inhibits aggregation and clumping of 10833 and USA300 JE-2

Aggregation and clumping is an important phenotype exhibited by many bacteria and this usually precedes successful biofilm formation. Various surface-associated proteins of *S. aureus* have been implicated in intercellular aggregation leading to biofilm formation. These include- *S. aureus* surface protein G (SasG) (Kuroda, Ito et al., 2008; Corrigan, Rigby et al., 2007), *S. aureus* surface protein C (SasC) (Schroeder K, Jularic et al., 2009) and extracellular adherence protein (Eap) (Thompson, Abraham et al., 2010). To directly implicate ClfB in the aggregation and clumping phenotype, we employed the use of a rabbit α-ClfB antibody (kindly provided by Dr. Gerald Pier, Harvard University, Boston, MA). The antibody was generated against the A domain of ClfB that has been implicated previously in binding to immobilized host matrix component fibrinogen (Perkins, Walsh et al., 2001).

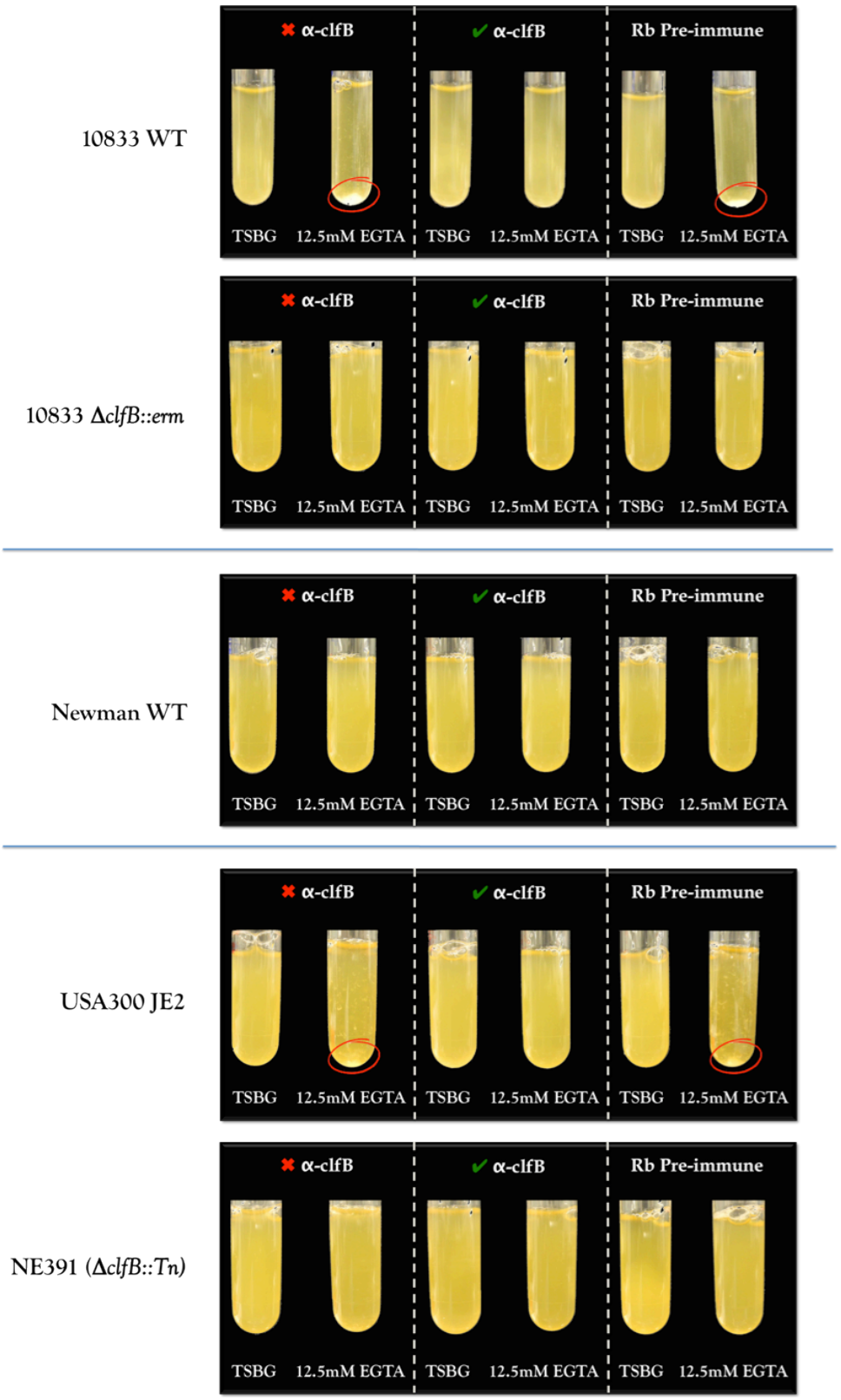
Overnight cultures of bacteria- 10833, Newman and USA300 JE2 and their isogenic *clfB* chromosomal deletion or transposon insertion mutants, standardized to ensure equal amount of bacteria, were inoculated into test tubes with fresh media i.e. TSBG or TSBG + 12.5 mM EGTA containing 4.0 μg/mL α-ClfB antibody, pre-immune antibody or No antibody. The tubes were incubated at 4 °C for 2 h, to ensure binding of

the antibody and thereafter transferred to 37 °C to incubate, without shaking, for 18 h. Tubes were removed, gently vortexed and observed for clumping and aggregation (**Figure 20**). In the absence of any antibody 10833 was observed to clump in EGTA, as previously observed in **Figure 11**. However, the addition of the α-ClfB antibody prevented this strain from clumping. The use of the rabbit pre-immune sera controls for the presence of any specific or non-specific α-staphylococcal antibody that would inhibit the strain from clumping. The pre-immune sera, similar to the no antibody treatment, permitted 10833 to clump in EGTA. The isogenic *clfB* mutant strain showed absence of any clumping in any of the conditions tested. Newman was previously observed, in **Figure 11**, to show no sign of clumping in either chelated or non-chelated media in the absence of any antibody. This was confirmed in this assay (**Figure 20**). Thus, the presence of the α-ClfB antibody also showed no signs of clumping in EGTA. Finally, strain USA300 JE2 showed similar trends as 10833, clumping in the absence of the antibody in EGTA and loss of this phenotype was with the addition of the α-ClfB antibody. The control strain NE391, displayed no apparent clumping under any condition tested. This data suggests a direct impact ClfB plays in promoting bacterial aggregation and clumping in chelated solutions, especially within strains like 10833 and USA300 JE2 and that this phenotype can be abrogated with the addition of the α-ClfB antibody.

α-ClfB antibody inhibits biofilm formation in 10833 and USA300 JE-2

To confirm the role for ClfB in biofilm formation, we utilized the α-ClfB antibody and analyzed its effects on biofilm formation in strains 10833 and USA300 JE2

Figure 20. 10833 and USA300 JE2 lose their clumping phenotype in EGTA with the addition of α-ClfB antibody. Strains 10833, Newman and USA300 JE2 were grown in the presence of TSBG or TSBG + 12.5 mM EGTA and 4.0 μg/mL of either the α-ClfB antibody, the rabbit pre-immune sera or absence of any added antibody. The cultures were added to test tubes and first allowed to incubate at 4 °C for 2 h to promote antibody binding to the cells and thereafter transferred to 37 °C for 18 h, without shaking. The tubes were briefly vortexed and then imaged. The red circle highlights the aggregative and clumped phenotype observed with strains. Control strains, including the two 10833 and Newman isogenic *clfB* chromosomal mutants and the NE391 transposon insertion mutant, were also tested.

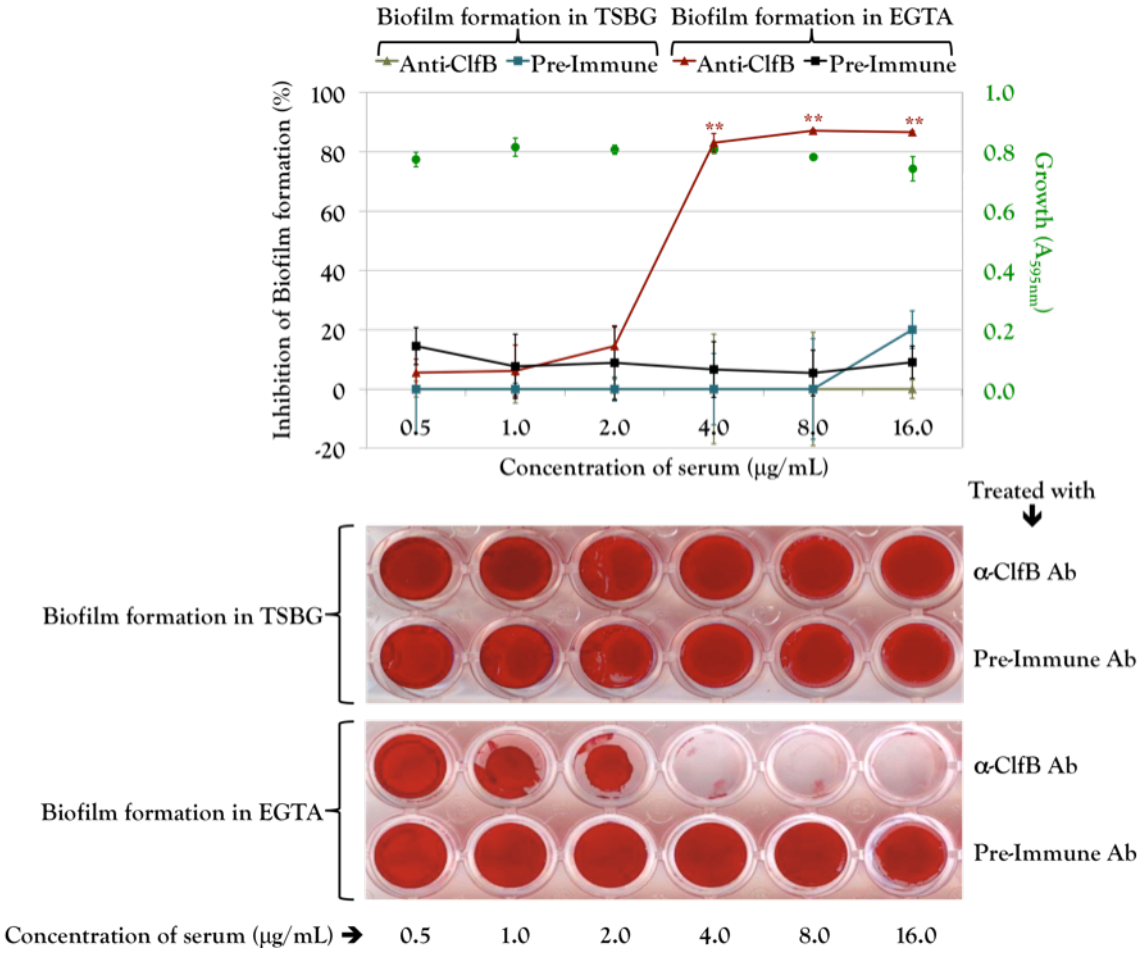


within chelated media. Overnight cultures of wildtype bacteria- 10833, Newman, and USA300 JE2 were diluted to an OD_{600} of 0.015 and added to fresh media, either TSBG or TSBG + 12.5 mM EGTA along with varying concentrations of α-ClfB antibody, pre-immune antibody (0.5 – 16.0 μg/mL) and compared biofilms formed in the absence of any antibody. The bacteria were inoculated into individual wells of a 96-well plate and incubated first for 2 h at 4 °C and then transferred to the 37 °C for 18 h. The wells were washed with water, to remove non-adherent bacteria, and stained with safranin to determine any detectable biofilm. The percent inhibition of biofilm was calculated using the formula: $[1- (Biofilm_{415nm\ (with\ antiserum)} / Biofilm_{415nm\ (without\ antiserum)}] \times 100$ (Hussain, Herrmann et al., 1997) (**Figure 21**).

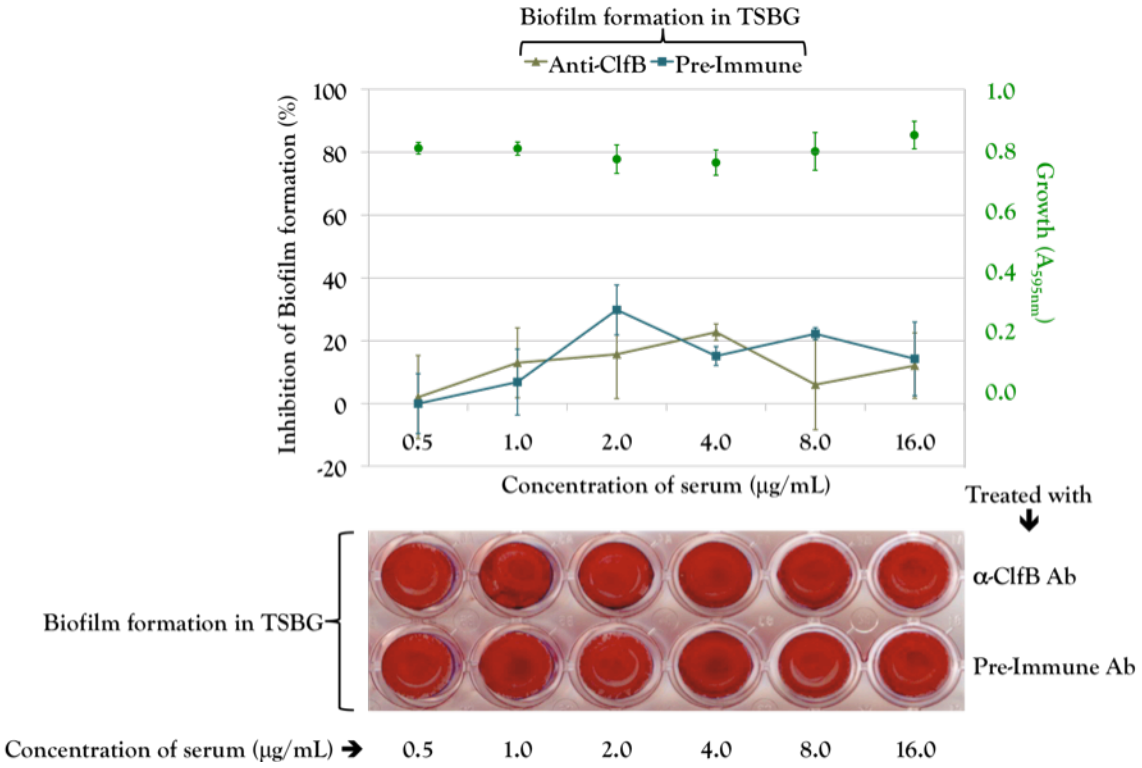
Biofilm formation by 10833 in EGTA (**Figure 21A**) was decreased with increasing concentrations of the α-ClfB antibody with concentrations greater than 4.0 μg/mL preventing any biofilm from being produced ($P<0.0001$). At high concentrations of the antibody (≥4.0 μg/mL) a significant loss of biofilm, previously obtained with lower concentrations (≤2.0 μg/mL), was observed (>65% inhibition, $P<0.0001$). To ensure high concentrations of the inhibiting α-ClfB antibody did not detrimentally affect cell growth, growth at A_{595nm} was assessed (**Figure 21A**, green dots). The α-ClfB antibody did not adversely affect growth of this strain within EGTA at any of the antibody concentrations used. In contrast, biofilms produced by this strain in TSBG alone remained robust and intact at every concentration of the α-ClfB antibody tested. The pre-immune control antibody remained ineffective in inhibiting biofilms produced by this strain in TSBG or EGTA. Strain Newman (**Figure 21B**) forms significant biofilms only in TSBG and thus

Figure 21. Biofilm formation by 10833 and USA300 JE2 is inhibited in EGTA with the addition of the α-ClfB antibody. Overnight cultures of 10883 (**A**), Newman (**B**), and USA300 JE2 (**C**) were diluted in fresh TSBG or TSBG + 12.5 mM EGTA and treated with varying concentrations of either α-ClfB antibody or rabbit pre-immune sera (0.5 – 16.0 μg/mL) or absence of any added antibody and added to wells of a 96-well polystyrene plate. The plates were incubated at 4 °C for 2 h to promote antibody binding and thereafter moved to the 37 °C for 18 h. Non-adherent cells were removed by washing with water and adherent bacteria were stained with safranin. Biofilms were quantified at A_{415nm} and the percent inhibition of biofilm formation was determined using the formula: $[1- (Biofilm_{415nm\ (with\ antiserum)} / Biofilm_{415nm\ (without\ antiserum)}] \times 100$. The representative biofilm images grown in either TSBG or supplemented EGTA treated with either α-ClfB antibody or rabbit pre-immune sera are shown below the graphs. Growth (A_{595nm}, green dots) was assessed for 10833 and USA300 JE2 (within EGTA) and Neman (within TSBG) in the presence of the α-ClfB antibody to ensure high concentrations of the antisera did not affect growth. $^{**}P<0.0001$ as determined by Student's t-test.

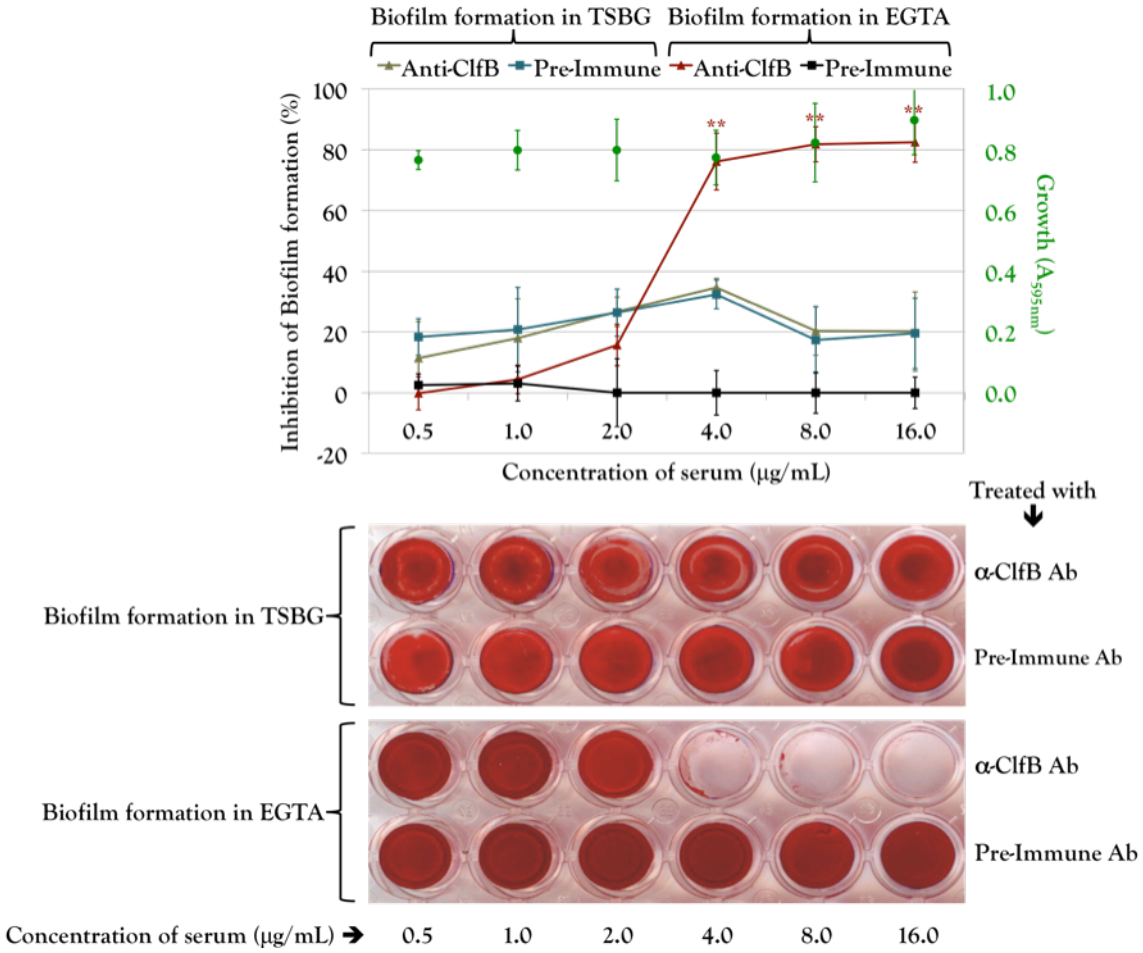
A. Strain: 10833 WT



B. Strain: Newman WT



C. Strain: USA300 JE2



only this condition was tested. Biofilms produced by Newman in TSBG remained unaffected and without any significant loss to the architecture in the presence of the α-ClfB antibody. **Figure 21C** shows the experiment performed with strain USA300 JE2. Similar results as 10833 were observed, with significant inhibition of biofilm formation in EGTA with ≥4.0 μg/mL α-ClfB antibody. Biofilms were significantly abrogated (>60% decrease) at concentrations greater than 4.0 μg/mL (P<0.0001) when this strain was grown in the presence of EGTA. In contrast, there no effect of the α-ClfB antibody when USA300 JE2 was grown in TSBG, or in either media condition tested supplemented with the pre-immune antibody. Results from **Figure 20** and **Figure 21** implicate ClfB, not only promoting clumping and aggregation within strains like 10833 and USA300 JE2 in chelated media but also confirm ClfB as playing a crucial role within these biofilms.

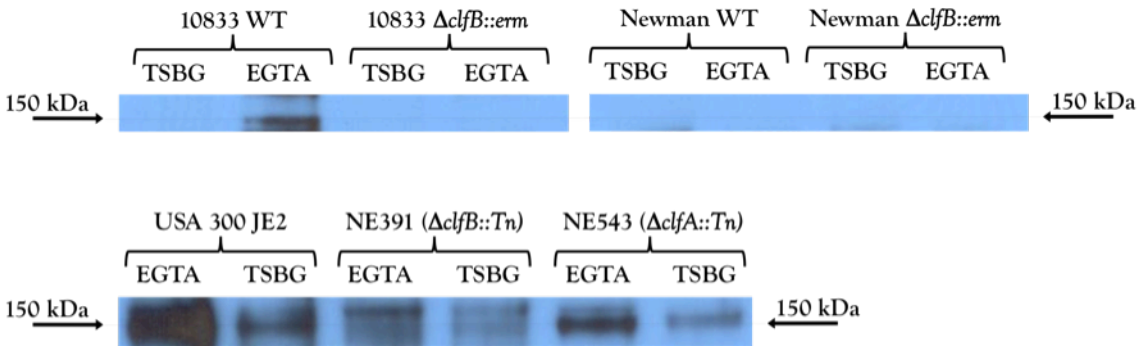
ClfB protein is expressed during aggregative stationary growth

Using the α-ClfB antibody, we investigated the relative expression of the ClfB protein across strains grown under the chelating condition. Overnight cultures of wildtype and isogenic *clfB* mutant bacteria were diluted in TSBG or media supplemented with 12.5 mM EGTA and added to test tubes. The cells were incubated under static conditions for 18 h, collected by centrifugation, pellets were standardized by weight and total surface protein extracted. The proteins were separated by SDS-PAGE, transferred to a PVDF membrane, probed using the polyclonal IgG rabbit α-ClfB antibody followed by a secondary goat α-rabbit HRP labeled antibody and developed using x-ray film.

Western blotting is a common technique used to detect and quantify proteins in *S. aureus*. However, protein A (Spa) secreted by 99% of *S. aureus* isolates (Goding, 1978) interferes with such assays by binding to the Fc region of most mammalian IgGs. To prevent interference Nguyen et al. demonstrated the use of increasing concentrations of diethylpyrocarbonate (DEPC) to successfully inhibited the interaction of protein A with the capturing antibody. Spa binding is mediated through an exposed histidine residue at position 435 in mammalian IgG (Deisenhofer, 1981). DEPC was originally shown to interfere with the binding of Spa to histidine-435 of mammalian IgG (Schroder, Nardella et al., 1987), however, more recent data has identified other residues including lysine, tyrosine, serine and threonine residues that can be modified by DEPC (Hnizda, Santrucek et al., 2008; Mendoza and Vachet, 2008). After numerous blots, we determined 5 mM DEPC along with 10% human serum, which naturally contains non-specific α-staphylococcal and α-Spa antibodies, supplemented to the primary α-ClfB antibody inhibited enough of the nonspecific binding by protein A such that the ClfB protein could be detected.

As shown in **Figure 22**, expression of the 150-kDa ClfB protein was much greater under chelated conditions. There was absence of any protein production from the isogenic *clfB* mutants under any condition tested. Wildtype Newman did not produce detectable levels of the protein under any condition. Finally, strain USA300 JE2 showed high expression of the protein when cultured with EGTA in relation to TSBG control. There were moderate levels of expression from the NE543 (Δ*clfA*) under both conditions and no expression from the NE391 (Δ*clfB*) mutant. This reconfirms our previous

Figure 22. Western blot analysis of ClfB protein by *S. aureus* strains grown under chelated versus non-chelated condition. *S. aureus* strains 10833, Newman, USA 300 JE2, and their isogenic chromosomal or tansposon insertion mutants were grown stationarily for 18 h in tubes within TSBG or added 12.5 mM EGTA. Total surface protein was extracted using 0.5 μg/mL of lysostaphin treatment and sonication and loaded into each well of a 4-12% NuPAGE SDS PAGE gel. Proteins were separated on the PAGE gel for 2 h at 200 V and then transferred to PVDF membranes for western blot analysis. The proteins were blocked in 5% skim milk overnight and probed thereafter with α–ClfB antibody (4.0 μg/mL). Molecular mass of ClfB (150kDa) is shown to the right and left (black arrows).



observations (**Figure 18B**) that ClfB and not closely related ClfA or the other fibronectin binding proteins promote chelator induced biofilm formation.

Discussion

Catheter-related bloodstream infections are a significant iatrogenic complication associated with the use of implanted intravascular devices, and incidence increases proportionally with the length of time that the catheter is left in place. Use of chelating agents such as NaCi in CLS have been previously shown to disrupt the process of bacterial adherence and biofilm formation on the synthetic surface, and can help prevent catheter contamination (Banin, Brady et al., 2006; Shanks, Sargent et al., 2006). However, catheter-related infections still occur, suggesting that bacteria can form biofilms even in the presence of antibiofilm CLS. This study elucidated the prevalence of certain strains of *S. aureus* that are adapted to becoming resistant to the antibiofilm effects of chelating agents and the results suggest that in certain cases, sub-inhibitory concentrations of the chelators could exacerbate the problem of biofilm formation.

We found that 12.5 mM EGTA and 12.5 mM NaCi were the minimum inhibitory concentrations required to effectively inhibit biofilm formation in 2 out of 4 laboratory strains (Newman and SA113) but augment it the others (10833 and USA300 JE2) (**Figures 8B**, **10**, **17B**). Furthermore, while biofilm formation in 5 out of 27 cardiac device-associated isolates was inhibited by both chelators, it was augmented by them in 8 suggesting that strains that cause biofilm-related infections may be more likely to produce biofilms under a variety of conditions (**Table 3**). These two pieces of data

combined reveal the innate variability that exists between the biofilm components of various, clinical and laboratory, strains. The concentrations of the chelators used in this study were sub-inhibitory, suggesting that they specifically affected the biofilm phenotype. The concentration of NaCi used in this study was lower than the concentrations in catheter lock solutions. However, sub-inhibitory doses would be expected during dialysis as the CLS is removed from the catheter lumen.

NaCi has been shown to prevent biofilm formation but very low doses can augment biofilm formation by serving as a TCA cycle intermediate (Shanks, Meehl et al., 2008; Shanks, Sargent et al., 2006). Therefore, to circumvent this concern and to ensure the results we observed were specifically due to metal ion chelation, we used EGTA in addition to NaCi. Closely related strains Newman and 10833, the focus of this study, were affected diametrically by both compounds, strongly suggesting that the effects were directly related to the sequestration of metal cations, especially Ca^{2+}. Many of the cardiac device isolates exhibited increased biofilm formation in both EGTA and NaCi, however, some were affected differently by the two compounds.

S. aureus biofilms are versatile, in that, they can be composed of varying components including polysaccharides like PNAG (O'Gara, 2007), surface-associated adhesins like the MSCRAMMs or other intercellular adhesin proteins like SasG or SasC (Corrigan, Rigby et al., 2007; Schroeder K, Jularic et al., 2009) and recently, the role for eDNA released from lysed bacterial cells has also been realized (Rice, Mann et al., 2007). Each of these components can play different relative roles depending on the strain of choice and the ever-changing environmental condition. Therefore, observations regarding

the variability between genetically related strains like 10833 and Newman in their capacity clump in chelated solutions (**Figure 11**) and the inherent microscopic differences between the biofilms produced by strain 10833 within the chelated versus non-chelated media (**Figure 12**), prompted the need for an investigation into each of these components. **Figure 13** indicated that 10833 exhibits increased adherence to catheter tubing within EGTA relative to control, which highlights the phenotypic variability among strains of *S. aureus* under various growth conditions such as metal ion chelation. *S. aureus* biofilms produced by strains like SA113 and MN8 appear to rely more heavily on PNAG production. However, 10833 was observed to produce very minimal amounts of this polysaccharide within EGTA indicating that these biofilms are formed independent of PNAG production (**Figure 14A**). The reduction in PNAG production by EGTA is not completely surprising since observations similar to this were previously recorded for the CoNS *S. epidermidis* where EDTA, another chelating agent, was shown to significantly reduce the amount of polysaccharide production (Ozerdem Akpolat, Elci et al., 2003).

Although we conducted preliminary studies on a number of *S. aureus* isolates, we focused our studies on strains 10833 and Newman. Due to their genetic similarity, we hypothesized that the contrasting and opposing effect of chelating agents on biofilm formation in these strains was based on the differential expression of just one or a few genes. Indeed, we found that biofilm formation by 10833 in the presence of EGTA or NaCi required expression of the *clfB* gene (**Figure 15**). Isogenic deletion of *clfB* in 10833 strongly repressed biofilm formation and further weakened the phenotype in strain

Newman in the presence of EGTA but did not affect biofilm formation in the absence of EGTA (**Figure 17A**, **B**). This suggests that, despite a decrease in *clfB* expression in the presence of EGTA in Newman, whatever ClfB is present on the surface of *S. aureus*, contributes to biofilm formation in these conditions. The finding that biofilm formation is unaffected by the *clfB* deletion in TSBG suggests that other biofilm-related factors contribute to biofilm formation in the absence of EGTA but that ClfB is the major determinant of biofilms formed in chelated media. Strain USA300 JE2 and two cardiac device associated isolates- Strains 19, and 24 that were observed to possess increased biofilms with EGTA and NaCi, lost this phenotype when *clfB* mutant constructs were made within these strains (**Figure 18B**, **19**). This reaffirmed our previous finding, highlighting ClfB as the sole surface-associated proteinaceous MSCRAMM that facilitates increased biofilms within the chelated media.

Biofilms produced by *S. aureus* are held together by a mixture of proteinaceous adhesins along with polysaccharides and eDNA. Because so many factors can contribute to the formation of a biofilm, the phenotype may be difficult to target pharmacologically, and agents with antibiofilm activity against certain strains may not inhibit biofilm formation in other strains. It also suggests that a further analysis of the effect of serum on biofilm formation as seen in Chapter 3, could potentially reveal the existence of strains that are able to form biofilms in serum. Strains that were observed to show increased aggregation and biofilm formation (10833 and USA300 JE2) in the presence of EGTA were significantly inhibited in this capacity with the introduction of a polyclonal IgG rabbit α-ClfB antibody (**Figures 20**, **21A**, **21C**). This significant finding would support

the idea of using surface-associated adhesins as potential vaccine candidates. A recent study conducted by Stranger-Jones et al., in which mice were immunized with each of 19 purified MSCRAMM adhesins including ClfB, ClfA and FnbA and then challenged by intraperitoneal infection, showed some degree of protection (Stranger-Jones, Bae et al., 2006).

In sum, *S. aureus* exhibits significant strain-to-strain variability in its ability to form a biofilm under different conditions. When Ca^{2+} is depleted by chelating agents, biofilm formation is abrogated in some strains, whereas it is retained or even augmented in others. ClfB has not been implicated in biofilm formation previously, but this study demonstrates a role for this surface protein in mediating biofilm formation under chelated / Ca^{2+} deplete conditions. Presence of a an EF-hand domain that it can coordinate divalent cations (Ni Eidhin, Perkins et al., 1998) and a putative protease cleavage SLAVA motif (McAleese, Walsh et al., 2001) within ClfB suggests regulation of ClfB may occur on multiple levels involving protein stability and activity. The regulation of the *clfB* gene will be discussed further in the next chapter.

CHAPTER 5

Regulation of Clumping factor B (ClfB) is mediated by calcium (Ca^{2+})

Introduction

It is estimated between 250,000 – 500,000 infections related to intravenous catheter use, 1-6 per 1,000 catheter days, occur every year in the U.S. (Crnich and Maki et al., 2005; Maki, Kluger et al., 2006; Ramos, Reitzel et al., 2010). *S. aureus* is one of the most common isolates (20%) and is responsible for a greater associated health-cost than any other species (Walz, Memtsoudis et al., 2010). Such infections occur, in part, because *S. aureus* forms resilient biofilms on the surface of the catheter. Intraluminal catheter lock solutions (CLS) composed of chemical chelators like trisodium citrate (NaCi), Ethylenediaminetetraacetic acid (EDTA), and Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), have been traditionally used to serve as effective anti-coagulants but also have inhibitory effects against bacterial growth and biofilm formation (Mermel, Farr et al., 2001; O'Grady, Alexander et al., 2002; Raad, Costerton et al., 1993; Arakawa, Saito et al., 2000; Raad and Hanna, 2002; Weijmer, Debets-Ossenkopp et al., 2002). Surprisingly, however, we have identified the prevalence of *S. aureus* strains that counter the inhibitory effects of chelators and actually form stronger biofilms in the presence of these agents. The molecular basis for this

"antibiofilm resistance" in strains was observed to depend upon the surface-associated fibrinogen-binding MSCRAMM clumping factor B (ClfB) (Chapter 4).

As described schematically in **Figure 23,** Clumping factor B is a large 150 kDa protein that bears a C-terminal LPETG motif which is used to covalently anchor the MSCRAMM to the cell wall peptidoglycan via a reaction catalyzed by sortase A (Schneewind, Mihaylova-Petkov et al., 1993). The A or ligand binding domain of this protein, mediates bacterial adherence to immobilized fibrinogen, blood clots, conditioned biomaterial *ex vivo*, and thrombin-damaged heart valves in a rat model of infective endocarditis (Vaudaux, Francois et al., 1995; McDevitt, Francois et al., 1994; Entenza, Foster et al., 2000; Francois, Schrenzel et al., 2000; Ni Eidhin, Perkin et al., 1998; O'Brien, Walsh et al., 2002). The A domain contains a short SLAVA motif, which is cleaved by the *S. aureus* metalloprotease- aureolysin (McDevitt D, Francois et al., 1994; McAleese, Walsh et al., 2001). Furthermore, the A region also contains a non-consensus abbreviated EF-Hand domain that is known to bind calcium ions (Ca^{2+}) specifically to promote fibrinogen binding (Ni Eidhin, Perkin et al., 1998). That said, it was rational for us to hypothesize the following: strains of *S. aureus* like 10833 that promoted biofilm formation within chelating agents utilize this domain to facilitate intercellular aggregation and clumping, which aid in establishing a biofilm. In contrast to ClfA, *clfB* is expressed *in vitro* during exponential growth (McAleese, Walsh et al., 2001). However, the specific molecular mechanisms that control its expression, stability, and activity, especially within biofilm formed within chelating agents, remains to be elucidated; as does the molecular basis for the phenotypic disparity between in EGTA or NaCi between strain pairs such as

Figure 23. Schematic representation of ClfB. Signal sequence (S), ligand binding domain (A), serine-aspartate repeat region (SD-Repeat), cell wall spanning domain (W), and the membrane-anchoring domain (M). The black box at residue 310 is the abbreviated EF-Hand domain. The shaded blue box is a proline rich region that separates the A domain from the SD Repeat region. The SLAVA motif spans residues 197 through 201, and the cell wall anchoring LPETG motif spans residues 874 to 878. The aureolysin cleavage site within the SLAVA motif, between residues Leu198 and Ala199, is shown (red arrows).

Newman and 10833.

S. aureus virulence factors including biofilm formation, expression of surface-associated and secreted proteins, and quorum sensing is controlled by the major global regulatory Agr system. The *agr* locus contains two divergent promoters, P2 and P3, which encode the RNAII and RNAIII transcripts respectively. RNAIII is the primary effector molecule of the *agr* response and is responsible for upregulating extracellular proteases and exoenzymes including *hla* (α- toxin) (Morfeldt, Taylor et al., 1995; Oscarsson, Kanth et al., 2006) and *sspA* (serine proteinase) (Oscarsson, Tegmark-Wisell et al. 2006), and downregulating of cell wall associated proteins, such as the fibronectin-binding proteins (FnbA and FnbB) and *spa* (protein A) (Novick, Ross et al., 1993) during the post-exponential phase of growth. The Agr system controls the size of the developing biofilm, and serves as a mechanism of biofilm dispersal, when the bacterial population reaches a certain density or "quorum" within a confined space.

Proteases are important virulence factors for a variety of microbial pathogens and may contribute to tissue degradation and resistance to the host defense system. *S. aureus* secretes 4 major extracellular proteases: staphylococcal serine protease (V8 protease) (SspA), a metalloprotease named aureolysin (Aur), a cysteine protease (ScpA)-staphopain, and a second cysteine protease (SspB) encoded within the same operon as SspA (Arvindson, 2000; Chan and Foster, 1998; Hofmann, Schomburg et al., 1993; Rice, Peralta et al., 2001). Aureolysin is a Ca^{2+} stabilizing, zinc (Zn^{2+})-dependent member of the thermolysin family of Zn^{2+}-dependent metalloproteases. Coordinaton of the one Zn^{2+} ion contributes to the catalytic pocket of the protease while binding of three

Ca^{2+} helps stabilizes the protease and augments its proteolytic activity. Little is known about the exact role of aureolysin in the pathogenicity of *S. aureus*. Recently though, aureolysin was shown to cleave ClfB at the SLAVA motif (**Figure 22**), shedding the molecule into the growth medium thereby explaining the inability of bacteria from stationary phase to bind to fibrinogen in a ClfB-dependent manner (McAleese, Walsh et al., 2001).

In eukaryotic cells, study of Ca^{2+} as a second messenger molecule has long been documented. An equally important role for this ion has been much harder to demonstrate in prokaryotes but is now becoming evident. Within prokaryotes, Ca^{2+} has been implicated in regulation of various cell processes including cell differentiation and spore formation in *Bacillus subtilis* (O'Hara and Hageman, 1990; Fry, Becker-Hapak et al., 1991), chemotaxis (Tisa, Olivera et al., 1993; Tisa and Adler, 1995; Matsushita, Hirata et al., 1988) and regulation of cell cycle processes like chromosome segregation and replication in *E. coli* (Botello and Nordstrom,1998) and adhesion and aggregation by dental plaque causing *Streptococcus mutans* (Rose, Dibdin et al., 1993). More recently, Ca^{2+} has also been shown to play a role in biofilm formation and PIA production in *P. aeruginosa* and *S. epidermidis* respectively (Sarkisova, Patrauchan et al., 2005; Ozerdem Akpolat, Elci et al., 2003; Banin, Brady et al., 2006). The divalent cation stimulates cell-cell adhesion and thus promotes aggregation of the organisms adhering to a finite surface, essential steps towards establishing a successful biofilm. More recently, alternative means of regulation by Ca^{2+} have been documented- acting in a primary extracellular fashion rather than a second messenger. Regulation of these proteins in such a fashion is

mediated through the presence of a Ca^{2+}-binding EF Hand domains. *S. aureus* proteins like ClfA, ClfB and Bap (biofilm-associated protein) bear similar EF-Hand domains and their innate functional capacity including binding to fibrinogen (ClfA and ClfB) or their multicellular biofilm formation (Bap), is severely hindered if not abolished completely when Ca^{2+} is bound to these sites (O'Connell, Nanavaty et al., 1998; Ni Eidhin, Perkin et al., 1998, Arrizubieta, Toledo-Arana et al., 2004)

Transcriptional regulation of *clfB* by major regulators Agr or SarA has only prevailed with contrasting knowledge. According to previous studies, it has been accepted that the extracellular surface-associated protein genes including *entA*, *cna*, and *clfA* are not regulated by *agr* (Novick, 2003). One study performed in *S. aureus* NCTC8325-4 indicated that much more ClfB was present in the *agr* mutants than in wildtype cells, suggesting that ClfB expression was negatively regulated by the Agr system (Frees, Sorensen et al., 2005). In addition, another study performed in *S. aureus* Newman suggested that mutation in neither *sarA* nor *agr* affected *clfB* transcription when measured by *lacZ* transcriptional fusions (Wolz, McDevitt et al., 1996). The goal of this study was to elucidate whether a post-translational level of regulation of ClfB- affecting protein stability and activity, especially during biofilm formation under chelating conditions occurred thereby augmenting biofilm formation in strains like 10833. Results from this study revealed ClfB regulation occurred through the independent or synergistic activities of the metalloprotease aureolysin and Ca^{2+}-binding to the EF Hand domain of the ClfB protein. This level of regulation shed light on how ClfB exhibited the diametric biofilm properties by differing strains like 10833 and Newman when cultured within

chelating agents like EGTA or NaCi.

Results

Agr expression is stronger within Newman than 10833

With the aim of investigating the differential biofilms phenotypes between strain pairs 10833 and Newman, we explored the role of Agr and its contribution within chelator based biofilms. The *agr* locus was shown to downregulate *S. aureus* adherence to fibrinogen and *agr* mutants showed increased adherence to immobilized fibrinogen and induction of bacterial aggregation (Shenkman B, Rubinstein et al., 2001). We therefore postulated that there could be a differential level of the Agr activity within these two strains. RNAIII, the major effector molecule of the *agr* response, is responsible for upregulating various extracellular proteases and exoenzymes including *hla* (α- toxin) (Morfeldt, Taylor et al., 1995; Oscarsson, Kanth et al., 2006) and *sspA* (serine proteinase) (Oscarsson, Tegmark-Wisell et al. 2006). It could therefore inhibit retention of proteins like ClfB on the surface of the cell or it could directly affect transcription of *clfB*.

S. aureus produces several membrane-damaging toxins that lyse various types of host cells. The most prominent examples of single-component toxins include the α-(*hla*), β-(*hlb*), and δ-toxins (*hld*). The δ-toxin gene (*hld*) is encoded within the RNAIII effector molecule. δ-Toxin can lyse a wide variety of mammalian cells including erythrocytes. Therefore, hemolytic activity on blood agar plates can serve as a measure of the Agr activity because δ-hemolysin (*hla*) is encoded within the agr locus. We tested a panel of strains for hemolytic activity by streaking them on Brucella blood agar (containing

sheep's blood) and allowing them to grow at 37 °C for 18 h (**Figure 24A**). RN450 and RN450 Δ*agr* serve as our positive and negative controls respectively. RN450 presented strong hemolytic activity represented by the zone of clearing around the bacterial streak. Though closely related, we observed Newman to exhibit stronger hemolytic activity than 10833. Hemolytic activity by 10833 was very similar to that displayed by RN450Δ*agr*.

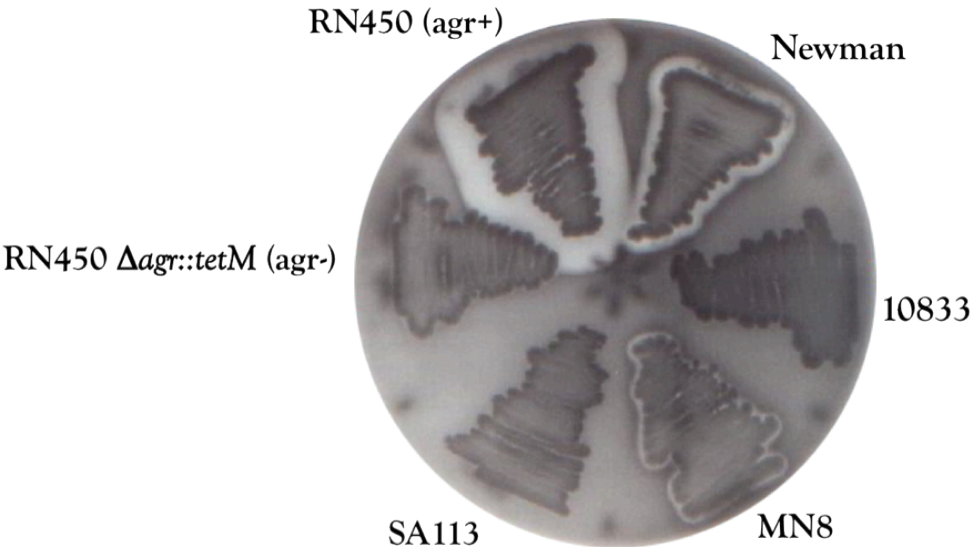
To confirm these results, total RNA was isolated from 10833 and Newman grown within chelated and non-chelated media and quantitative realtime RT-PCR analysis of the RNAIII transcript levels, encoded within the *agr* locus, was determined (**Figure 24B**). Under both media conditions tested, Newman displayed greater RNAIII transcript levels versus that in 10833. This lead us to hypothesize the increased protease activity, associated with increased expression of Agr, could prevent strains like Newman from retaining ClfB on the surface and thus explaining the decreased biofilm phenotype of this strain within chelated media.

Deletion of *agr* does not restore Newman biofilms within EGTA

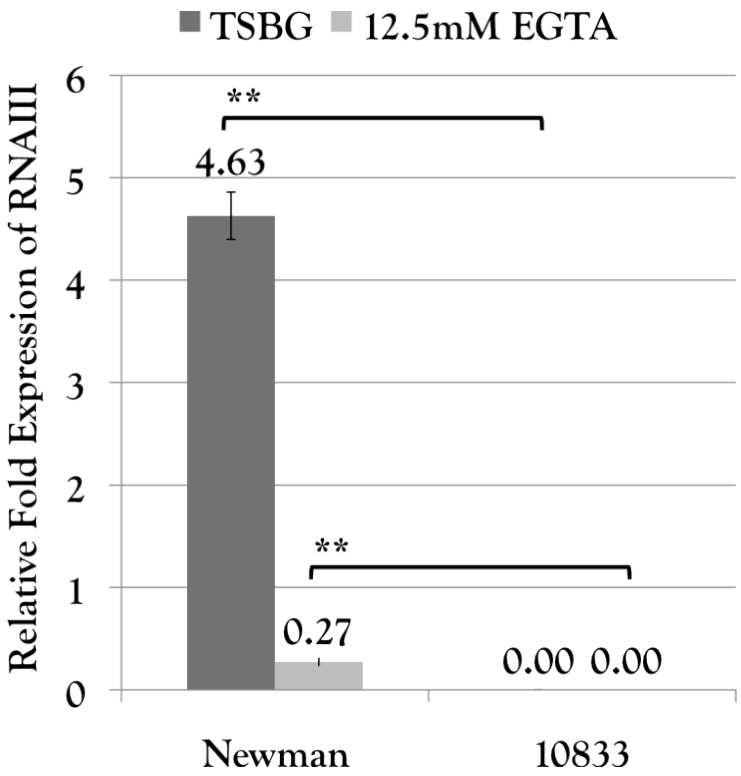
Identifying increased expression and hemolytic activity of the *agr* locus within Newman, we decided to delete the *agr* locus from Newman and thereafter assess whether loss of this locus, and its potential downstream effects, could restore biofilm formation of Newman within chelated media. Deletion mutation of *agr* within Newman was generated by transducing the mutation from the RN450Δ*agr::tetM* strain, utilizing phage 80α. The resultant transductant colonies (Newman Δ*agr::tetM*) were picked and rescreened on tetracycline plates. To confirm the loss of the hemolytic activity, contributed by δ-toxin

Figure 24. Expression and activity of Agr by 10833 and Newman. (**A**). *S. aureus* strains SA113, MN8, RN450, RN450Δ*agr::tetM*, Newman and 10833 were streaked onto Brucella blood agar plate containing sheep's blood. A zone of clearing around the bacterial streak is a positive test for hemolysis. (**B**) Relative expression of RNAIII was determined by isolating total RNA from Newman and 10833 grown to late exponential phase (5 h) within TSBG or TSBG + 12.5 mM EGTA. Quantitative realtime PCR analysis was performed and the relative fold expression of the RNAIII transcript, normalized to the 16S rRNA, is shown graphically. $^{**}P<0.0001$ as determined by Student's t-test.

A.



B.

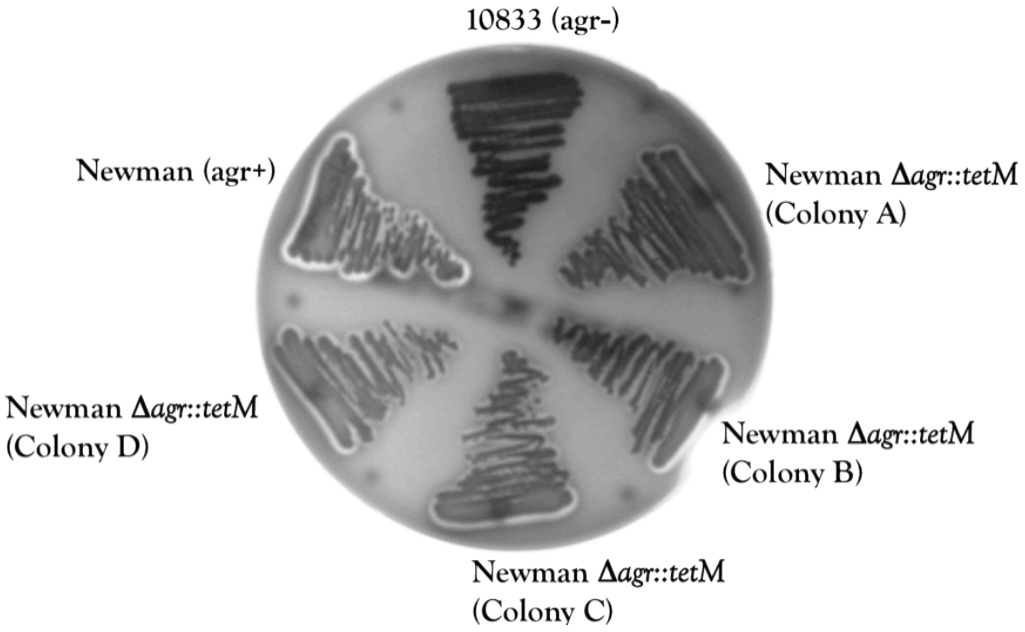


gene (*hld*), four transductants (Colonies A-D), along with 10833 and Newman wildtype, were streaked onto Brucella blood agar (**Figure 25A**). Compared to our wildtype Newman strain (positive control) the various isogenic *agr* mutant transductants relieved the strength of hemolysis. Compared to 10833 (negative control), the *agr* mutants continued to show residual amounts of hemolytic activity. This can be explained knowing deletion of *agr* only represses the δ-hemolytic activity; the other hemolytic genes [α-(*hla*), β-(*hlb*) and γ-(*hlgA, hlgB, hlgC*) toxins] could contribute to the trace hemolytic activity observed. Thus, though they do not compare exactly to 10833, there was a decrease in the amount of hemolysis produced by the NewmanΔ*agr* transductants with Colony A showing the most significant amount of repression of this phenotype.

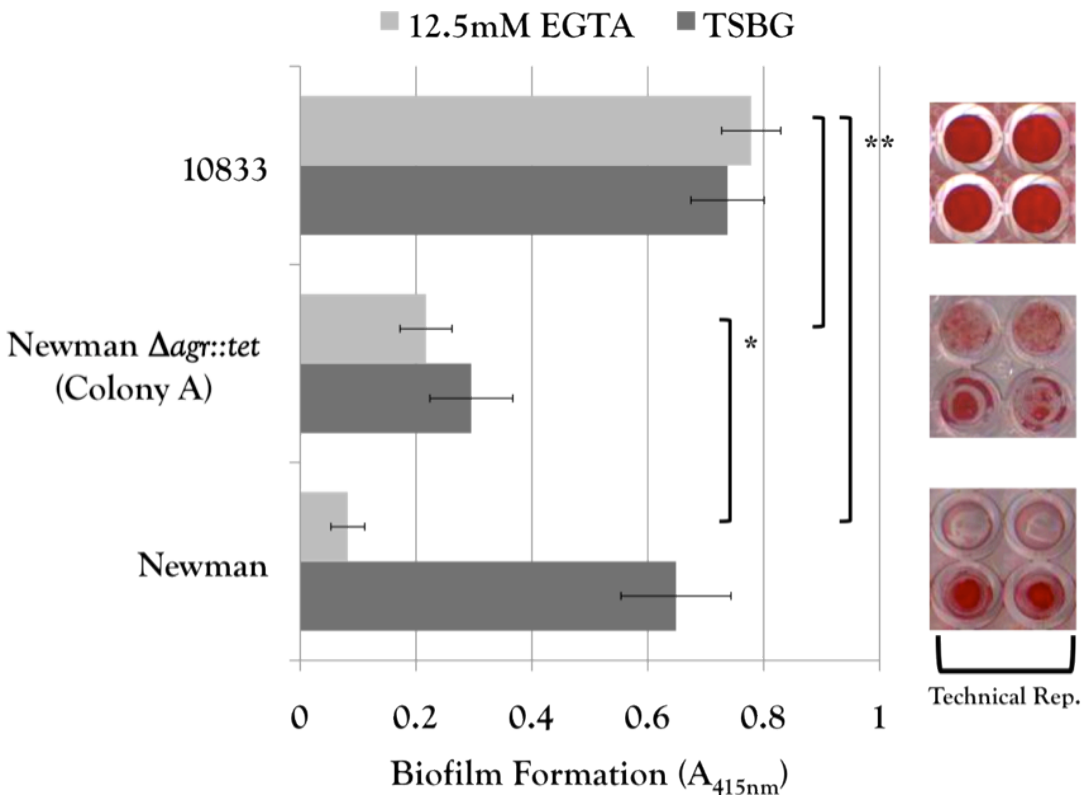
To determine whether *agr* inhibits Newman from forming biofilm within chelated media, we assayed the newly acquired *agr* mutant for biofilm formation under chelating and non-chelating conditions (**Figure 25B**). Compared to the wildtype, deletion of *agr* lead to a decrease in biofilm formation in TSBG. However, NewmanΔ*agr* showed a 13% increase in biofilm formation within EGTA ($P<0.01$). Nonetheless, these biofilms within EGTA were still significantly lower than what has been observed with 10833 biofilms in the same chelated conditions. In sum, while a direct role for Agr was not determined by this study, results suggest that the *agr* locus may play an indirect role in mediating biofilm formation under these Ca^{2+} depleted/chelated conditions- possibly through the increased expression and activity of secreted proteases.

Figure 25. Deletion of *agr* restores Newman biofilms minimally. (**A**) Phage transductants (Colonies A – D) of NewmanΔ*agr::tetM* were streaked on Brucella blood agar plates, along with wildtype Newman and 10833, to confirm loss of the *agr* locus through depreciated hemolytic activity. Colony A showed the most, visually discernible decrease within hemolytic activity. (**B**). Newman Δ*agr::tetM* (Colony A) was tested for augmented biofilms within chelated media and compared to wildtype Newman and 10833 by diluting overnight cultures of strains in TSBG or TSBG + 12.5 mM EGTA, added to wells of a 96-well plate and assayed for biofilm formation. The biofilms were washed for non-adherent bacteria and stained and quantified for any detectable biofilm. The experiment was performed in biologic triplicate and each triplicate contained four technical replicates for statistical analysis. * $P<0.01$ and ** $P<0.0001$ as determined by Student's t-test.

A.



B.

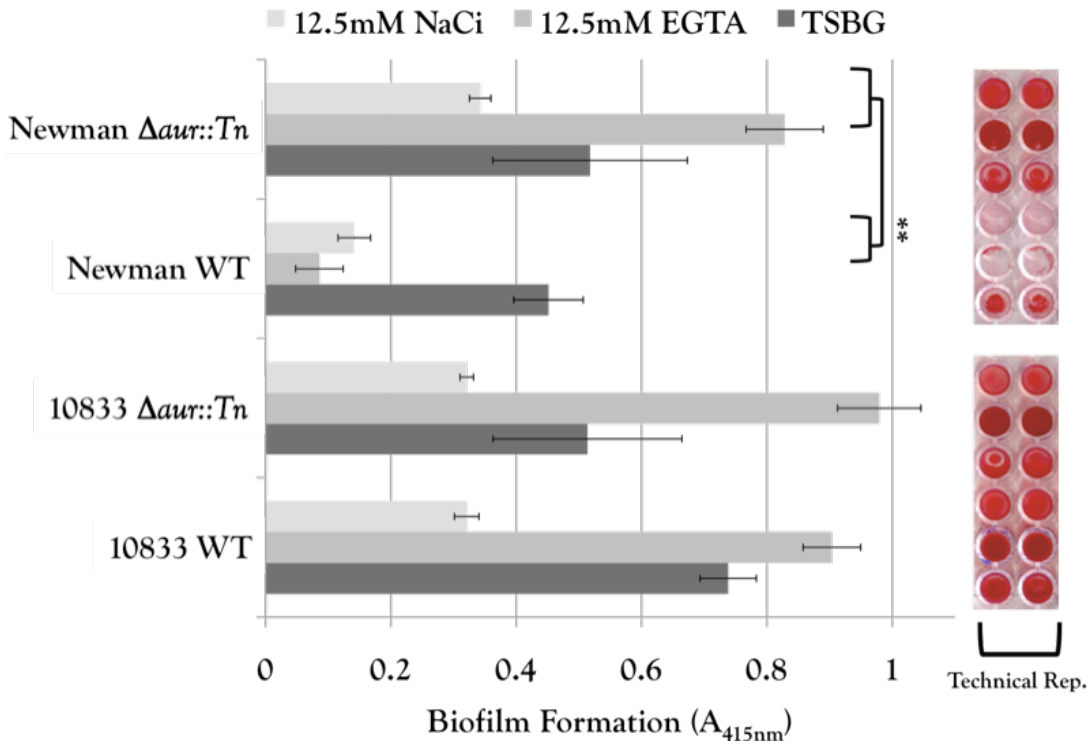


Aureolysin deletion augments Newman biofilms

Early work performed on identifying the regulatory network associated with ClfB identified a role for the protease aureolysin (McAleese, Walsh et al., 2001). Aureolysin cleaves ClfB between Ser197 and Leu198 within the SLAVA motif, located within Region A of the protein, thereby preventing the protein from binding immobilized fibrinogen (McAleese, Walsh et al., 2001; Perkins, Walsh et al., 2001) (**Figure 22**). Additionally, previous observations showing a possible role for *agr* mediated regulation of biofilm through increased protease activity provided the necessary leverage to determine the role aureolysin played in mediating biofilms within chelated media. Aureolysin is a Zn^{2+} dependent metalloprotease and is stabilized by three Ca^{2+} ions. Its activity would therefore be expected to respond to chelating agents and could therefore be involved in regulating biofilm formation in the presence of chelators. To test this hypothesis, we phage transduced the transposon insertion mutation of *aur* from the Nebraska Tn Library strain NE163, into wildtype 10833 and Newman and tested for biofilm formation under chelating and non-chelating conditions (**Figure 26**).

Deletion of *aur* from 10833 revealed similar results as the wildtype strain, biofilm formation being augmented under chelating conditions. If aureolysin was responsible for cleaving ClfB from the surface of cells then the aureolysin mutant should be rendered ineffective against biofilm production within strains like 10833 where ClfB is the predominant proteinaceous adhesin promoting this process. Surprisingly, in contrast with wildtype Newman where ablated or diminished biofilms were detected, deletion of *aur* from this strain reversed the biofilm phenotype under chelating conditions. Deletion of

Figure 26. Aureolysin deletion mutant fully restores Newman biofilms within chelated media. The *aur* transposon insertion mutation was phage transduced from strain NE163 into 10833 and Newman and tested for biofilm formation with TSBG or TSBG + 12.5mM EGTA or TSBG + 12.5 mM NaCi. Diluted cultures of the wildtype or isogenic mutant strain were inoculated into wells of a 96-well plate and grown for biofilms. Safranin stained biofilms (right) were quantified at A_{415nm} (left). Statistical data was obtained from three biologic replicates; each with four technical replicates. $^{**}P<0.0001$ as determined by Student's t-test.



Newman *aur* increased biofilm formation significantly within EGTA and NaCi (P<0.0001); similar to what was observed with 10833 and its isogenic *aur* mutant. These results suggest a role for the aureolysin protease playing a role in increased biofilms within chelated media and potentially cleaving ClfB, within that putative protease recognition SLAVA motif, preventing ClfB-dependent biofilms within chelated media from forming. No observable difference between wildtype and the *aur* mutant of 10833 suggests variable aureolysin activity in this strain versus that in Newman, which could account for the opposing biofilm phenotypes that exists between the related strains.

Synthesized aureolysin disperses established *S. aureus* biofilms

If our hypothesis is correct, that aureolysin is responsible for the lack of biofilm formation in strain Newman under chelating conditions and that the biofilm positive phenotype in 10833 is due to reduced production of aureolysin, then we should be able to add aureolysin exogenously to 10833 and to the *aur* deletion mutants and reduce biofilm formation in the presence of EGTA. Thus, we next investigated the role aureolysin played in specifically dispersing *S. aureus* pre-established biofilms. *S. aureus* strains 10833, 10833Δ*aur::Tn*, and Newman Δ*aur::Tn*, the only strains that permit biofilm formation within chelated media, were grown for biofilms within TSBG or added 12.5 mM EGTA. Established biofilms were thereafter washed to remove non-adherent bacteria, and treated with varying concentrations of recombinant aureolysin synthesized from *S. aureus* strain V8-bc10, diluted in deionized water to prevent any added growth, and allowed to incubate for another 18 h stationarily. The biofilms were observed

visually and quantified for retention of the pre-established biofilm (**Figure 27**).

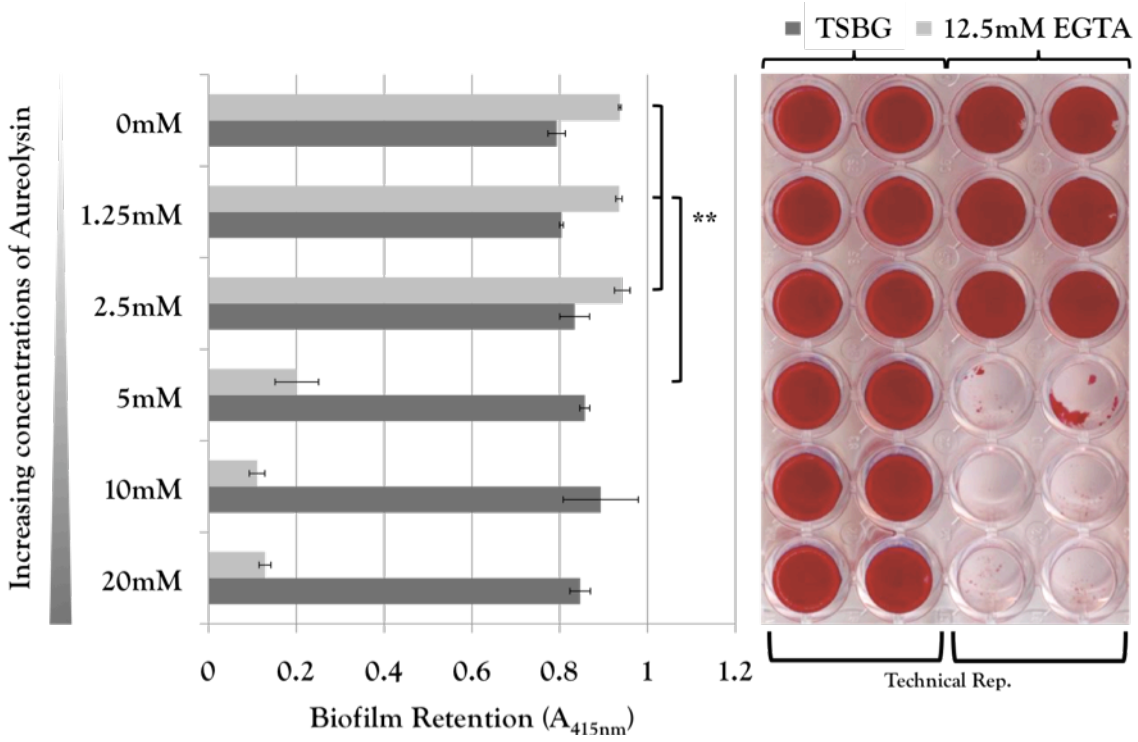
Strain 10833 (**Figure 27A**) displayed adherent biofilms when grown, in either TSBG alone or with 12.5 mM EGTA, in the absence of any aureolysin. However, increasing concentrations of aureolysin promoted dispersion of the retained biofilm within EGTA. Concentrations ≥5 mM aureolysin prevented any detectable biofilm from being retained within EGTA ($P<0.0001$). In contrast, biofilms pre-established within TSBG continued being retained at all concentrations of the protease tested. This reinforces the concept that while 10833 biofilms formed in TSBG are likely composed of various components including exopolysaccharide and other surface-associated proteinaceous adhesins, biofilms produced within chelated media are largely ClfB-dependent. Additionally, this confirmed aureolysin protease cleaving ClfB from the surface of the cells thereby preventing intercellular aggregation and biofilm retention. Strains 10833Δ*aur::Tn* (**Figure 27B**) and Newman Δ*aur::Tn* (**Figure 27C**) showed identical trends as wildtype 10833, that is, dispersion of any retained biofilm within EGTA with ≥5 mM aureolysin. The use of Newman Δ*aur::Tn* was an important control not only showing the importance of aureolysin in cleaving ClfB from the surface but the biofilms produced by this isogenic mutant reaffirms the evolving hypothesis that variability does exist between 10833 and Newman aureolysin.

Newman aureolysin confers the biofilm-negative phenotype on 10833 in EGTA

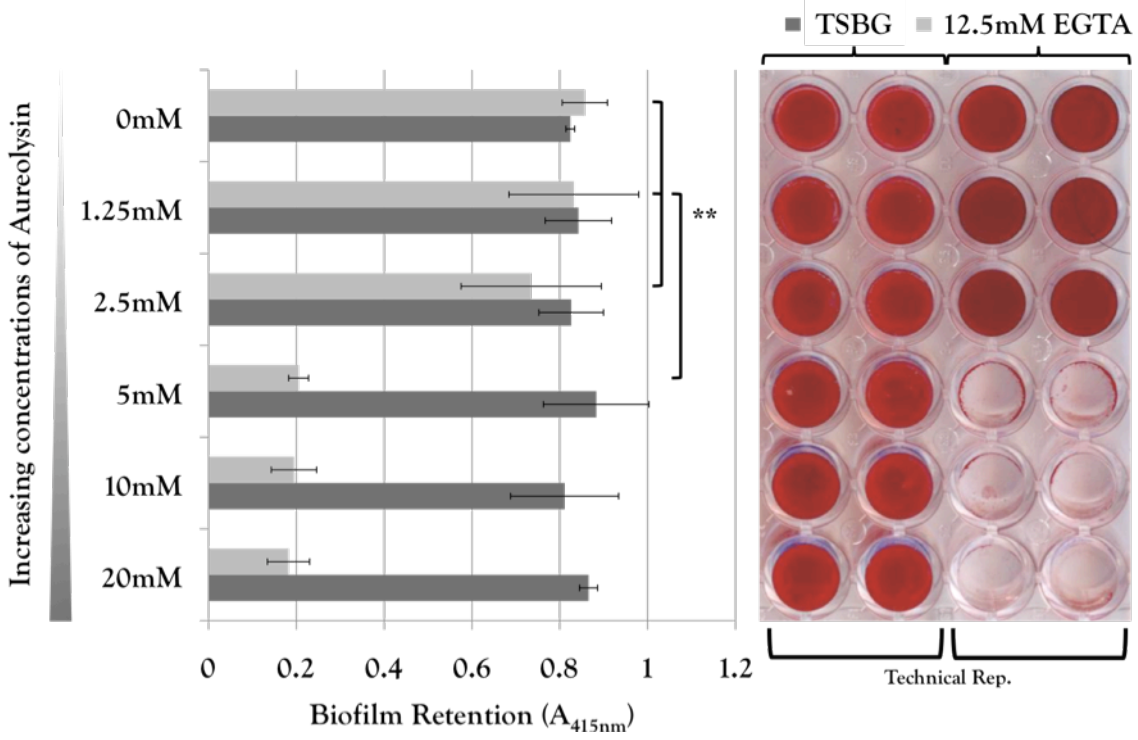
Thus far we have identified the metalloprotease aureolysin as being responsible for cleaving ClfB from the surface of cells thereby preventing cells from aggregating and,

Figure 27. Dispersion of pre-established chelator induced biofilms is mediated by aureolysin. Biofilms pre-established by *S. aureus* strains 10883 (**A**), 10833 Δ*aur::Tn* (**B**), and Newman Δ*aur::Tn* (**C**) within TSBG or TSBG + 12.5 mM EGTA were treated with varying concentrations (0 – 20 mM) of synthesized aureolysin recombinant protein, diluted in water, and incubated for 18 h at 37 °C. Dispersed or retained biofilms were stained with safranin and imaged (right). Quantified assessment of the biofilms was generated by dissolving the stain in acetic acid and measuring at A_{415nm} (right). Three biologic replicates, each with four technical replicates was used to generate the statistical analysis. $^{**}P<0.0001$ as determined by Student's t-test.

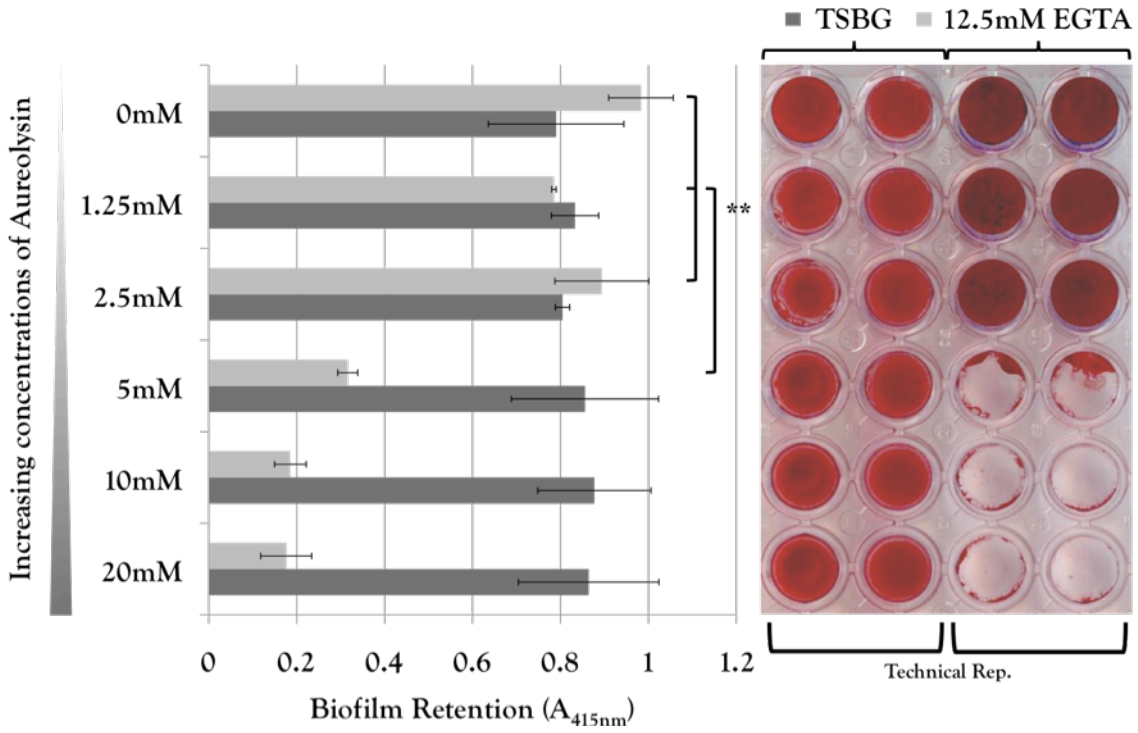
A. Strain: 10833 WT



B. Strain: 10833 Δ*aur::Tn*



C. Strain: Newman Δ*aur::Tn*



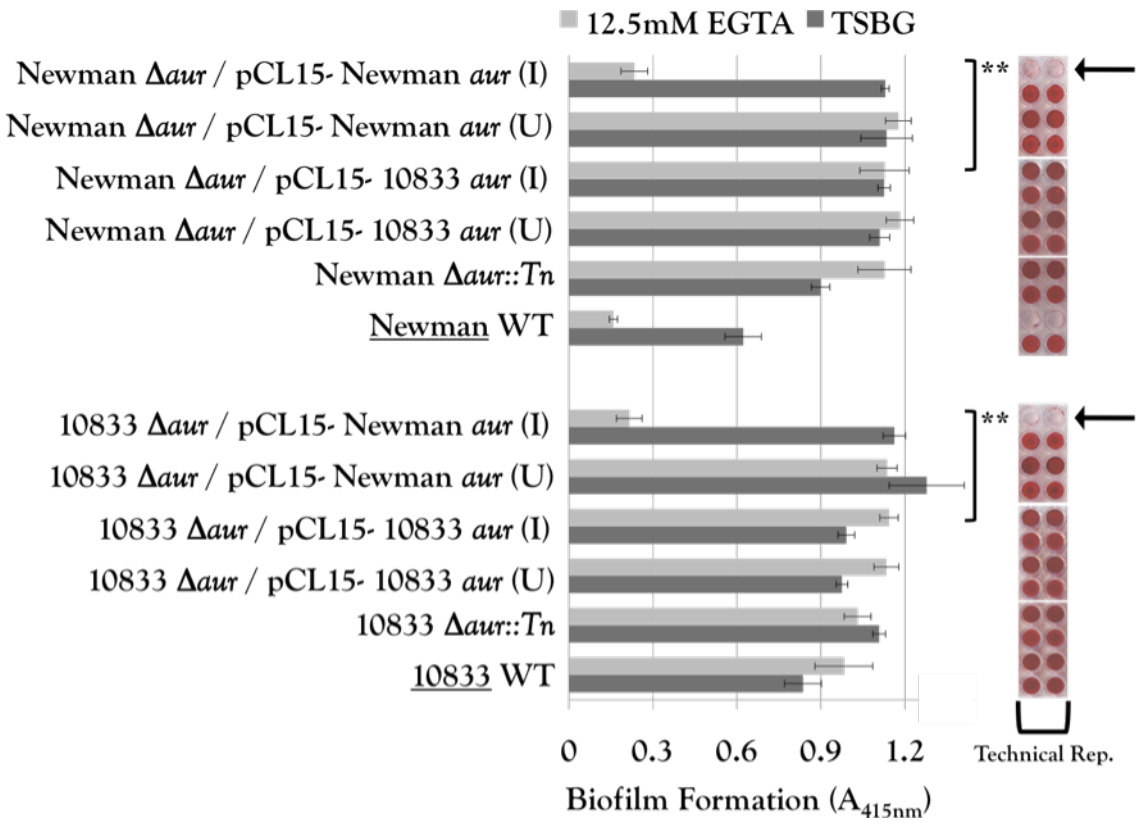
in turn, developing into biofilms. However, the opposing biofilm phenotypes exerted by 10833 and Newman within chelated media; augmented biofilms by 10833 and repressed or ablated phenotype within Newman, suggested variability between the native aureolysin activity between 10833 and Newman. Increased activity of the protease within Newman could prevent biofilm formation of this strain within chelated media. Therefore, to specifically identify a differing level of activity of this protease between the two strains we used the IPTG inducible expression vector pCL15 to complement either the 10833 or the Newman aureolysin gene (*aur*) into each of the isogenic *aur* deletion strains and thereafter test for biofilm formation (**Figure 28A**).

Biofilm assay of 10833Δ*aur* and NewmanΔ*aur* complemented in trans with the 10833-aureolysin gene formed strong biofilms in EGTA. In contrast, complementation of the *aur* deletion mutants with the Newman aureolysin gene not only prevented biofilm formation within the Newman background but also barred biofilms within strain 10833 in EGTA. These results suggest that a significant variability exists between the aureolysin proteins of 10833 and Newman. Newman aureolysin disallowed biofilm formation of either strain in EGTA, whereas 10833 aureolysin did not.

To confirm these results, we performed aggregation and clumping assays using these deleted and complemented strains and determined if complementation with Newman aureolysin would prevent clumping of bacteria when static cultures were grown in TSBG or added 12.5 mM EGTA (**Figure 28B**). The top panel of this figure shows aggregation of the bacteria within the 10833 background. Deletion of *aur* from 10833 was observed to promote a strong clumping phenotype similar to wildtype within the

Figure 28. Variability within the aureolysin protease activity between Newman and 10833 affects biofilm formation and aggregation. (**A**) To highlight the variability that exists between the native Aur activity between 10833 and Newman, strains were complemented with either 10833 or Newman *aur* gene within their respective *aur* mutant background and tested for biofilm formation within chelated versus non chelated conditions. Complementation constructs were induced (I) in the presence of 1 mM IPTG. Uninduced samples (U) were not supplemented with any IPTG. Statistical analysis was performed using three independent biologic replicates, each containing four technical replicates. The arrow highlights the effectiveness of complementation in trans with the Newman-*aur* gene. ***P*<0.0001 as determined by Student's t-test. (**B**) Aggregation and clumping assay was performed to confirm the biofilm phenotype observed in (**A**). Wildtype, *aur* mutant and *aur* complementation strains of 10833 and Newman were grown in test tubes with either TSBG or TSBG + 12.5 mM EGTA and allowed to incubate stationarily for 18 h at 37 °C. The tubes were vortexed briefly and then imaged. Complemented strains were induced with the addition of 1mM IPTG. The red circle highlights a clumped phenotype observed within the volume of the liquid.

A.



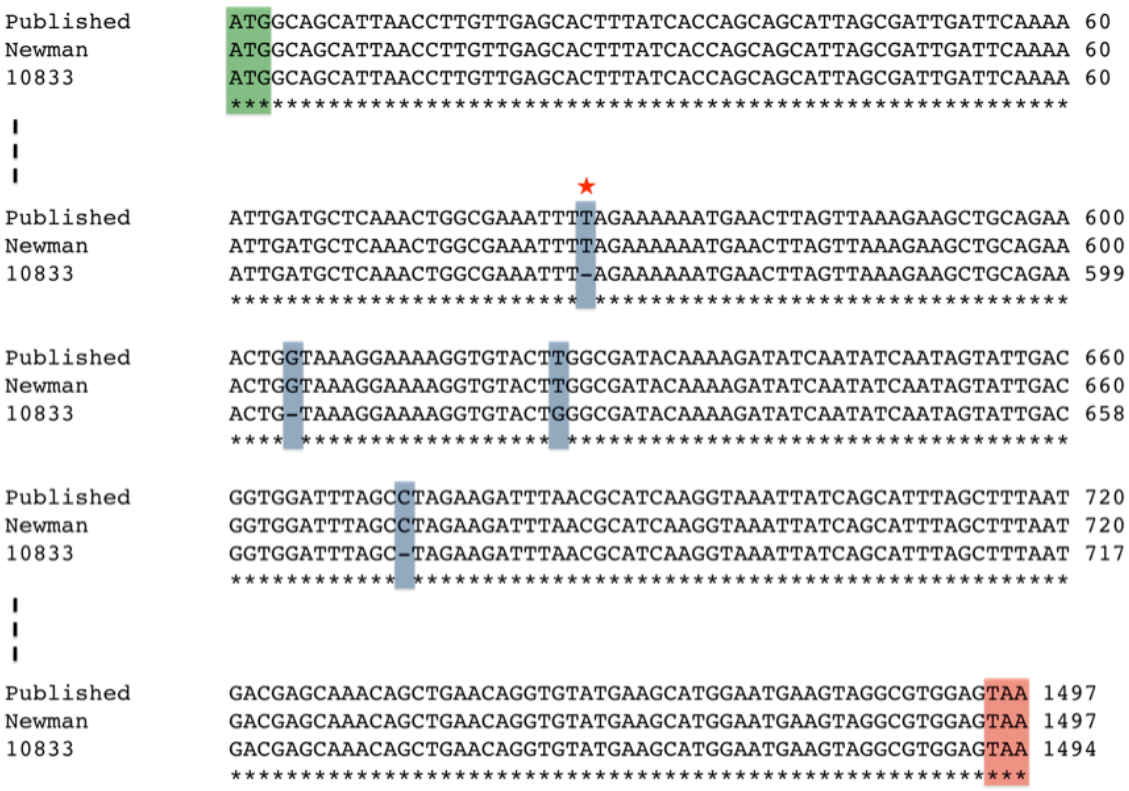
B.

chelated condition. Clumping was also observed when 10833 *aur* mutants were complemented with its native copy of *aur*. However, complementation with Newman *aur* prevented any aggregation of the bacteria. Aggregation assays with the Newman background (bottom panel, **Figure 28B**) revealed clumping of bacteria within the *aur* mutant and loss of this phenotype when the *aur* mutant of Newman was complemented with the Newman-*aur* gene. Taken together, we confirmed the variability in the aureolysin activity between 10833 and Newman- Newman possess the aureolysin protease in a fully active and functional form that is sufficient to cleave ClfB from 10833 preventing this strain from inducing biofilms within chelated media.

10833 does not encode functional aureolysin

To confirm the phenotypic effects of Newman aureolysin we sequenced the *aur* gene from 10833 and Newman and compared these sequence results to the *aur* published sequence from strain Newman (Baba, Bae et al., 2008) (**Figure 29**). Two sets of primers were designed to provide full coverage of the *aur* gene. Sequence results from Newman *aur* revealed 100% identity and similarity to the published aureolysin sequence. Analysis of 10833 *aur* showed 100% identity to the region upstream of the protein coding open reading frame. However, 10833 *aur* protein coding sequence (1493 bp) contained 3 deletion mutations relative to Newman *aur* (1497 bp). The nucleotides at positions 565, 604, and 671 were absent in the 10833 *aur* sequence and there was one substitution mutation at nucleotide 623. Deletion of thymine 565 resulted in a frame shift of the protein coding sequence resulting in an inframe TAG (DNA) or UAG (RNA) that

Figure 29. Sequence analysis of the 10833 *aur* gene reveals insertion of a premature stop codon. The aureolysin (*aur*) gene was amplified from 10833 and Newman genomic DNA sequenced and compared to the published protein coding *aur* sequence from Newman (Published). The green and red box highlights identical start and stop codons across all three sequences. The blue boxes highlight the 4-nucleotide polymorphisms-three deletions (at nucleotides 565, 604, and 671) and one substitution (at nucleotide 623) that exists between 10833 and the sequenced or published Newman *aur*. 100% identify was observed between the Newman *aur* sequenced and the published Newman *aur* sequence. The dashed lines signify the intermediate sequence between all three that were 100% identical. The red star identifies the deleted nucleotide (thymine 565) from 10833 that causes a frame shift in the open reading frame resulting in the insertion of a premature stop codon.



encodes for a stop codon. Thus, the 10833 *aur* protein coding sequence would not be expected to encode a functional aureolysin protein. These results were highly significant and confirmed previously observed phenotypic data showing 10833 aureolysin having lower protease activity versus that from Newman.

EF-Hand domain and calcium mediated regulation at the level of ClfB protein activity

While aureolysin may account for the differences between strains in their capacity to form biofilms within chelated media, we also aimed to investigate the role of the helix-loop EF-Hand domain that has been characterized to bind Ca^{2+} very specifically. Several lines of evidence have implicated Ca^{2+} in the regulation of various bacterial processes including chemotaxis, cell differentiation, and adherence and aggregation (O'Hara and Hageman, 1990; Fry, Becker-Hapak et al., 1991; Tisa, Olivera et al., 1993; Tisa and Adler, 1995; Matsushita, Hirata et al., 1988; Rose, Dibdin et al., 1993). These domains, within *S. aureus*, have been identified to regulate processes like intercellular aggregation, adherence to host proteins, and biofilm formation (O'Connell, Nanavaty et al., 1998; Arrizubieta, Toledo-Arana et al., 2004). Clumping factor B has an EF-hand domain, suggesting that it can coordinate divalent cations, and its autoaggregative activity and capacity to bind fibrinogen is inhibited by Ca^{2+} and Mn^{2+} (Ni Eidhin, Perkin et al., 1998). Therefore, an additional level of regulation, affecting protein activity, would be suspected.

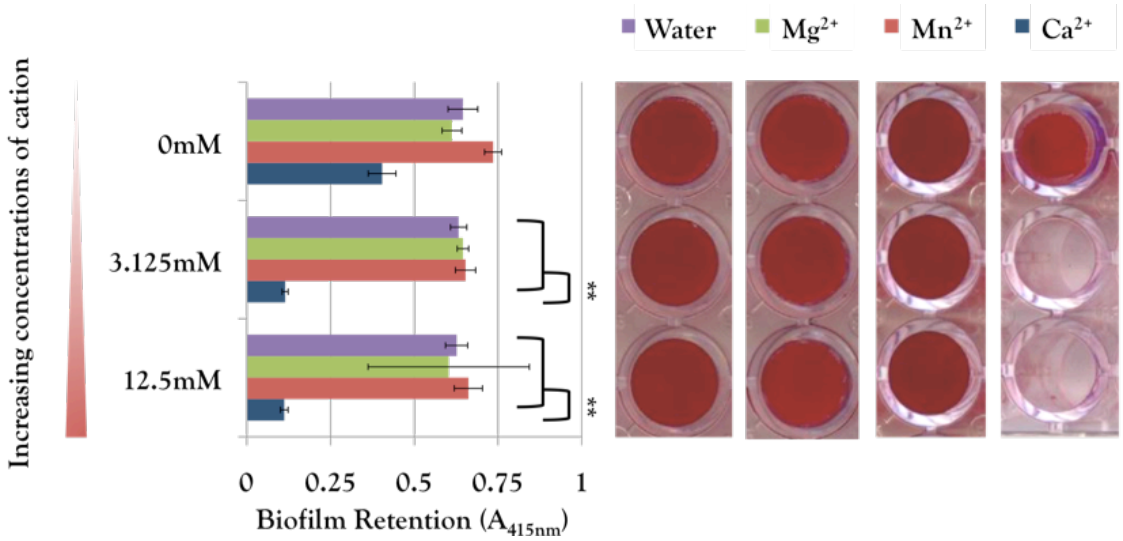
To assess the role for divalent cations like Ca^{2+} or Mn^{2+} in dispersing biofilms we

performed dispersion assays where 10833 biofilms were pre-established in 12.5 mM EGTA, and once established, were treated with varying concentrations of metallic cations including Ca^{2+}, Magnesium (Mg^{2+}), and Manganese (Mn^{2+}) and tested for biofilm retention (**Figure 30A**). The ions were diluted in deionized water to prevent any added growth of the bacteria thus highlighting the role for the divalent cations, alone, on biofilm dispersion or retention. Deionized water served as the positive control and prevented any loss of the biofilm. However, high concentrations of Ca^{2+} prevented biofilm retention with 3.125 mM Ca^{2+} being the minimum biofilm eradication concentration (MBEC) ($P<0.0001$). Neither Mg^{2+} nor Mn^{2+} adversely affected the phenotype; biofilms treated with these ions were fully retained similar to the water control.

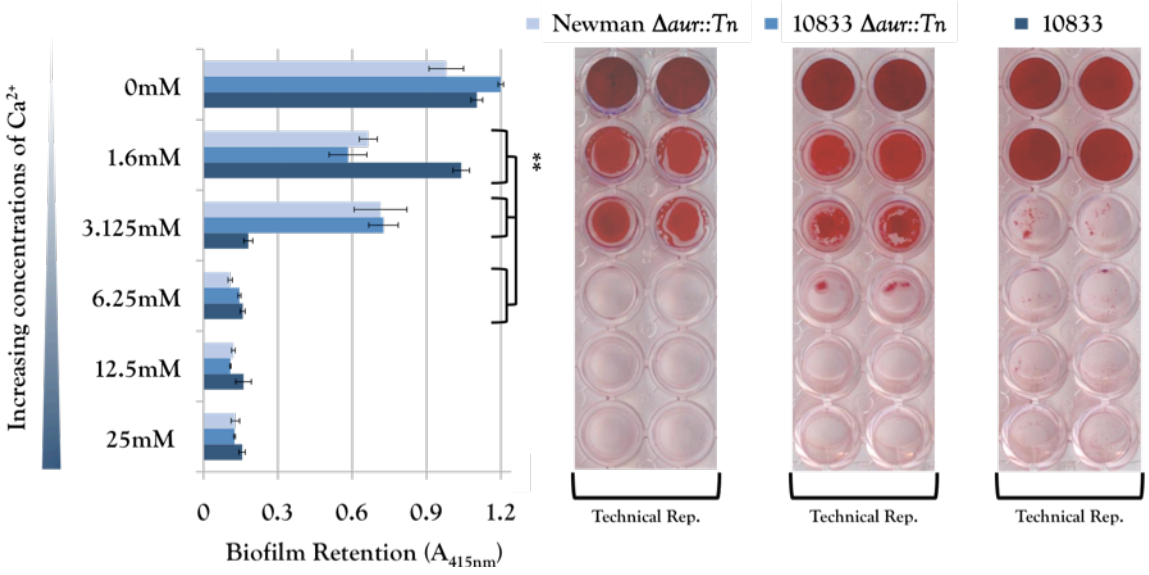
While the effect of Ca^{2+} could potentially be attributed to an increase in aureolysin activity there is also the possibility that this effect is due to Ca^{2+} binding to the EF-Hand domain of ClfB, preventing the bacteria from aggregating and being retained as a biofilm. To determine whether the effect of Ca^{2+} was mediated through aureolysin or through the EF-Hand domain of ClfB, we tested similar dispersion/retention biofilm assays using biofilms pre-established by 10833Δ*aur::Tn* and Newman Δ*aur::Tn* within EGTA and then treated with increasing concentrations of Ca^{2+} (**Figure 30B**). As previously observed in **Figure 30A**, ≥3.125 mM Ca^{2+} prevented 10833 wildtype biofilms in EGTA from being retained. However, both *aur* mutants were observed to prevent biofilm formation at ≥6.25 mM Ca^{2+} ($P<0.0001$). This would suggest, while the effect of aureolysin on ClfB stability may play a more predominant role in preventing the protein from being retained on the bacterial surface, to promote aggregation and biofilm

Figure 30. High concentrations of Ca^{2+} ions disperse pre-established *S. aureus* biofilms. (**A**) To assess the relative contribution divalent cations and their effect through the ClfB EF-Hand domain, biofilms were pre-established by 10833 within 12.5 mM EGTA. The biofilms were thereafter treated with varying concentrations (12.5 mM – 0 mM) of divalent cations including Mg^{2+}, Mn^{2+} and Ca^{2+} or deionized water. The plates were incubated once again for 18 h at 37 °C and then washed for non-adherent or dispersed bacteria, stained (right) and quantified at A_{415nm}. (left). (**B**) The Ca^{2+} mediated effect on the ClfB EF-Hand domains, independent of aureolysin, was determined by pre-establishing biofilms by 10833 wildtype or 10833 and Newman *aur* mutants in 12.5 mM EGTA which were thereafter treated with varying concentrations (25 mM – 0 mM) of Ca^{2+}. The experiments were performed in biologic triplicate with each condition being tested in technical quadruplets. $^{**}P<0.0001$ as determined by Student's t-test.

A. Strain: 10833 WT



B.



formation, inhibitory effects of Ca^{2+} binding to the EF-Hand domain is stimulated at higher concentrations of Ca^{2+} and can function independent of aureolysin. These results present a novel post-translational regulatory network associated with ClfB-dependent biofilms, where Ca^{2+} binding to the EF-Hand domain affects ClfB activity and its capacity to form biofilms within chelated media.

Discussion

Biofilm formation by *S. aureus* is an important virulence mechanism associated with this species, and as a result, *S. aureus* is a leading cause of catheter-related BSIs, a significant and growing worldwide concern. The use of catheter lock solutions containing chemical chelators like NaCi, EDTA, and EGTA have been shown to provide an anti-thrombotic function, as well as, an antimicrobial and antibiofilm effect (Arakawa, Saito et al., 2000; Raad and Hanna, 2002; Weijmer, Debets-Ossenkopp et al., 2002). However, we have shown previously, that a subgroup of strains including 10833, a close relative of sequenced strain Newman, grown within such chelating agents, surprisingly, augment biofilms. This mechanism of increased biofilm formation was promoted through the use of a specific MSCRAMM protein- clumping factor B (ClfB). The goal of this study was to chracterize the regulatory mechanism associated with this protein in aiding 10833 biofilms within chelated media and to determine the basis for the different phenotype observed in Newman.

ClfB is large surface-associated protein, which, *in vivo*, binds fibrinogen as its native ligand. However, we have shown ClfB to promote intercellular aggregation i.e.

clumping between bacteria, a phenotype that precedes the development of robust biofilms and this occurs in the absence of fibrinogen. As with any developmental process, biofilm formation is tightly regulated via the coordinated and sequential activities of SarA and Agr. Expression of SarA is dependent upon growth phase, with its greatest expression observed during the late exponential growth phase. Thus, it can regulate virulence determinants in a temporal manner; upregulating surface-associated proteins involved in initial colonization and biofilm formation like the fibronectin-binding proteins (FnbA and FnbB) and downregulating expression of extracellular proteases and exoenzymes (Karlsson and Arvidson, 2002; Chan and Foster, 1998). Mutants with this locus have been usually identified with lower levels of biofilms (Beenken, Blevins et al., 2003). Agr, on the other hand, is usually identified with quorum sensing and bacterial communication, is also associated with the late stages of biofilm development i.e. biofilm dispersal. This is brought about through the decreased expression of surface proteins (Novick, Ross et al., 1993) and increased expression of secreted proteases and exoenzymes (Morfeldt, Taylor et al., 1995; Oscarsson, Kanth et al., 2006; Oscarsson, Tegmark-Wisell et al. 2006). Our findings that Agr activity was elevated in Newman but low in 10833, and that deletion of *agr* in Newman augmented biofilm formation in EGTA, suggested a role for Agr as a negative regulator of biofilm formation in EGTA, although whether this effect was direct, through repression of *clfB* gene expression, or indirect, possibly through increase protease production was not determined in this study.

Previous work accomplished by McAlesse and colleagues identified a secreted metalloprotease- aureolysin (*aur*) to cleave ClfB resulting in decreased binding to

fibrinogen (McAleese, Walsh et al., 2001). This suggested the possibility of a post-translational means of regulating this protein. Our hypothesis was confirmed when we deleted *aur* from Newman, which restored biofilm formation of the strain similar to that observed with 10833 (**Figure 26**). A similar deletion with 10833 did not affect biofilm formation within EGTA. This supported the likelihood that aureolysin cleaves ClfB from the surface of the cell thereby preventing Newman biofilms in EGTA or NaCi. Thus, deletion of this gene from strains like 10833, which already augment biofilms within chelated media, would remain unaffected. Addition of recombinant aureolysin protease, exogenously to pre-established biofilms within EGTA using the *aur* mutants confirmed this hypothesis. Biofilms pre-established by 10833 and Newman *aur* mutants were successfully dispersed with high concentrations of aureolysin (**Figure 27**), thus confirming a role of the aureolysin protease specifically cleaving ClfB and implicating a novel post-translation regulatory mechanism associated with strains that displayed increased biofilms within chelating agents.

While identifying aureolysin as the protease that cleaves ClfB from the surface of strains that promote ClfB-dependent biofilms, we had yet to answer the paramount question of how related strains Newman and 10833 exhibit opposing biofilm phenotypes within chelating agents. Hypothesizing a variable level of aureolysin activity between 10833 and Newman we constructed complementation *aur* mutants using Newman or 10833's copy of the gene, and transformed these constructs into the 10833 or Newman *aur* deletion mutants. Biofilm and clumping assays revealed strains that were complemented with Newman *aur* prevented biofilm formation and aggregation of both

10833 and Newman *aur* mutants. In contrast, complementation with 10833 *aur* did not affect biofilm formation or clumping and aggregation in either 10833 or Newman *aur* mutants (**Figure 28A**, **B**). These findings were better understood during sequence analysis of the 10833 and Newman *aur* gene. While only four nucleotide polymorphisms were observed with the 10833 *aur* gene, the deletion of thymine nucleotide at position 565 brought about a frame shift inserting a premature stop codon (**Figure 29**). Thus, while strain Newman generated a functional protease, strain 10833 did not. Therefore, the genotypic analysis provided the appropriate rationale behind Newman *aur* complementation preventing biofilm formation and clumping while complementation with 10833 *aur* did not.

The role of aureolysin as a major regulator of ClfB decisively explains the variability that occurs between strains like 10833 and Newman and their differential biofilm phenotype within chelated media. Chelators like EGTA are specific for Ca^{2+} cations. Dispersal of pre-established 10833 biofilms within EGTA within increasing concentrations of Ca^{2+} suggested an additionally level of ClfB regulation- at the level of protein activity. EF-Hand domains in *S. aureus* proteins like Bap have been observed to bind Ca^{2+} and prevent aggregation and biofilm formation (Arrizubieta, Toledo-Arana et al., 2004). Similar domains with ClfB suggested a role for Ca^{2+} mediated regulation of biofilm formation. Dispersion of biofilms produced by strains that were deleted for *aur* with high concentrations of Ca^{2+} confirmed this hypothesis. This suggested an added level of regulation whereby, binding of Ca^{2+} to these helix-loop domains destabilizes the

ClfB protein and thus prevents intercellular aggregation and biofilm formation and retention.

Virulence properties of prokaryotes mediated through Ca^{2+} is an area of research that is still in its infancy. This chapter has highlight a novel pair of regulatory networks associated with the variable biofilm phenotype associated with strains like 10833 and Newman when cultured with chelators like EGTA or NaCi. Understanding that these biofilms are promoted using the sole surface-associated MSCRAMM ClfB, we have shown that the decreased retention of the protein on surface of strains like Newman is mediated by the protease aureolysin. The discrepancy between the biofilm phenotypes is explained knowing that while Newman encodes a functional aureolysin protease which cleaves ClfB off the surface of the cell, 10833 does not. Thus wildtype and *aur* mutants of 10833 continue to promote strong biofilms within chelated media. Finally, the role for Ca^{2+} binding to the EF-Hand domains affecting ClfB activity i.e. its capacity to aggregate and thus promote biofilms, was a secondary level of regulation identified and this level of regulation was observed to in concert or independent of aureolysin.

CHAPTER 6

Conclusions and Future Perspectives

Bacterial nosocomial infections have become more prevalent in recent years with the increased use of prosthetic biomedical implants. Chronic infection of a prosthetic implant can serve as a septic focus for bacteremia, which can then lead to metastatic or disseminated infections, sepsis, and death, particularly in immunocompromised patients (Christensen, Baldassarri et al., 1994; Gristina, 1987). *Staphylococcus aureus* is a leading cause of implant-associated infections. While it is has a commensal relationship with close to half of healthy individuals, it is opportunistic in nature, and can cause a broad range of infections from purulent self-limiting skin infections to more severe, potentially fatal conditions including infective endocarditis and catheter-related BSIs. Colonization of implanted medical devices including catheters occurs when *S. aureus* forms tenacious biofilms, a consortium of sessile cells that tightly adhere to the implant surface and each other. Once a biofilm has been established it can be very difficult to treat clinically since the bacteria on the interior of the biofilm are well protected from the host immune response as well as antibiotic agents (Hoyle and Costerton, 1991). The major aim of this work was to investigate the effects that compounds to which *S. aureus* would be exposed during infection, have on its capacity to form a biofilm. More specifically, the project elucidates the effects of serum, and thereafter, chelating agents that comprise catheter

lock solutions on biofilm formation by *S. aureus*.

Serum-mediated inhibition of *Staphylococcus aureus* biofilm formation

S. aureus is an exceptionally adaptable organism and responds rapidly to changes in its environment by modulating the expression of appropriate genes. This flexibility in bacterial gene expression permits survival in rapidly changing environments and conditions. Sudden shifts in temperature and osmolarity, mechanical stimulation, carbon and metal ion availability are some noteworthy environmental conditions which can induce global changes in *S. aureus* gene expression that initiate the switch to a sessile biofilm mode of growth. An important environmental factor to which *S. aureus* is exposed during certain types of infections is blood but the effects of blood on biofilm formation have not been well defined.

The acellular component of blood following removal of clotting factors is serum. Pre-coating polystyrene with serum is frequently used as a method to stimulate biofilm formation. The effect of pre-coating with serum is due to the deposition of extracellular matrix components onto the polystyrene, which are then recognized by MSCRAMMs. We hypothesized that if serum were included with the culture medium rather than removed prior to culture, that it would induce biofilm formation. Surprisingly, when the concentration of human or fetal bovine serum was 10% or more, planktonic growth was supported but the serum was a potent inhibitor of biofilm formation. Thompson et al. have since confirmed these results when they observed *S. aureus* biofilms were promoted only in the presence of ≤5% serum; such biofilms were dependent on the *S. aureus*

secreted extracellular adherence protein (Eap) (Thompson, Abraham et al., 2010). We found that the inhibitory serum component had a molecular weight less than 3,000 Da. Precedence for the role of larger molecular weight host serum components including lactoferrin, apo-transferrin, and albumin that effectively inhibit *S. aureus* adherence to polyurethane surfaces and biofilm formation has been well established (Ardehali, Shi et al., 2002; Ardehali, Shi et al., 2003; Singh, Parsek et al., 2002; Hammond, Dertien et al., 2008); however, a role for a small molecular weight component/s has never been established. Furthermore, the inhibitory LMWF component was protease-resistant and heat stable. We therefore hypothesize that the LMWF component could be a carbohydrate, a small, heat-stable protein such as an antimicrobial peptide, a nucleic acid fraction, or a cellular metabolite. Future experiments could determine whether the compound is a carbohydrate by treating this fraction of serum using sodium metaperiodate, which oxidizes polysaccharides. To gain some perspective on the role of AMPs or other low molecular weight heat stable proteins, we could perform SDS PAGE analysis on untreated versus heat inactivated fractions. Heating should destroy a large number of proteins however those that continue to remain on the gel could be isolated and analyzed by mass spectrometry.

We hypothesize, because serum inhibits biofilm formation and yet *S. aureus* elaborates biofilms during catheter-related infections that biofilm formation *in vivo* may be "selected for" rather than "induced" at the level of transcription by serum. It is possible that there is a stochastic component to the expression of biofilm-related genes and that some cells reflect a planktonic gene expression profile, while others, express a

biofilm mode of growth. Alternatively, a sub-population may encounter different "micro-environmental conditions" leading to the expression of biofilm-related genes. In either case, we hypothesize that blood components induce the planktonic mode of growth *in vivo* to maximize its growth potential in a nutrient-rich environment, but advantages of *in vivo* planktonic survival are outweighed by the numerous disadvantages including exposure to antimicrobials and easier access by host immune defenses. Thus, the few bacteria that happen to adhere to the device would have a selective advantage over the planktonic population.

The variability between the transcript levels of genes associated with biofilm formation or regulation between distinct strains SA113 and Newman in the presence of the serum component suggests that the response to serum varies between strains. Thus we can also postulate a strain-based variability to biofilm formation *in vivo*. With the large degree of genetic variability that exists between strains it is possible that there are some strains of *S. aureu*s like 10833 that could be better suited for biofilm formation versus strains SA113 or Newman. *In vivo* such strains may 'sense' themselves in an environment where a biofilm mode of growth would be preferred.

Whichever the case, these results have suggested serum-mediated inhibition of biofilm formation may represent a previously uncharacterized aspect of host innate immunity and provide a novel outlook into host immunity against the expression of a key *S. aureus* virulence factor: the ability to establish a resistant biofilm.

Chelator based catheter lock solutions augment biofilm formation in a subset of *S. aureus* strains in a ClfB-dependent fashion

Catheter-related BSIs are a significant iatrogenic complication associated with the use of implanted intravascular devices, and incidence increases proportionally with the length of time that the catheter is left in place. To combat the burgeoning numbers of catheter-related BSIs, metal ion chelators like NaCi, EGTA and EDTA are frequently chosen to lock intravenous catheters. While serving a critical anti-thrombotic function, these chemical compounds additionally provide added antimicrobial and antibiofilm properties (Banin, Brady et al., 2006; Shanks, Sargent et al., 2006). With the availability of these improved solutions, there has been a significant decrease in catheter-related bacteremia within patients (Weijmer, van den Dorpel et al., 2005). Nonetheless, catheter-related infections still occur, suggesting that bacteria continue to form biofilms even in the presence of antibiofilm CLS. In contrast to our original hypothesis that CLS had uniform antibiofilm properties across all strains of *S. aureus*, we observed this to be true to only a certain number of strains including SA113 and Newman and 5 out of 27 clinical isolates. Observing a striking increase in biofilms within 10833, genetically related to Newman, and others including USA300 JE2 and 8 out of 27 clinical isolates, we reasoned, similar to our discussion above, strains of *S. aureus* differ in their capacity to form biofilms within CLS and this variability is directly related to the strains' capacity to form varied types of biofilms, some which are more resistant or tolerant to the antibiofilm chelators, while others that are specifically targeted by these compounds. We hypothesized that *S. aureus* could become resistant or that certain strains may be innately

resistant to the antibiofilm effects of chelating agents, countering their inhibitory effects, thus exacerbating the problem of biofilm-associated infections. In the strains tested, we found that resistance to the antibiofilm effects of CLS was due to expression and retention of a surface-associated *S. aureus* adhesin ClfB.

S. aureus exhibits significant strain-to-strain variability in its ability to form a biofilm under different conditions. **Figure 31** describes schematically, a simplified model of what we believe to occur *in vivo* during *S. aureus* colonization and biofilm formation on catheter surfaces. Pharmaceutically available CLS such as Citra-Lock ™, Dura-Lock-C ™, and Loxxit ™, composed solely of the chelating agents like NaCi are advertised to target established biofilms and promote their dispersal (top). At sub-inhibitory concentrations, the antibiofilm properties of chelators, at least in part, would seem to be mediated through repressed polysaccharide (PNAG) expression and production. Therefore, we believe strains like SA113 that rely more heavily on polysaccharides within their biofilms (Cramton, Gerke et al., 1999), are potentially more susceptible to the antimicrobial and antibiofilm effects of CLS. However, certain strains rely more heavily on proteinaceous adhesins rather than polysaccharides for biofilm formation (Corrigan, Rigby et al., 2007; O'Neill, Pozzi et al., 2008; Hennig, Nyunt Wai et al., 2007). Strains like 10833 and USA300 JE2, among others, when cultured with chelating agents, retain the ability to form a biofilm through the coordinated increased expression and retention of surface-associated ClfB. Once anchored to the bacterial cell surface, the

Figure 31. Schematic for chelator-based induction of ClfB-dependent *S. aureus* biofilms. *S. aureus* biofilms can be composed for varying components including an exopolysaccharide (PNAG), proteinaceous adhesins (MSCRAMMs, intercellular adhesin proteins like SasG, SasC, Bap) and eDNA. Each of these components play mutually exclusive roles in promoting biofilm formation depending on the nature of the strain and the changing environmental conditions. (**Top**) Strains like SA113 rely primarily on exopolysaccharides like PNAG to facilitate an adherent biofilm phenotype. The addition of chelators, at least in part, represses PNAG expression and production and thus targets biofilms to become dislodged. (**Bottom**) In contrast, strains like 10833, USA300 JE2, VF Strains #19, and #24, rely on the surface-associated MSCRAMM ClfB, which actually functions more efficiently when calcium is chelated. Thus, chelators stimulate biofilm formation especially when proteinaceous adhesins like ClfB are the major component.

protein promotes intercellular aggregation, resulting in a clumped phenotype, which progresses towards establishing a biofilm.

Table 4. summarizes our overall results from chapters 3 and 4. Identifying ClfB as a protein determinant promoting increased biofilm formation in some strains we next sought to answer two questions: first, "what is the role of ClfB in biofilms formed under chelating versus non-chelating conditions?" and second, "why do strains 10833 and Newman differ so dramatically in their response to the chelating agents?"

In response to the first question, we hypothesize from the results of our study, that, under non-chelating conditions, *S. aureus* strains such as Newman and 10833 form biofilms that do not require ClfB. Instead, these biofilms may depend upon different surface proteins, polysaccharides and/or possibly eDNA. However when these bacteria are grown under chelating conditions, 10833 and Newman depend upon expression and activity of ClfB. Chromosomal and insertional inactivation of *clfB* confirmed that biofilms formed in the presence of EGTA or NaCi require ClfB, however ClB is expendable for biofilm formation when the strains are grown under non-chelating conditions. We also hypothesize, based on our results that the reason for this dependence is that chelators interfere with other biofilm mediators such as other surface adhesins and polysaccharide either at the level of transcription or possibly by interfering with surface expression or even charge. However, ClfB was actually activated, rather than inhibited by chelating conditions. We found that calcium led to the dispersal of biofilms that had been pre-established in EGTA. This was presumably because calcium inactivated the EF-hand

Table 4. Biofilm formation by Newman and 10833 under chelating versus non-chelating conditions

Strain	**- Chelator (TSBG)**	**+ Chelator (EGTA / NaCi)**
Newman	++	+/-
Newman Δ*clfB::erm*	++	-
Newman Δ*aur::Tn*	++	+++
10833	++	+++
10833 Δ*clfB::erm*	++	-
10833 Δ*aur::Tn*	++	+++

Biofilm formation scores shown indicate (+++),very strong biofilms; (+/-), diminished biofilms; (-), no biofilms detected

domain while calcium removal by EGTA activated the autoaggregative capacity of the EF-hand domain.

In answer to the second question regarding the difference between strains 10833 and Newman; this variability between the two strains occurs as a result of the effects of the aureolysin protease. ClfB would presumably be active and contribute to biofilm formation in both strains, however, in strain Newman, aureolysin prevents this; likely through cleavage of ClfB from the bacterial cell surface. Therefore, we hypothesize, that this major mediator of chelator-grown biofilms is removed by aureolysin in strain Newman and no biofilm is able to form. In contrast, due to a mutation within the gene, aureolysin is not produced in strain 10833 and the ClfB accumulates on the cell surface and is highly active under chelating conditions.

Thus ClfB activity is regulated in two ways. Its aggregative activity is turned on in the absence of calcium but aureolysin can destroy this activity. **Figure 32** better elucidates this schematically. Knowledge that ClfB regulation occurs through two independent and/or synergistic post-translational networks affecting protein activity and protein stability can be used as vital information in generating effective therapeutics. Conjugative therapeutics- combining the anticoagulant properties of chelating agents in concert with superior proteases like aureolysin, would be ideal, and could be used to promote disintegration and thereafter prevention of biofilm formation; however, the enzymatic activity of secreted *S. aureus* proteases *in vivo* would have detrimental effects within the host. Aureolysin degrades antibodies and possibly other host immune effectors. Recently *S. aureus* proteases- staphopains (SspA and SspB) were shown to

Figure 32. Regulatory networks of Clumping factor B during biofilm formation. Shown is a schematic representation of the ClfB protein identifying the signal sequence (S), ligand binding domain (A), the long serine-aspartate repeat region (SD-Repeat), cell wall spanning domain (W), and the membrane anchoring domain (M). The black box at residue 310 is the abbreviated EF-Hand domain and the shaded blue box is a proline rich region that separates the A domain from the SD Repeat region. The SLAVA motif spans residues 197 through 201, and the cell wall anchoring LPETG motif spans residues 874 to 878. Post-translational regulation of ClfB occurs via the independent or synergistic activities of aureolysin and Ca^{2+} ions binding to the EF-Hand domain. High concentrations of Ca^{2+} ions binding to the EF-Hand affect ClfB activity and thus prevent aggregation and intercellular adhesion. The metalloprotease aureolysin, on the other hand, affects ClfB activity. The proteases cleaves ClfB at the SLAVA motif within the A domain, between residues Leu198 and Ala199 (red arrows), preventing aggregation and biofilm formation.

promote degradation of host fibrinogen and collagen, and suggest an involvement of staphopains in the clotting impairment and tissue destruction caused by staphylococcal infection (Ohbayashi, Irie et al., 2011). Our studies, while identifying a proteinaceous adhesin that plays a predominant role in biofilm formation and possibly in bacterial resistance to CLS, were limited to two lab strains and eight cardiac device-associated isolates. The clinical gravity of this novel phenotype would suggest it be prudent to gain a better perspective of the prevalence of the novel phenomenon within catheter-related bacteremia isolates. Genetic and phenotypic polymorphisms observed with related strains like 10833 and Newman would suggest an even wider spread among clinical strains *S. aureus*. Nonetheless, it would be interesting to compare the *aur* sequences from the eight cardiac isolates that demonstrated increased biofilm formation within EGTA and NaCi and compare them to the five that were repressed for the phenotype.

This research brings to light data regarding strains of *S. aureus*, *in vivo*, encountering a variety of environmental signals that could inhibit or augment the capacity to form biofilms. We highlight the role for a novel small molecular weight host derived serum component that successfully prevents biofilm formation. *S. aureus* being one of the leading causes of catheter-related BSIs, subinhibitory levels of catheter lock solutions, contrary to their advertized antimicrobial and antibiofilm properties, may augment biofilm formation within a subset of *S. aureus* strains and this phenotype is promoted by the surface-associated MSCRAMM- ClfB. This significant finding validates and further emphasizes the clinical burden and downstream hematogenous manifestations *S. aureus* catheter-related infections bear. The scientific community is highly interested in the

enzymatic degradation of bacterial biofilms, as biofilm formation is a cause of many medical, industrial and environmental problems (Chaignon, Sadovskaya et al., 2007). Therefore, biological or chemical agents that could disintegrate the biofilm, releasing the bacteria into the environment, would allow antibiotics and host immune defense to eliminate the infection with improved efficiency (Chaignon, Sadovskaya et al., 2007). Clinicians and investigators have found eradication of *S. aureus* colonization and infection a daunting task. Nevertheless, the more knowledge gained from studies as such adds to the wealth of information in hopes of identifying the 'magic bullet' that could prevent staphylococcal biofilm-related infections.

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VITA

Nabil Mathew Abraham was born on August 20th, 1985 in Bangalore, India. He is citizen of India. He graduated from Cathedral and John Connon School, Mumbai, India in 2003. He received his Bachelor of Arts degree in Biology and Chemistry from Hiram College, Hiram, OH in 2007. His avid interest in microbiology and microbial pathogenesis led him to Virginia Commonwealth University in the Fall of 2007. He received his doctoral degree from the Department of Microbiology and Immunology at VCU in 2011. A brief list of his accomplishments, while at VCU, is listed below.

Publications:

Thompson KM, **Abraham N**, Jefferson KK. Staphylococcus aureus extracellular adherence protein contributes to biofilm formation in the presence of serum. FEMS Microbiol Lett. 2010. 305(2):143-7

Abraham NM, Jefferson KK. A low molecular weight component of serum inhibits biofilm formation in *Staphylococcus aureus*. *Microb Pathog*. 2010. 49(6):388-91

Manuscripts in preparation and review:

Abraham NM, Lamlertthon S, Fowler VG, Jefferson KK. Chelating agents exert diametric effects on biofilm formation in different strains of *Staphylococcus aureus*: role for Clumping factor B.

Abraham NM, Jefferson KK. *Staphylococcus aureus* chelator-induced ClfB-dependent biofilm formation is regulated at the post-translational level.

Abstracts and Presentations:

Abraham NM. Effect of chelating agents on biofilm formation in *Staphylococcus aureus*. The Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA), Reston, VA. 2011. (Presentation)

Abraham NM. Role for Calcium in Biofilm formation in *Staphylococcus aureus.* American Society of Microbiology- Virginia Branch, VCU, Richmond VA. 2009. (Presentation)

Abraham NM, Jefferson KK. Effect of chelating agents on *Staphylococcus aureus* biofilm formation. 111th General Meeting American Society of Microbiology, New Orleans, LA. 2011. (Poster)

Abraham NM, Jefferson KK. Effect of chelating agents on biofilm formation in *Staphylococcus aureus*. Daniel T. Watts Symposium, VCU, Richmond, VA. 2010. (Poster)

Abraham NM, Jefferson KK. Effect of chelating agents on biofilm formation in *Staphylococcus aureus*. International Conference on Gram Positive Pathogens, Omaha, NE. 2010. (Poster)

Abraham NM, Jefferson KK. Small molecular weight compound(s) within Fetal Bovine Serum inhibit biofilm formation in *Staphylococcus aureus*. Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA. 2009. (Poster)

Awards and Honor Societies:

Travel Award, International Conference on Gram Positive Pathogens, Omaha, NE- 2010
Phi Kappa Phi Scholarship- 2010
Charles C. Clayton Award- 2008
Golden Key International Honor Society- 2008- Present
Phi Kappa Phi National Honor Society- 2008-Present